

A 3' UTR sequence stabilizes termination codons in the unspliced RNA of Rous sarcoma virus

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ABSTRACT

Eukaryotic cells target mRNAs to the nonsense-mediated mRNA decay (NMD) pathway when translation terminates within the coding region. In mammalian cells, this is presumably due to a downstream signal deposited during pre-mRNA splicing. In contrast, unspliced retroviral RNA undergoes NMD in chicken cells when premature termination codons (PTCs) are present in the *gag* gene. Surprisingly, deletion of a 401-nt 3' UTR sequence immediately downstream of the normal *gag* termination codon caused this termination event to be recognized as premature. We termed this 3' UTR region the Rous sarcoma virus (RSV) stability element (RSE). The RSE also stabilized the viral RNA when placed immediately downstream of a PTC in the *gag* gene. Deletion analysis of the RSE indicated a smaller functional element. We conclude that this 3' UTR sequence stabilizes termination codons in the RSV RNA, and termination codons not associated with such an RSE sequence undergo NMD.

Keywords: NMD; RNA surveillance; translation termination; Rous sarcoma virus

INTRODUCTION

Eukaryotic cells employ a quality control mechanism to eliminate mRNAs bearing premature termination codons (PTC) (Hillgren and Parker 1999; Gonzalez et al. 2001; Maquat 2004; Wilkinson 2005). This mechanism of post-transcriptional control may have evolved not only to eliminate aberrant mRNAs, but also to regulate the level of some mRNAs (Lewis et al. 2003; Neu-Yilik et al. 2004). In mammalian cells, the nonsense-mediated mRNA decay (NMD) machinery recognizes a termination codon as premature if it is more than 50 nucleotides (nt) upstream of the last exon-exon boundary (Zhang et al. 1998a; Sun et al. 2000). During splicing, an exon junction complex (EJC) is deposited on the RNA 20–24 nt upstream of splice junctions (Le Hir et al. 2000a). This complex includes RNA splicing, export, cytoplasmic localization, and NMD factors (Le Hir et al. 2000a,b, 2001; Lykke-Andersen et al. 2000; Kim et al. 2001a,b; Chan et al. 2004). When a termination codon is encountered, the terminating ribosome and its associated factors recognize a downstream EJC through interactions with the Upf proteins, and the RNA is targeted for rapid destruction by the NMD machinery (Lykke-Andersen et al.

2000; He and Jacobson 2001; Lykke-Andersen 2002). In turn, naturally intronless genes are immune to NMD in mammalian cells (Maquat and Li 2001; Brocke et al. 2002).

Conversely, in yeast very few genes contain introns (Lopez and Seraphin 2000), and these unspliced mRNAs readily undergo NMD in a Upf-dependent fashion (Hillgren and Parker 1999; Gonzalez et al. 2001). This decay appears to be directed either by a coding region destabilizing element or distance from the poly(A) tail (for review, see Hillgren and Parker 1999). Recent experiments in which the poly(A) binding protein was tethered downstream of a PTC resulted in stabilization of the NMD substrate (Amrani et al. 2004).

In contrast to the paradigm in mammalian cells that NMD is linked to splicing, Rous sarcoma virus (RSV) RNA undergoes NMD in chicken cells in the absence of splicing (Barker and Beemon 1991, 1994; LeBlanc and Beemon 2004). The 9.3-kb unspliced viral RNA is exported to the cytoplasm where it serves as both the genome of progeny virions and the mRNA template for the Gag and the Gag-Pol polyproteins (Swanstrom and Wills 1997). In most cases, only the first gene, *gag*, is translated, resulting in a 7-kb 3' untranslated region (UTR) (Fig. 1A). Approximately 5% of the time, a -1 programmed ribosomal frameshift moves the ribosome into the reading frame of the second viral gene, *pol*, resulting in synthesis of a Gag-Pol fusion protein (Swanstrom and Wills 1997). Barker and Beemon (1991, 1994) reported that premature termination codons (PTCs) in the *gag* gene lead to a substantial decrease in unspliced cytoplasmic viral RNA. This decay diminishes

