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A Premature Termination Codon Mutation at the C Terminus of Foamy Virus Gag Downregulates the Levels of Spliced *pol* mRNA▽

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Foamy viruses (FV) comprise a subfamily of retroviruses. Orthoretroviruses, such as human immunodeficiency virus type 1, synthesize Gag and Pol from unspliced genomic RNA. However, FV Pol is expressed from a spliced mRNA independently of Gag. FV *pol* splicing uses a 3′ splice site located at the 3′ end of *gag*, resulting in a shared exon between *gag* and *pol*. Previously, our laboratory showed that C-terminal Gag premature termination codon (PTC) mutations in the 3′ shared exon led to greatly decreased levels of Pol protein (C. R. Stenbak and M. L. Linial, J. Virol. 78:9423–9430, 2004). To further characterize these mutants, we quantitated the levels of unspliced *gag* and spliced *pol* mRNAs using a real-time PCR assay. In some of the PTC mutants, the levels of spliced *pol* mRNA were reduced as much as 30-fold, whereas levels of unspliced *gag* RNA were not affected. Substitutions of a missense codon in place of a PTC restored normal levels of spliced *pol* mRNA. Disrupting Upf proteins involved in nonsense-mediated mRNA decay (NMD) did not affect Pol protein expression. Introduction of an exonic splicing enhancer downstream of the PTC mutation restored *pol* splicing to the wild-type level. Taken together, our results show that the PTC mutation itself is responsible for decreased levels of *pol* mRNA but that mechanisms other than NMD might be involved in downregulating Pol expression. The results also suggest that normal *pol* splicing utilizes a suboptimal splice site seen for other spliced mRNAs in most retroviruses, in that introduced exonic enhancer elements can increase splicing efficiency.

Foamy viruses (FV) comprise one of the two subfamilies of retroviruses. FV replication strategy differs in many respects from that of orthoretroviruses. A major difference is in the mode of Pol expression, regulation, and encapsidation into virions. Orthoretroviruses, such as human immunodeficiency virus type 1 (HIV-1) and avian sarcoma and leukosis virus (ASLV), synthesize Pol as a Gag-Pol fusion protein from the unspliced genomic RNA. A frameshift at the C terminus of Gag occurs to produce the Gag-Pol fusion protein at approximately 5% of Gag production (10), and the Gag-Pol fusion protein coassembles into virus particles with self-assembling Gag. This mechanism of Pol incorporation results in about 50 to 100 Gag-Pol proteins per particle (35). However, FV express Pol independently of Gag from a separate spliced mRNA that is not very abundant (40). The polymerase activity of FV reverse transcriptase (RT) is more active and processive than that of HIV-1 RT (3). Two genomic RNA sequences are required for Pol packaging into virus particles (27). These findings support the hypothesis that FV particles may contain as few as two to four Pol molecules. This raises two fundamental issues about how FV Pol expression is regulated and how Pol is selectively packaged into virions.

FV are complex retroviruses. The FV genome contains the open reading frames for Gag, Pol, and Env originating from the long terminal repeat promoter and other open reading frames for the nonstructural proteins Tas and Bet at the 3′ end of the genome that are derived from an internal promoter (Fig. 1A). Alternative splicing utilizing a single 5′ splice site (5′ss) and multiple 3′ss generates different subgenomic mRNAs from full-length pre-mRNA. In higher eukaryotes, accurate splicing requires exon-intron junctions that are defined by conserved intronic *cis*-acting elements. The conserved motifs include the nearly invariant GU and AG dinucleotides at the 5′ss and 3′ss, respectively; a polypyrimidine tract preceding the 3′ss AG; and an A residue that serves as a branch point (reviewed in reference 39). These elements are necessary, but are by no means sufficient, to define exon-intron boundaries. There are many other sites that match the consensus sequence as well as or better than true splice sites but that are never recognized as exons by the splicing machinery (33). The additional information needed to delineate exons is thought to reside in the exons themselves. These elements are called the exonic splicing enhancer (ESE) and exonic splicing silencer. They can act by stimulating (ESE) or repressing (exonic splicing silencer) splicing, and they seem to influence both the efficiency of splicing and the regulation of alternative splicing (4). ESE sequences are generally not well conserved, so delineating the presence of an ESE in any sequence is difficult. However, there are several ESEs that have been characterized in HIV-1 and other viruses (32, 43). In ASLV, a 24-bp linker insertion just upstream of the suboptimal *env* 3′ss greatly enhanced splicing and resulted in a replication defect caused by an insufficient amount of unspliced RNA

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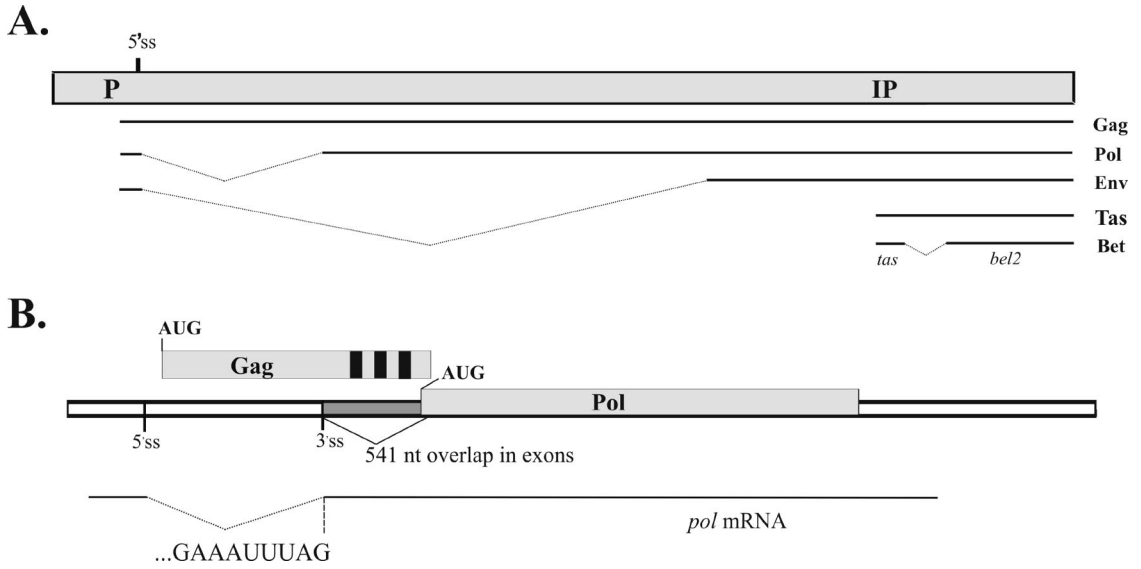

FIG. 1. (A) Diagram of the FV genome representing unspliced and spliced mRNAs derived from a major promoter (P) at the 5′ long terminal repeat and an internal promoter (IP). (B) Splice sites for *pol* mRNA shown with respect to the open reading frames of Gag and Pol. Pol is synthesized by a −1 frameshift at the 3′ end of a *gag* gene. There is a 541-nt overlap in the two exons, shown in gray, which includes three glycine-arginine-rich (GR) boxes at the C terminus of Gag, shown in black.

(13). Replication-competent revertants were identified and found to have second-site suppressors either in the linker or downstream of the splice site. These sequences define the ESE, a 33-nucleotide (nt) purine-rich sequence downstream of the 3′ss, which has been shown to be transportable, since it functions when it is placed in *Schizosaccharomyces pombe* genes (37).

A balance between unspliced and spliced mRNAs is essential for retroviral replication. The level of splicing must be controlled to preserve sufficient amounts of full-length RNA, which serves as genomic RNA and mRNA for both Gag and Gag-Pol proteins. One of the mechanisms that control the balanced splicing is the use of suboptimal processing signals to direct inefficient splicing in many retroviruses, such as HIV-1 (25), Rous sarcoma virus (13, 42), and equine infectious anemia virus (28). In the case of FV, *pol* splicing uses a 5′ss upstream of the Gag translation start site and a 3′ss located at the 3′ end of *gag*, resulting in a 541-nt exon shared between *gag* and *pol* (Fig. 1B). The 3′ss has a weak polypyrimidine tract containing only three U residues compared to 8 to 10 pyrimidine residues in canonical motifs.

Eukaryotic cells have evolved mechanisms to ensure the fidelity of gene expression. One such mechanism, called mRNA surveillance, ensures that functional mRNAs are available for translation in the cytoplasm. Aberrant mRNAs containing premature termination codons (PTC) are recognized and degraded by nonsense-mediated mRNA decay (NMD) (reviewed in references 1, 21, and 36). Such a quality control mechanism prevents the synthesis of truncated proteins that can have deleterious effects. Factors that regulate NMD were originally identified in yeast but are highly conserved in other organisms, including humans. They include the proteins Upf1, Upf2, and Upf3 (17). Mutations in any of these proteins can significantly decrease the decay rate of PTC-containing mRNAs. The NMD pathway is closely linked with steps of transcription, pre-mRNA splicing, mRNA export, and translation. NMD factors and other parts of the cellular machinery involved in posttranscription work in concert to proofread newly made transcripts in both the nucleus and cytoplasm (11, 15).

The Gag protein of FV differs from those of orthoretroviruses. Gag is not cleaved into the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins found in mature orthoretroviruses. FV protease cleaves only once at the C terminus of Gag to release a p3 peptide. In orthoretroviruses, NC contains one or two copies of a Cys-His (CH) motif, and clustered basic residues flanking the CH motif(s). These structural motifs are required for essential steps of virus replication, including reverse transcription, integration, RNA packaging, viral assembly, and infectivity (reviewed in reference 6). FV Gag lacks the conserved CH motif(s), but instead, it contains three regions of Gly- and Arg-rich motifs (GR boxes) at the C terminus of Gag. GR box 1 has a nucleic acid binding activity in vitro, and GR box 2 contains a nuclear localization signal (29, 41). Overall, the C terminus of Gag contains domains important for RNA packaging and expression, cleavage, and packaging of the Pol protein (30).

In previous studies, we found that truncation mutations at the C terminus of Gag unexpectedly decreased Pol expression, which is normally very low, to a great extent (30). In this study, we examined the mechanism by which Pol expression is downregulated by PTC mutations at the C terminus of Gag in order to better understand how FV Pol expression is regulated. The PTC mutation itself played important roles in downregulating the levels of spliced *pol* mRNA. Introduction of an ESE from another virus was shown to increase levels of splicing in the PTC mutants, as well as in the wild type (wt), suggesting that suppression of *pol* splicing is a mechanism to regulate the level of FV Pol expression and to keep it low.

MATERIALS AND METHODS

DNA mutagenesis and cloning. Mutations at the C terminus of Gag were constructed in the context of full-length primate FV containing a cytomegalovirus intermediate-early promoter (pcHFV) (30). The ESE sequence from the *env* gene of ASLV (13) was introduced 6 nt downstream of the GR (−)st mutation (see below). All of the site-directed deletion, insertion, and substitution mutations at the C terminus of Gag were obtained by two rounds of PCR using four oligonucleotides. Two outer oligonucleotides were designed to anneal to the 5′ or 3′ end of a *gag* or *pol* gene with the addition of two engineered unique restriction sites at each end. Two inner mutagenic oligonucleotides (in either forward or reverse orientation) were designed to be complementary to the Gag sequences except for the desired mutations. The first round of DNA amplification was done with a pair of oligonucleotides consisting of either the 5′ outer oligonucleotide and the reverse inner mutagenic oligonucleotide or the forward inner mutagenic oligonucleotide and the 3′ outer oligonucleotide. The amplified DNAs were then used for the second round of PCR with two outer oligonucleotides. The resultant PCR-amplified *gag* mutated sequences were digested with flanking restriction enzymes and ligated to DNA restriction fragments of the full-length proviral vectors. Each mutant construct was sequenced to confirm the presence of the correct mutational changes. Primer sequences will be supplied upon request.

Cell cultures and transfections. 293T cells, human kidney embryonic cells expressing the large T antigen, were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Transient transfection was done using 1 mg/ml polyethyleneimine (PEI) (Polysciences, Warrington, PA) as previously described (7). For the Upf cotransfection experiments, PolyFect transfecting reagent (Qiagen) was used according to the manufacturer's protocol. 293T cells were transfected with either pcHFV proviruses or β-globin test plasmid (34), along with either wt or dominant-negative (DN) mutant (RR857AA) (22) hUpf1 at a 1:1 molar ratio. Both β-globin and hUpf 1 constructs were kindly provided by Karen Beemon (Johns Hopkins University). Cell lysates were prepared between 45 and 48 h posttransfection by scraping cells in antibody buffer (20 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholic acid, 0.5% sodium dodecyl sulfate [SDS], 0.5% aprotinin, 1 mM EDTA [pH 8.0]), shearing the cells with a 23-gauge needle, and centrifuging them at 138,000 × *g* for 10 min in a table-top microcentrifuge.

Western blot analysis. The cell lysates were prepared with 1× SDS sample buffer (12.5% 4× Tris-HCl/SDS [pH 6.8], 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.01% bromophenol blue) prior to being loaded onto SDS-10% polyacrylamide gels. Proteins were separated by electrophoresis and transferred to Immobilon-P membranes (Millipore). The membranes were blocked and hybridized with antibodies in a 5% milk-PBS solution containing 0.05% Tween-20 at either 1:5,000 dilution for polyclonal rabbit anti-Gag antibody (2) or 1:800 dilution for monoclonal mouse anti-Pol antibody (30). Monoclonal mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Abcam) was used to normalize samples. Enhanced chemiluminescence reagents (Amersham) were used for signal detection with X-ray film. In one experiment, the Odyssey detection method (Li-Cor) using infrared-wavelength fluorescence was used when more sensitivity and quantitation were required, according to the manufacturer's protocol.