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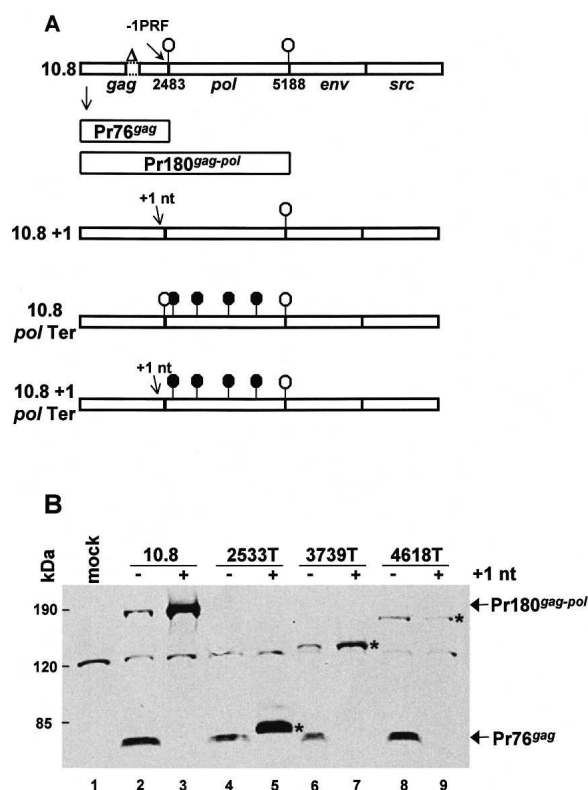


FIGURE 1. A 1-nucleotide (nt) insertion before the *gag* termination codon forces readthrough to the *pol* gene. (A) The 10.8 plasmid contains a full-length copy of the Prague C strain of RSV with a deletion in the nucleocapsid region of the *gag* gene (Δ). All viral clones in this study originated from the 10.8 background. The *gag* gene is translated to generate a 76-kDa precursor protein (Pr76^{gag}). A -1 programmed ribosomal frameshift (-1 PRF) leads to the synthesis of a 180 kDa Gag-Pol fusion polypeptide (Pr180^{gag-pol}). 10.8 +1 has a 1-nt insertion at position 2482, putting *gag* and *pol* in the same reading frame. 10.8 *pol* Ter constructs have a PTC in the *pol* gene, and 10.8 +1 *pol* Ter constructs have the same PTC in the 10.8 +1 background. The white stop signs designate the *gag* and *pol* natural termination codons. The black stop signs represent PTCs in *pol*. (B) Western blot of CEFs transiently transfected with 10.8 *pol* Ter and 10.8 +1 *pol* Ter constructs. The position of the termination codon in *pol* is given in nt (i.e., 2533T). The intermediate sized bands (denoted by *) in lanes 5, 7, and 9 are truncated Gag-Pol polypeptides due to the inserted termination codon. Viral proteins were detected using an antibody against AMV p19 (Matrix).

gradually when the PTC is in the final 150 nt of the *gag* gene, approaching the natural termination codon (Barker and Beemon 1994). Additionally, LeBlanc and Beemon (2004) showed that this decay depends on translation and the critical NMD factor Upf1, implicating the NMD machinery. Since this regulation occurs on an unspliced RNA, it contrasts with previous reports in higher eukaryotes that splicing is necessary to distinguish a PTC from a normal termination codon (Maquat 2004).

In this report, we have characterized a region of the RSV genome found after the *gag* termination codon, which stabilizes the full-length unspliced RNA in chicken embryo

fibroblasts (CEFs). We have termed this region the RSV stability element (RSE). We uncovered this element when examining termination codons in the *pol* gene. We observed that PTCs 500 nt or more into the *pol* gene only caused a lower steady state level of RNA when *pol* was placed in the same reading frame as the *gag* gene. Conversely, a PTC at the beginning of *pol* (within the RSE) did not result in a lower accumulation of RNA. Interestingly, a deletion downstream of the normal *gag* termination codon (the RSE region) led to a lower accumulation of unspliced RNA. This instability required translation and the protein Upf1, suggesting that deletion of the RSE leads to recognition of the *gag* natural termination codon as premature and induces NMD. In turn, insertion of the RSE immediately downstream of a PTC in *gag* stabilized the RNA. We conclude that there is a stability element found after the *gag* termination codon in RSV, and this region can stabilize an immediately preceding termination event.

RESULTS

Translation of PTCs in *pol* destabilize the unspliced RNA

Previous results have shown that termination codons placed throughout the *gag* coding region destabilize the unspliced RSV transcript (Barker and Beemon 1991) by the NMD pathway (LeBlanc and Beemon 2004). Knowing that the *pol* gene is translated much less frequently than *gag* (Swanstrom and Wills 1997), we asked whether termination codons in *pol* would cause degradation of the unspliced RNA.