RT-PCR and quantitative real-time PCR. Total cellular RNA was isolated by lysing cells with TRIzol reagents (Invitrogen) according to the manufacturer's protocol. In order to obtain cDNA, isolated RNA was reverse transcribed using ThermoScript RT (Invitrogen) and poly(A) · poly(dT)$_{12}$ as a primer, according to the manufacturer's protocol. PCR amplification was performed using a forward primer specific for unspliced *gag* or spliced *pol* mRNA and the same reverse primer for both RNAs. To measure the levels of β-globin mRNA, a 90-nt spliced mRNA was PCR amplified using a forward primer annealed to exon 1 and a reverse primer specific for the junction between exon 1 and exon 2. The levels of *gapdh* mRNA were used to normalize samples before running the RT-PCRs. Quantitation of unspliced and spliced mRNAs was performed using the 7900HT Fast Real-Time PCR system (Applied Biosystems) with a Sybr Green PCR mix. To obtain standard curves, either unspliced or spliced cDNA obtained from pcHFV-transfected cells was cloned into pCR 4-TOPO vector (Invitrogen), and RNA was synthesized by in vitro transcription.

RESULTS

PTC mutations at the C terminus of Gag affect expression of the Pol protein. A series of C-terminal Gag truncation mutations were engineered by introducing PTC downstream of GR box 3 at the p3 cleavage site (GR 3st), downstream of GR box 2 (GR 2st), downstream of GR box 1 (GR 1st), and upstream of GR box 1 [GR (−)st] (30) (Fig. 2A). Gag and Pol protein expression levels were compared to those of the wt by Western blot analysis using either anti-Gag or anti-Pol antibody (Fig. 2B). The proteolytic processing of wt Gag by FV protease (PR) produces two proteins (71 kDa and 68 kDa) by partial cleavage of a p3 peptide at the C terminus. PR also cleaves the 127-kDa precursor Pol protein (PrPol; PR-RT-IN) once to release an 80-kDa Pol protein (Pol; PR-RT) and a 40-kDa IN protein. All of the PTC mutants efficiently expressed the predicted truncated Gag proteins. However, the levels of Pol expression differed in the mutants. The GR 3st mutant lacking the p3 peptide produced wt amounts of Pol protein, whereas decreased levels of Pol were produced in cells transfected with GR 1st and 2st. The GR (−)st mutant lacking all three of the GR boxes showed the greatest reduction in the level of Pol protein.

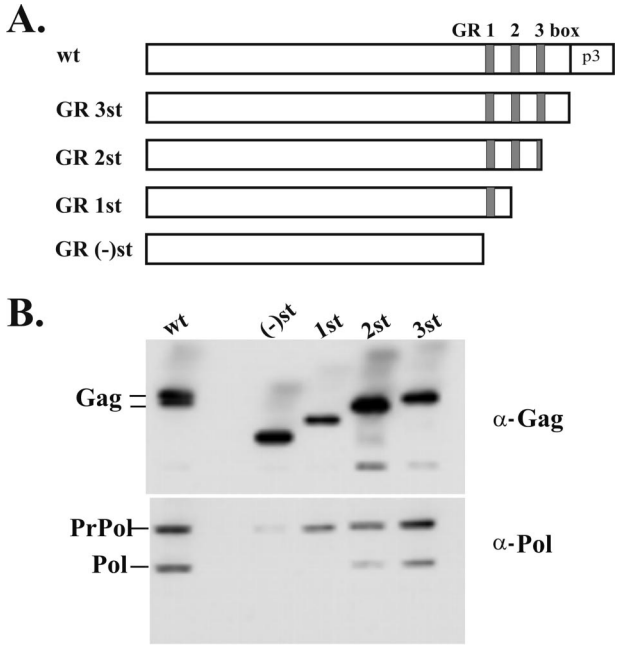

FIG. 2. (A) C-terminally truncated Gag proteins. Stop codons (UAA) were introduced at the C terminus of Gag with respect to the three GR boxes, shown in gray. (B) Gag and Pol proteins expressed in the GR st mutants were examined by Western blot analysis with either anti-Gag (α-Gag) or α-Pol antibody.

One possible reason for the low levels of Pol synthesis seen in the GR (−)st, 1st, and 2st mutants is that wt Gag protein itself regulates Pol synthesis at either the transcriptional or posttranscriptional level. In this case, addition of wt Gag in *trans* should rescue the Pol synthesis defect. To test this, a plasmid encoding wt Gag protein (pGag) was cotransfected into 293T cells with each of the Gag PTC mutant proviruses, and the amount of Gag or Pol was compared to that of cells transfected with the mutant alone (Fig. 3). Transfection of pGag alone yielded a single Gag band, as expected (Fig. 3A, lane 2). Singly transfected cells are shown in Fig. 3A, lanes 3 to 7. Cotransfected cells efficiently produced both wt Gag and the

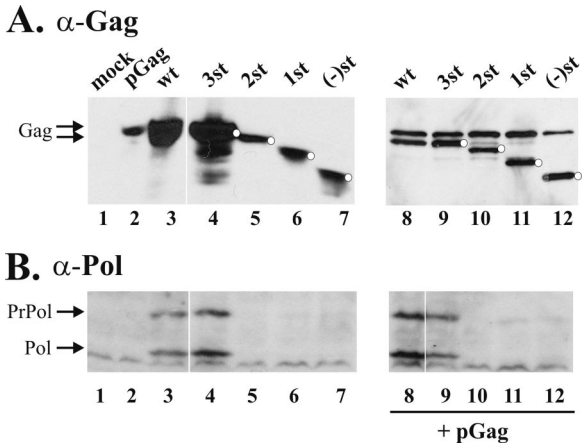

FIG. 3. Gag and Pol proteins expressed in the Gag PTC mutants. Mutant proviruses were transfected into 293T cells, proteins were extracted 2 days posttransfection, and the Western blot was probed with anti-Gag (A) or anti-Pol (B) antibody. Lane 2 was transfected with the pGag plasmid alone. In lanes 8 to 12, the wt or C-terminally truncated Gag mutants were cotransfected with pGag at a 1:1 molar ratio. The open circles indicate the C-terminally truncated Gag proteins expressed by mutants.

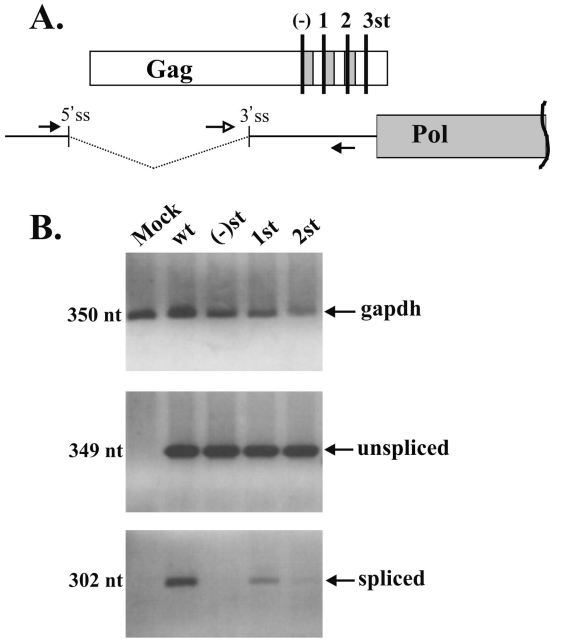

FIG. 4. Levels of unspliced *gag* mRNA and spliced *pol* mRNA in the Gag PTC mutants analyzed by RT-PCR assays. (A) PTC mutations are indicated by black lines at the C terminus of Gag. The 5′ss and 3′ss are marked at the 5′ untranslated region of *pol*. The solid arrow on the right was used to amplify spliced RNA, and the open arrow on the right was used to amplify unspliced RNA in RT-PCR assays. The same reverse primer shown in the solid arrow on the left was used for amplifying both unspliced and spliced mRNAs. (B) 293T cells were transfected with each of the Gag PTC mutant proviruses. Total cellular RNA was isolated and subjected to reverse transcription to make cDNA, and mRNA levels were determined by PCR.

truncated Gag proteins (Fig. 3A, lanes 8 to 12). Addition of wt Gag did not increase the low levels of Pol in the Gag PTC mutants (Fig. 3B, lanes 5 to 7 versus lanes 10 to 12), showing that full-length Gag is not required for Pol expression and suggesting that the truncated Gag proteins do not negatively regulate Pol synthesis.

Levels of spliced *pol* mRNA were greatly reduced in some of the Gag PTC mutants. To investigate whether the decreased expression of Pol protein in the PTC mutants is caused by reduced levels of *pol* mRNA, we examined RNA levels by RT-PCR. 293T cells were transfected with the Gag PTC mutant proviruses. Two days posttransfection, total cellular RNA was extracted and reverse transcribed using poly(A) · poly(dT)$_{12}$ primers. For cDNA PCR amplification, the forward primers that annealed to the sequences near the splice sites in *pol* were designed to distinguish spliced *pol* mRNA from unspliced *gag* mRNA (Fig. 4A). *gapdh* mRNA was used to normalize the RNA before reactions were run. We found that all of the Gag PTC mutants efficiently expressed wt levels of unspliced *gag* mRNA (Fig. 4B). The levels of spliced *pol* mRNA in all of the Gag mutants were much lower than those of unspliced mRNA. The levels of spliced mRNA in GR (−)st and 2st mutants were below the limit of visual detection on the gel. We then quantitated mRNA levels by a real-time PCR analysis using a standard-curve method, as described in Materials and Methods. Quantitation of the spliced and unspliced mRNAs was normalized to the wt (Table 1). Both GR (−)st and 2st mutants reduced the levels of spliced *pol* mRNA by approximately 25-fold. Although GR 1st showed higher levels of unspliced RNA than the wt, the ratio of spliced to unspliced RNA was also depressed about eightfold. These results show that Pol synthesis is regulated by the level of *pol* mRNA in the PTC mutants.

Substitutions of a missense codon for the GR (−)st mutation restored the levels of spliced *pol* mRNA. To test whether the PTC mutations themselves were responsible for downregulating the levels of spliced *pol* mRNA, we substituted missense codons for a PTC in the GR (−)st mutant (Fig. 5A). The m-ATC mutation encodes the same amino acid as in the wt (isoleucine); the m-GTG and m-GCA mutations resulted in substitution of valine or alanine. 293T cells were transfected with the mutant proviruses, and total cellular RNA was extracted and used in RT-PCR assays. All of the mutants had unspliced RNA levels equivalent to those of the wt. The levels of spliced mRNAs in two of the missense mutants (Fig. 5B, lanes 4 and 6) were increased relative to GR (−)st (Fig. 5B, lane 3) to about the wt level (Fig. 5B, lane 2). The level in mutant ATC was increased, but to a lesser extent (Fig. 5B, lane 5). Quantitation of mRNA levels using real-time PCR showed

TABLE 1. Quantitation of GR st mutant mRNA levels by real-time PCR

Construct	Level[a]		Ratio of spliced to unspliced
	Spliced	Unspliced	
Mock	ND	ND	
Wt	1.00	1.00	1.0
GR (−)st	0.05 ± 0.01	1.62 ± 0.40	0.03
GR 1st	0.42 ± 0.20	3.38 ± 0.56	0.12
GR 2st	0.04 ± 0.01	1.83 ± 0.11	0.02

[a] The levels of mutant mRNA are normalized to that of the wt. The numbers are averages ± standard deviations from three independent assays. ND, not detected.

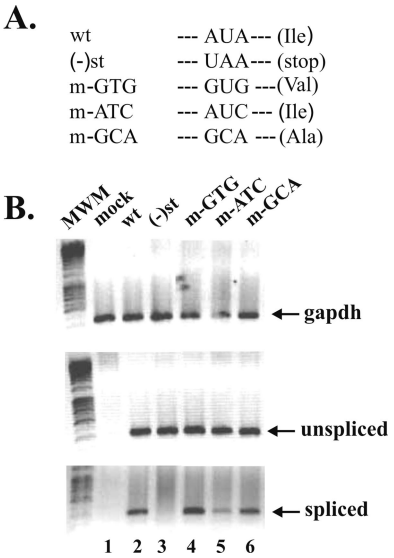

FIG. 5. (A) Missense mutations substituted for the PTC mutation in the GR (−)st mutant. (B) Levels of mRNA using primers shown in Fig. 4A as measured by RT-PCR assays.

significant increases in the levels of spliced *pol* mRNA for all three of the missense mutants, as well as in the ratio of spliced to unspliced RNA (Table 2). Thus, the PTC mutation itself is responsible for downregulating the levels of spliced *pol* mRNA.