Toward this end, we used the 10.8 viral construct, a clone of the Prague C strain of RSV, harboring a deletion in the nucleocapsid region of the *gag* gene (Meric et al. 1988). This deletion prevents packaging of the viral unspliced RNA. Barker and Beemon (1994) reported that a termination codon placed early in the *pol* gene (nt 2598) had no substantial effect on unspliced RNA stability. However, this could be due to the infrequent -1 frameshift, which leads to infrequent translation of the *pol* gene. To address the question of whether engineered termination codons throughout *pol* cause NMD, we constructed the plasmid 10.8 +1; 10.8 +1 contains a single adenine insertion at nt 2482, immediately upstream of the *gag* natural termination codon. This insertion places *gag* and *pol* in the same reading frame, ensuring efficient translation of the Gag-Pol fusion polypeptide (Fig. 1A). We then made a series of constructs, each possessing a termination codon within the *pol* gene, in either the 10.8 or the 10.8 +1 background (Fig. 1A). CEF cells were transiently transfected with the above constructs, and 2-d post-transfection protein and RNA were harvested.

A Western blot using anti-p19 (Matrix) antibody to detect RSV Gag proteins showed that the majority of protein translated from the 10.8 transcript was the Gag protein, Pr76^{gag}, with a smaller amount of Pr180^{gag-pol} readthrough product (Fig. 1B, lane 2). In contrast, translation of 10.8 +1

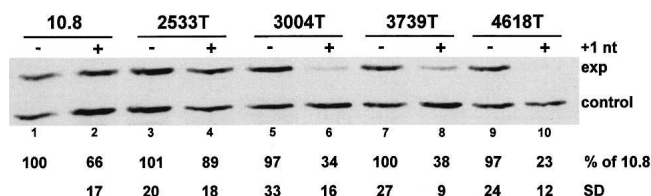


FIGURE 2. PTCs in *pol* have different effects on RNA level. RNase protection assay of RNA harvested from transiently transfected CEFs analyzed with a probe that detects the *gag* gene of RSV. The top band is the protected fragment corresponding to the unspliced viral RNA (exp). The bottom band is a stable viral loading control that gives a different size protected fragment using the same probe (LeBlanc and Beemon 2004). Quantification of bands was carried out by Phosphor-Imager analysis. A ratio was determined between the experimental and the control band in each lane, and normalized to 10.8, which was set at 100%. Standard deviations (SD) are noted. Values represent the average of at least four experiments.

RNA led to production of only the Gag-Pol polyprotein, Pr180^{*gag-pol*} (Fig. 1B, lane 3). As predicted, truncated readthrough protein products (denoted by an *) of approximately 78, 123, and 154 kDa were observed when a PTC was placed within the *pol* gene at nt 2533, 3739, and 4618, respectively (Fig. 1B, lanes 4–9).

We next assayed the relative levels of unspliced RNA transcribed from each of the constructs bearing a PTC in *pol* by an RNase protection assay (Fig. 2). We co-transfected a stable viral loading control, and then probed the sample using a viral *gag* probe that can distinguish between the two viral RNAs (LeBlanc and Beemon 2004). We found that the 10.8 +1 transcript, which terminates translation at the end of *pol*, only accumulated 66% as much RNA as the 10.8 wild-type construct (Fig. 2, lanes 1,2). Interestingly, RNA bearing a termination codon at nt 2533, ~ 50 nt downstream of the *gag* natural termination codon, accumulated at wild-type levels in both the 10.8 and the 10.8 +1 constructs (Fig. 2, lanes 3,4). In contrast, termination codons placed further downstream, at nt 3004, 3739, and 4618 in the *pol* gene, led to RNA accumulation at the wild-type level in the 10.8 background (Fig. 2, lanes 5,7,9). However, only 23%–38% as much RNA as wild type accumulated in the 10.8 +1 background (Fig. 2, lanes 6,8,10). This experiment suggests that PTCs in *pol* that are infrequently translated do not induce NMD. However, when readthrough from *gag* is efficient, these same PTCs now cause a lower level of the unspliced RNA. The observation that RNA bearing a PTC just downstream of the *gag* natural termination codon still accumulated to high levels in the 10.8 +1 context (Fig. 2, lane 4) led us to examine this region more closely.

A deletion of 400 nt immediately after the *gag* termination codon elicits NMD

Previous data indicate that various deletions downstream of the *gag-pol* junction lead to lower levels of unspliced viral RNA (Barker and Beemon 1994). The data presented above

led us to propose that this region may be directing proper *gag* translation termination and stabilization of the RNA. RNA with a termination codon ~ 50 nt downstream of the *gag* natural termination codon accumulated at wild type levels, even when translated 100% of the time (Fig. 2, lane 4). When the termination codon was moved 500 nt or more downstream in the same background, there was much less steady state RNA (Fig. 2, lanes 6,8,10).