DN Upf protein did not affect Pol expression in the GR (−)st mutant. Downregulation of *pol* spliced mRNA could result from mRNA degradation mediated by the NMD pathway. In order to examine this, we looked at the effects of human Upf1 (hUpf1), an essential protein in the NMD pathway, on the levels of mRNA and protein expression of *gag* and *pol*. It has been well documented that overexpression of the DN hUpf1 protein reproducibly enhanced the levels of reporter mRNAs containing PTCs (17, 34). We first did these experiments using the PEI transfecting reagent (see Materials and Methods) used in all of the other experiments. Interestingly, we noticed that expression of endogenous hUpf1 was upregulated after PEI treatment (data not shown). Therefore, we used the PolyFect reagent, which did not affect the levels of endogenous hUpf1 (data not shown). As a control, mRNA levels of a test construct β-globin containing a PTC (Ter-39) (34) were examined in the presence of hUpf1 overexpression. Either wt or DN hUpf1 (22) was cotransfected into 293T cells with wt or PTC-containing β-globin plasmid. In cells transfected without hUpf1, the PTC-containing mRNA (Ter-39) was greatly reduced compared to the wt (Fig. 6A, lane 1 versus lane 4), although the levels of *gapdh* mRNA were equivalent, consistent with previously published results (34). Levels of wt β-globin mRNA were not changed in the presence of either wt or DN hUpf1 (Fig. 6A, lanes 2 to 3). However, the low level of Ter-39 mRNA was restored to about the wt level upon DN Upf1 overexpression (Fig. 6A, lane 6).

Either wt or DN hUpf1 DNA was cotransfected into 293T cells, along with wt or mutant FV provirus. The levels of unspliced *gag* RNA were about the same in all samples (Fig. 6B, lanes 1 to 6). The addition of wt or DN hUpf1 did not greatly change the levels of wt spliced *pol* mRNA (Fig. 6B, lanes 2 and 3). DN hUpf1 did not increase the levels of spliced *pol* mRNA, as would be predicted if NMD was involved (Fig. 6B, lanes 4 to 6). Both Gag and Pol protein expression levels were also examined by Western blot analysis (Fig. 6C and D) and quantitated using the Odyssey Western detection system (Table 3). The levels of wt Gag in cells transfected with either wt or DN hUpf1 were comparable to that of cells without the exogenous hUpf1 (Fig. 6C, lane 2 versus lanes 3 and 4). Levels of wt Pol were also not changed in the presence of either wt or DN hUpf1 (Fig. 6D, lane 9 versus lanes 10 and 11), although wt Pol seemed to be cleaved less in the presence of hUpf1. Thus, in the wt, neither Gag nor Pol expression was changed in cells coexpressed with hUpf1. Levels of Gag protein in the GR (−)st mutant transfected with wt hUpf1 were comparable to those without hUpf1 (Fig. 6C, lane 5 versus lane 6). GR (−)st Pol expression was not changed and stayed low in expression of either wt or DN hUpf1 (Fig. 6D, lanes 12 to 14). These results suggest that downregulation of Pol in the GR (−)st mutant is unlikely to be mediated by the classical NMD pathway. Unexpectedly, the level of GR (−)st Gag was increased about sixfold in the presence of DN hUpf1 (Fig. 6C, lane 7, and Table 3) without an increase in the level of Pol.

Introduction of an ESE restored normal levels of *pol* splicing in the GR (−)st mutant. The FV *pol* 3′ss does not contain an upstream polypyrimidine tract (reviewed in reference 39) (Fig. 1B), suggesting that it is suboptimal. This raises the possibility that the splice site has ESE sequences that are fortuitously interrupted by the PTC mutations at the C terminus of Gag. If this were the case, we would expect that addition of an exogenous ESE would restore the splicing efficiency of the GR (−)st mutant. We introduced a transportable ESE (37) downstream of the GR (−)st mutation (Fig. 7A) and determined the levels of spliced *pol* mRNA by RT-PCR. As a control, the ESE was also introduced into the wt sequence. The results are shown in Fig. 7B. We found that the ESE did not change the levels of unspliced mRNA or *gapdh* mRNA. However, cells transfected with the wt/ESE provirus had an increased amount of spliced *pol* mRNA compared to that of the wt. In addition, introducing ESE into the GR (−)st mutant restored the level of *pol* splicing to that of the wt. Real-time PCR assays

TABLE 2. Quantitation of GR (−)st missense mutant mRNA levels by real-time PCR

Construct	Level[a]		Ratio of spliced to unspliced
	Spliced	Unspliced	
Mock	ND	ND	
Wt	1.00	1.00	1.0
GR (−)st	0.03 ± 0.01	0.95 ± 0.20	0.03
m-GTG	0.81 ± 0.18	1.03 ± 0.21	0.79
m-ATC	1.44 ± 0.38	1.01 ± 0.18	1.43
m-GCA	0.50 ± 0.11	0.77 ± 0.31	0.65

[a] The levels of mutant mRNA are normalized to that of the wt. The numbers are averages ± standard deviations from four independent assays. ND, not detected.

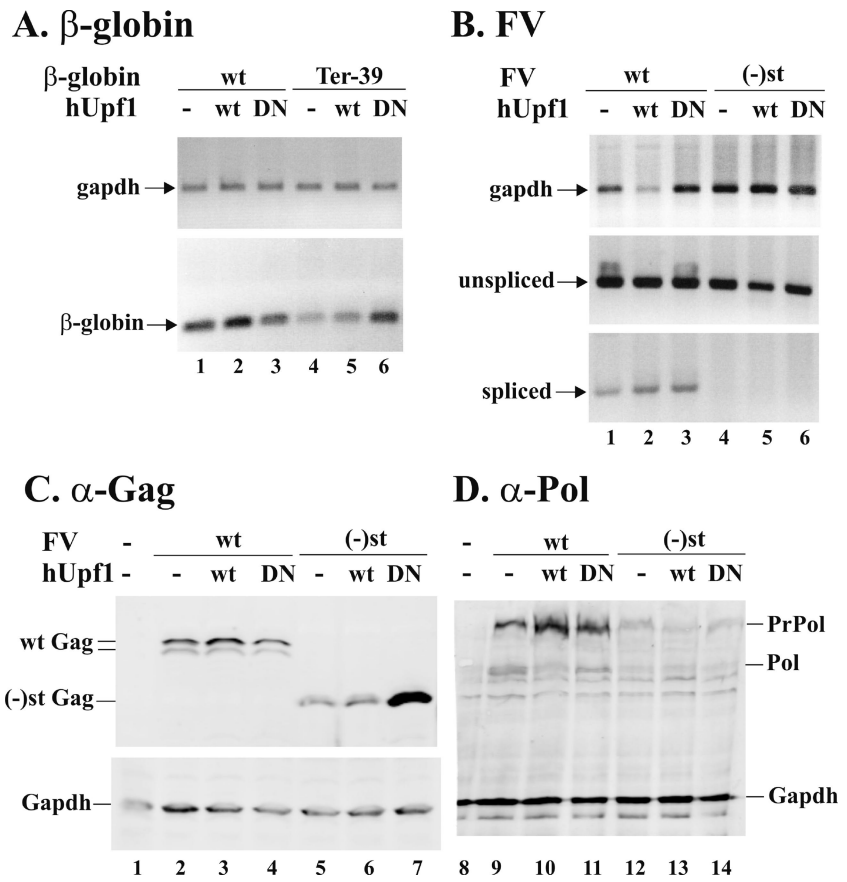

FIG. 6. Levels of mRNA expressed in cells transfected with either a test construct β-globin (A) or FV (B) upon coexpression of hUpf1 by RT-PCR. Either β-globin plasmid or foamy proviruses were transfected, along with either wt or a DN mutant hUpf1 at a molar ratio of 1:1. Ter-39 is a PTC-containing β-globin construct (34). Levels of Gag and Pol proteins produced in wt FV or the GR (−)st mutant upon coexpression of hUpf1 were examined by Odyssey Western blot analysis. At 48 h posttransfection, cell lysates were prepared to run on 10% SDS-polyacrylamide gel electrophoresis. The transferred membranes were probed with anti-Gag (C) or anti-Pol (D) antibody for detection. GAPDH was used to normalize the samples.

were performed to quantitate the levels of mRNA (Table 4). The GR (−)st mutant had an approximately 50-fold increase in the levels of spliced *pol* mRNA in the presence of the ESE, raising *pol* mRNA to wt levels. Addition of the ESE to the GR (−)st mutant increased the ratio of spliced to unspliced RNA about 5-fold more than in the wt and 160-fold more than in GR (−)st alone. However, the addition of an ESE to the wt also improved the efficiency of *pol* splicing about 14-fold, suggesting that the *pol* 3′ss has evolved to be suboptimal to limit the level of wt *pol* mRNA.

TABLE 3. Gag and Pol expression in the presence of hUpf1

FV	hUpf1	Gag[a]	Pol[a]
Wt		1	1
Wt	Wt	1.4	1.3
Wt	DN	1.2	1.4
(−)st		1	1
(−)st	Wt	1	0.7
(−)st	DN	5.9	0.7

[a] Quantitation of Gag and Pol proteins was done using the gels shown in Fig. 6C and D, as well as additional data. Gapdh was used to normalize the samples. The levels of Gag and Pol are relative to those of cells transfected without hUpf1.

DISCUSSION

In this study, we examined the posttranscriptional regulation of FV Pol expression. Retroviruses have mechanisms to control the balance between their requirements for large amounts of their structural protein, Gag, and for lesser amounts of their enzymatic protein, Pol. Orthoretroviral Pol is synthesized as a Gag-Pol fusion protein, and regulation of the Pol protein level is translational. However, FV Pol is expressed from a separate spliced mRNA, implying that regulation is at the level of transcription and/or posttranscription rather than translation. Unspliced *gag* mRNA is much more abundant than spliced *pol* mRNA (40) (Fig. 4, 5, and 7). Retroviruses have developed a number of strategies to limit RNA splicing. For some retroviruses, specialized mechanisms ensure efficient transport of intron-containing RNA from the nucleus so that unspliced mRNA is separated from the splicing machinery and is not further processed. Orthoretroviruses use either an autonomous nuclear export signal (the constitutive transport element for Mason-Pfizer monkey virus [8]; the direct repeats for ASLV [26]) or a viral protein specifically bound to viral sequences for direct transport of unspliced mRNA from the nucleus (HIV-1 Rev protein) (18, 24). There have been at-

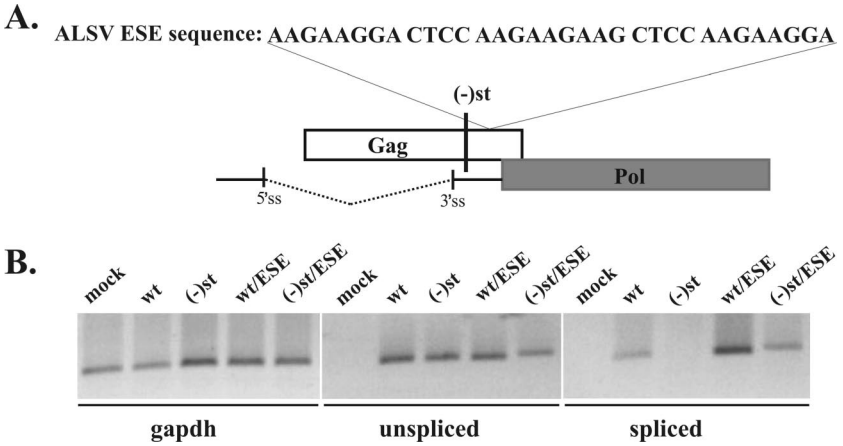

FIG. 7. (A) Sequence of the ASLV ESE introduced downstream of the PTC mutation in the GR (−)st mutant, as well as in the wt. (B) Levels of mRNA measured by RT-PCR. 293T cells were transfected with the proviral vectors encoding ESE either in a wt (wt/ESE) or in a GR (−)st mutant [(−)st/ESE] background. Total cellular RNA was extracted to prepare cDNA and run on PCR amplification using the primers specific for unspliced or spliced mRNAs shown in Fig. 4A. The levels of *gapdh* mRNA were used to normalize the RNA before reactions were run.

tempts to show that Gag protein binds in the vicinity of splice sites and acts as a negative or positive regulator for splicing (31). However, no correlation between Gag binding and changes in the extent of viral RNA splicing was found. We investigated the possibility that the Gag PTC mutant proteins negatively affected Pol synthesis. We found that wt Gag protein added in *trans* did not increase Pol expression in the GR (−)st mutant. Although the model cannot yet be definitely ruled out, we have no evidence for a role of FV Gag in the regulation of *pol* splicing.

Each of the PTC mutations at the C terminus of Gag affected *pol* mRNA expression to a different extent. GR 3st affected Pol expression very little, and GR 1st had a minimal effect on decreasing the level of *pol* mRNA (an eightfold decrease compared to the wt), whereas both GR (−)st and 2st showed much great defects in *pol* mRNA levels. Thus, the PTC mutations affected Pol expression in a position-dependent manner. One possibility is that the TAA mutations in the GR st mutants could fortuitously interrupt an ESE. There is evidence for nonsense-associated altered splicing in that PTC can elicit exon skipping by disrupting ESE, leading to alternative splicing using the next 3′ss or cryptic splice site (4, 19, 22). If this is the case in FV, we would expect increased splicing for *env*, the next exon using the *pol* 5′ss (Fig. 1A). However, we observed no difference in the levels of Env protein between the wt and the PTC mutants (data not shown). Moreover, this hypothesis is not consistent with the fact that several missense mutants at the same place as the GR (−)st PTC did not abolish the expression of *pol* mRNA, which would be expected if the mutations disrupt an ESE. Further, although we found that addition of a transportable ESE increased the level of *pol* mRNA in the GR (−)st mutant (Fig. 7 and Table 4), the ESE also greatly increased the level of *pol* mRNA in wt virus (Fig. 7 and Table 4). Thus, we do not believe that the PTC disrupts a natural ESE.