We tested this hypothesis by deleting the putative RSV stability element (RSE) (nt 2486–2886) immediately after the natural termination codon in the 10.8 context, to generate the construct Δ RSE (Fig. 3A). After transient transfection in CEFs, the amount of viral RNA generated was assayed by RNase protection and found to be about fourfold less abundant than wild-type 10.8 RNA (Fig. 3B). This reduced level of viral RNA was similar to that seen previously with viral

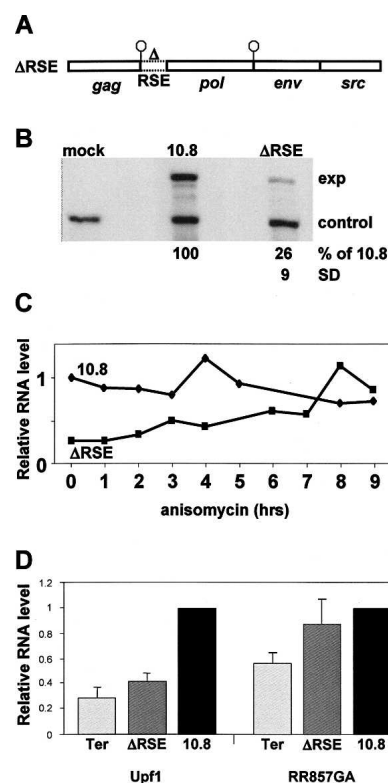


FIGURE 3. Deletion of the RSE causes NMD of the unspliced RNA. (A) Construct Δ RSE contains a deletion of the first 401 nt of the *gag* 3' UTR, termed the "RSE" (nt 2486–2886) in the 10.8 viral clone. (B) A representative RNase protection assay of unspliced RNA derived from this deletion construct. The same loading control and probe as in Figure 2 were used, and the values are the average of at least five experiments, normalized and quantitated as in Figure 2. (C) CEFs were transiently transfected with the 10.8 and Δ RSE constructs, and RNA was harvested from 1 to 9 h after addition of 25 μ g/mL of the translation inhibitor anisomycin (Calbiochem). RNA levels were assayed as in Figure 2 and normalized to the average values from B. (D) RNA from both the Δ RSE construct and a construct bearing a termination codon at nt 1924 in *gag* (Ter) were stabilized compared with wild-type constructs in the presence of a dominant negative Upf1 mutant (RR857GA). The average of three experiments is shown, normalized and quantitated as in Figure 2.

RNAs containing a termination codon in *gag* (LeBlanc and Beemon 2004). The previous observation that RNAs with PTCs in the last 150 nt of the *gag* gene, near the RSE, are increasingly more abundant than RNAs with PTCs in the rest of the *gag* gene should be noted (Barker and Beemon 1994).

To determine if the decrease in unspliced RNA with the Δ RSE construct was due to the NMD pathway, we tested its dependence on translation and the protein Upf1, both previously shown to be required for NMD (Leeds et al. 1991; Belgrader et al. 1993; Carter et al. 1995). Translation was inhibited by addition of anisomycin. Over a 9-h period, we saw up to a fourfold increase in the RNA level of the Δ RSE construct (Fig. 3C). The level of 10.8 RNA was fairly uniform over this same time period, decreasing to $\sim 75\%$ of its original level after 9 h (Fig. 3C).

We also blocked NMD by co-transfection of a dominant negative mutant of hUpf1, RR857GA (Mendell et al. 2002). When the Upf1 mutant was co-transfected with Δ RSE, we saw a twofold increase in the unspliced RNA level, normalized to 10.8 RNA in each experiment (Fig. 3D). In the same experiment, we saw a similar increase in the amount of a viral RNA bearing a PTC at nt 1924 (Ter) (Barker and Beemon 1994). Co-transfection with either wild-type Upf1 or RR857GA caused no significant change in the level of the control 10.8 RNA to which the mutants are standardized. We conclude that deletion of this 3' UTR region, the RSE, causes the natural termination codon to behave like a PTC.