TABLE 4. Quantitation of mRNA levels in the presence of ESE by real-time PCR

Construct	Level[a]		Ratio of spliced to unspliced
	Spliced	Unspliced	
Mock	ND	ND	
Wt	1.00	1.00	1.0
GR (−)st	0.03 ± 0.01	0.95 ± 0.20	0.03
Wt/ESE	8.43 ± 1.50	0.60 ± 0.22	14.1
(−)st/ESE	1.52 ± 0.30	0.31 ± 0.14	4.9

[a] The levels of mutant mRNA are normalized to that of the wt. The numbers are averages ± standard deviations from three independent assays. ND, not detected.

Another possible mechanism for the observed PTC effects is that disruption of reading frames by PTC causes inefficient RNA splicing. Nonsense codons acquired during normal lymphocyte development have been shown to accumulate incompletely spliced or unspliced pre-mRNA for several genes in the nucleus (immunoglobulin κ and μ and T-cell receptor β) (16, 23). It has also been shown that PTC, but not missense mutations, inserted into the genome of the parvovirus minute virus of mice (MVM) inhibited nuclear excision of an intron upstream of a PTC-containing exon, leading to the retention of the upstream intron (9). This is somewhat similar to the FV case, since our PTC mutations are in the exon downstream of the *pol* 3′ss and MVM also has a poor polypyrimidine tract in the 3′ss. However, there is a difference between MVM and FV. In MVM, the mRNAs involved are singly and doubly spliced rather than unspliced and singly spliced as in FV. Gersappe and Pintel concluded that the PTC can interfere with the function of ESE to improve the 3′ss in an open reading frame-dependent manner, suggesting that there is translational scanning in the nucleus that affects RNA splicing. In an MVM PTC mutant, reduction of doubly spliced mRNA was independent of mRNA stability in the nucleus or cytoplasm, ruling out an NMD pathway, reminiscent of the failure of Upf1 to affect the FV PTC mutant. If this mechanism is operant in FV, levels of unspliced *gag* mRNA should be increased in the PTC mutant. However, the increase caused by the PTC mutation would be insignificant compared to the level of unspliced *gag* mRNA, which is normally much more abundant than spliced *pol* mRNA. If inhibition of splicing is dependent on the reading

frame to recognize nonsense codons, a defect caused by the PTC mutation should be suppressed by frameshift mutations to correct the *pol* open reading frame. This has not been tested.

We found that a series of missense mutations at the same site as the PTC in the GR (−)st mutant led to wt levels of spliced *pol* mRNA, suggesting that the PTC itself plays important roles in downregulating levels of *pol* mRNA. Transcripts containing a PTC are rapidly degraded by NMD. As a general rule, a PTC located more than ~50 nt upstream of the last exon-exon junction is recognized and elicits degradation (21, 36). However, our PTC mutations do not fit the expectations for classical NMD, since they reside upstream of the Pol translation initiation site. However, if a PTC within pre-mRNA is recognized in the nucleus before or concomitant with splicing, nucleus-associated NMD can explain decreased levels of *pol* mRNA in the mutants. There are a number of observations supporting open reading frame scanning in the nucleus (12, 38). If NMD is operant, the DN mutant of Upf, but not wt Upf, should increase the level of spliced *pol* mRNA in the PTC mutants. Conversely, the level of unspliced *gag* mRNA should not be changed by Upf, since unspliced RNAs are generally not targets of NMD (20), although there are some examples of unspliced RNA being subject to NMD (14). We found that there was no increase in the levels of GR (−)st *pol* mRNA and protein in the presence of DN Upf1. However, surprisingly, DN Upf was shown to increase the expression of Gag approximately sixfold in the PTC mutant. We examined mRNA turnover by RT-PCR assays after treatment with actinomycin D for 7.5 h. Unspliced *gag* mRNA was found to be stable in both the wt and the GR (−)st mutant during this time, similar to the control *gapdh* mRNA (data not shown), which has a half-life of about 8 h (5). Therefore, we do not have a satisfactory explanation for how GR (−)st Gag expression is increased in the presence of DN Upf.

In summary, the results presented here show that insertion of PTC mutations at the C terminus of FV Gag greatly decreases splicing efficiency. The PTC itself appears to be important for the downregulation, as it does not occur with several missense mutations inserted at the same place. Our data are not consistent with RNA degradation through cytoplasmic NMD mechanisms, although we cannot rule out NMD occurring in the nucleus. However, in our case, the PTC-mediated downregulation does not seem to involve the NMD protein Upf1. During the course of our experiments, we found that the level of *pol* mRNA present in wt viral infection can be greatly increased by insertion of an ESE from another retrovirus. This suggests that FV *pol* splice sites are deliberately poor so that the levels of *pol* mRNA and protein remain low. This notion is consistent with the very low levels of packaged Pol required for viral infection.

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REFERENCES

1. **Amrani, N., M. S. Sachs, and A. Jacobson.** 2006. Early nonsense: mRNA decay solves a translational problem. Nat. Rev. Mol. Cell Biol. **7:**415–425.
2. **Baldwin, D. N., and M. L. Linial.** 1998. The roles of Pol and Env in the assembly pathway of human foamy virus. J. Virol. **72:**3658–3665.
3. **Boyer, P. L., C. R. Stenbak, P. K. Clark, M. L. Linial, and S. H. Hughes.** 2004. Characterization of the polymerase and RNase H activities of human foamy virus reverse transcriptase. J. Virol. **78:**6112–6121.
4. **Cartegni, L., S. L. Chew, and A. R. Krainer.** 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet. **3:**285–298.
5. **Dani, C., M. Piechazyk, Y. Audigier, S. El Sabouty, G. Cathala, L. Marty, P. Fort, J. M. Blanchard, and P. Jeanteur.** 1984. Characterization of the transcription products of gluceraldehyde 3-phosphate-dehydrogenase gene in HeLa cells. Eur. J. Biochem. **145:**299–304.
6. **Darlix, J.-L., M. L.-T., H. de Rocquigny, and B. P. Roques.** 1995. First glimpses at structure-function relationship of the nucleocapsid protein of retroviruses. J. Mol. Biol. **254:**523–537.
7. **Durocher, Y., S. Perret, and A. Kamen.** 2002. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. **30:**e9.
8. **Ernst, R. K., M. Bray, D. Rekosh, and M. L. Hammarskjold.** 1997. A structured retroviral RNA element that mediates nucleocytoplasmic export of intron-containing RNA. Mol. Cell. Biol. **17:**135–144.
9. **Gersappe, A., and D. J. Pintel.** 1999. A premature termination codon interferes with the nuclear function of an exon splicing enhancer in an open reading frame-dependent manner. Mol. Cell. Biol. **19:**1640–1650.
10. **Goff, S. P.** 2007. Retroviridae: the retroviruses and their replication, p. 1999–2069. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott Williams and Wilkins, Philadelphia, PA.
11. **Iborra, F. J., A. E. Escargueil, K. Y. Kwek, A. Akoulitchev, and P. R. Cook.** 2004. Molecular cross-talk between the transcription, translation, and nonsense-mediated decay machineries. J. Cell Sci. **117:**899–906.
12. **Iborra, F. J., D. A. Jackson, and P. R. Cook.** 2001. Coupled transcription and translation within nuclei of mammalian cells. Science **293:**1139–1142.
13. **Katz, R. A., and A. M. Skalka.** 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. Mol. Cell. Biol. **10:**696–704.
14. **LeBlanc, J. J., and K. L. Beemon.** 2004. Unspliced Rous sarcoma virus genomic RNAs are translated and subjected to nonsense-mediated mRNA decay before packaging. J. Virol. **78:**5139–5146.
15. **Lejeune, F., and L. E. Maquat.** 2005. Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Curr. Opin. Cell Biol. **17:**309–315.
16. **Lozano, F., B. Maertzdorf, R. Pannell, and C. Milstein.** 1994. Low cytoplasmic mRNA levels of immunoglobulin kappa light chain genes containing nonsense codons correlate with inefficient splicing. EMBO J. **13:**4617–4622.
17. **Lykke-Andersen, J., M. D. Shu, and J. A. Steitz.** 2000. Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. Cell **103:**1121–1131.
18. **Malim, M. H., J. Hauber, S. Y. Le, J. V. Maizel, and B. R. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature **338:**254–257.
19. **Maquat, L. E.** 2002. NASty effects on fibrillin pre-mRNA splicing: another case of ESE does it, but proposals for translation-dependent splice site choice live on. Genes Dev. **16:**1743–1753.
20. **Maquat, L. E., and X. Li.** 2001. Mammalian heat shock p70 and histone H4 transcripts, which derive from naturally intronless genes, are immune to nonsense-mediated decay. RNA **7:**445–456.
21. **Maquat, L. E.** 2004. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. Nat. Rev. Mol. Cell Biol. **5:**89–99.
22. **Mendell, J. T., C. M. J. ap Rhys, and H. C. Dietz.** 2002. Separable roles for rent1/hUpf1 in altered splicing and decay of nonsense transcripts. Science **298:**419–422.
23. **Muhlemann, O., C. S. Mock-Casagrande, J. Wang, S. Li, N. Custodio, M. Carmo-Fonseca, M. F. Wilkinson, and M. J. Moore.** 2001. Precursor RNAs harboring nonsense codons accumulate near the site of transcription. Mol. Cell **8:**33–43.
24. **Najera, I., M. Krieg, and J. Karn.** 1999. Synergistic stimulation of HIV-1 rev-dependent export of unspliced mRNA to the cytoplasm by hnRNP A1. J. Mol. Biol. **285:**1951–1964.
25. **O'Reilly, M. M., M. T. McNally, and K. L. Beemon.** 1995. Two strong 5′ splice sites and competing, suboptimal 3′ splice sites involved in alternative splicing of human immunodeficiency virus type 1 RNA. Virology **213:**373–385.
26. **Paca, R. E., R. A. Ogert, C. S. Hibbert, E. Izaurralde, and K. L. Beemon.** 2000. Rous sarcoma virus DR posttranscriptional elements use a novel RNA export pathway. J. Virol. **74:**9507–9514.
27. **Peters, K., T. Wiktorowicz, M. Heinkelein, and A. Rethwilm.** 2005. RNA and protein requirements for incorporation of the Pol protein into foamy virus particles. J. Virol. **79:**7005–7013.
28. **Rosin-Arbesfeld, R., A. Yaniv, and A. Gazit.** 2000. Suboptimal splice sites of equine infectious anaemia virus control Rev responsiveness. J. Gen. Virol. **81:**1265–1272.
29. **Schliephake, A. W., and A. Rethwilm.** 1994. Nuclear localization of foamy virus Gag precursor protein. J. Virol. **68:**4946–4954.

30. **Stenbak, C. R., and M. L. Linial.** 2004. Role of the C terminus of foamy virus Gag in RNA packaging and Pol expression. J. Virol. **78:**9423–9430.
31. **Stoltzfus, C. M.** 1988. Synthesis and processing of avian sarcoma retroviruses RNA. Adv. Virus Res. **35:**1–38.
32. **Stoltzfus, C. M., and J. M. Madsen.** 2006. Role of splicing elements and cellular RNA binding proteins in regulation of HIV-1 alternative RNA splicing. Curr. HIV Res. **4:**43–55.
33. **Sun, H., and L. A. Chasin.** 2000. Multiple splicing defects in an intronic false exon. Mol. Cell. Biol. **20:**6414–6425.
34. **Sun, X., H. A. Perlick, H. C. Dietz, and L. E. Maquat.** 1998. A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. Proc. Natl. Acad. Sci. USA **95:**10009–10014.
35. **Vogt, V. M., and M. N. Simon.** 1999. Mass determination of Rous sarcoma virus virions by scanning transmission electron microscopy. J. Virol. **73:**7050–7055.
36. **Wagner, E., and J. Lykke-Andersen.** 2002. mRNA surveillance: the perfect persist. J. Cell Sci. **115:**3033–3038.
37. **Webb, C. J., C. M. Romfo, W. J. van Heeckeren, and J. A. Wise.** 2005. Exonic splicing enhancers in fission yeast: functional conservation demonstrates an early evolutionary origin. Genes Dev. **19:**242–254.
38. **Wilkinson, M. F., and A. B. Shyu.** 2002. RNA surveillance by nuclear scanning? Nat. Cell Biol. **4:**e144–e147.
39. **Will, C. L., and R. Luhrman.** 2006. Spliceosome structure and function, p. 369–400. *In* R. F. Gesteland, T. R. Cech, and J. F. Atkins (ed.), The RNA world. Cold Spring Harbor Laboratory Press, Cold spring Harbor, NY.
40. **Yu, S. F., D. N. Baldwin, S. R. Gwynn, S. Yendapalli, and M. L. Linial.** 1996. Human foamy virus replication—a pathway distinct from that of retroviruses and hepadnaviruses. Science **271:**1579–1582.
41. **Yu, S. F., K. Edelmann, R. K. Strong, A. Moebes, A. Rethwilm, and M. L. Linial.** 1996. The carboxyl terminus of the human foamy virus gag protein contains separable nucleic acid binding and nuclear transport domains. J. Virol. **70:**8255–8262.
42. **Zhang, L., and C. M. Stoltzfus.** 1995. A suboptimal src 3′ splice site is necessary for efficient replication of Rous sarcoma virus. Virology **206:**1099–1107.
43. **Zheng, Z. M.** 2004. Regulation of alternative RNA splicing by exon definition and exon sequences in viral and mammalian gene expression. J. Biomed. Sci. **11:**278–294.