The RSE can stabilize a PTC in *gag*

Since the wild-type RNA has the RSE downstream of the natural termination codon, we examined the effect of placing the RSE downstream of a PTC in the *gag* gene. To test this, we used the Ter construct with a PTC at nt 1924 in *gag* (Fig. 4A; Barker and Beemon 1994). The unspliced RNA from this PTC-containing RNA accumulated at 19% of wild-type RNA levels (Fig. 4A, lane 1). We then made a deletion in the *gag* gene, immediately downstream of the PTC insertion, extending to the natural termination codon. This deletion effectively positioned the RSE immediately downstream of the PTC. RNA from this construct accumulated to $\sim 62\%$ of the wild-type level (Fig. 4A, lane 4). Subsequent deletion of the RSE from this construct resulted in decreased RNA levels (27% of wild type) (Fig. 4A, lane 5), further implicating the RSE in stabilization.

Another interpretation of these results is that we were deleting some destabilizing element in the coding region. To address this issue, we first moved sense (forward) or anti-sense (reverse) forms of the 401-nt RSE into the *gag* coding region, immediately after a termination codon at nt 1250 (Fig. 4B, Ter for/rev Δ RSE). We observed approximately wild-type levels of unspliced RNA with the forward, but not the reverse insertion, suggesting that this element can stabilize the RNA with a termination codon at nt 1250 (Fig. 4B lanes 6,7). To ensure that the RNA containing only the reverse copy of the RSE was not less abundant simply because it now has no sense copy of the RSE, we left the natural copy of the RSE in place (~ 1200 nt downstream of the PTC) and inserted another copy of the RSE after the PTC at nt 1250 (Ter for/rev). We again observed sense orientation-specific accumulation of the unspliced RNA (Fig. 4B, lanes 3,4). Since the RSE had this effect when it was found immediately downstream of a PTC, but not 1200 nt away, we conclude that the RSE acts on an immediately preceding translation termination codon.

To ask whether the observed decrease in RNA level was due to RNA instability, we used the drug actinomycin D to inhibit bulk cellular transcription in CEFs transiently transfected with 10.8, Ter (1924) (Fig. 4A), Ter for Δ RSE, and

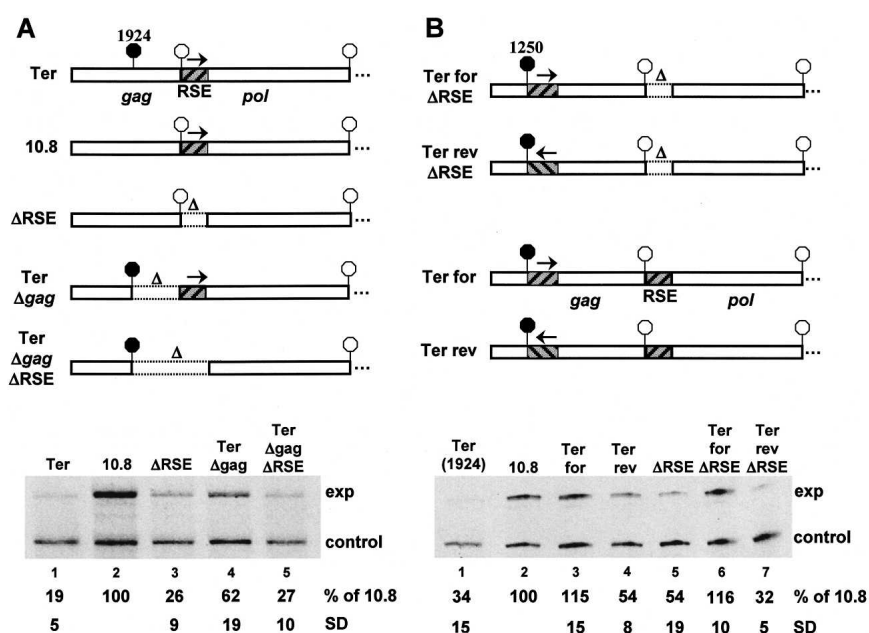


FIGURE 4. Movement of the RSE next to a premature termination codon increases RNA levels. (A) Constructs were created to examine the effect of the RSE on PTC-containing RNAs. A partial deletion of the *gag* coding region (Ter Δ gag) positioned the RSE immediately 3' of a PTC at nt 1924. In this diagram the RSE is indicated by a hatched box. A representative RNase protection assay is shown. Relative RNA levels were normalized and quantitated as in Figure 2, and the average of five experiments is shown. (B) Forward and reverse RSE fragments (forward and reverse hatched boxes) were inserted downstream of a PTC at nt 1250. Constructs were tested both with and without the normal RSE downstream of the *gag* termination codon. A representative RNase protection assay is shown, with quantification from at least four experiments, normalized and quantitated as in Figure 2.

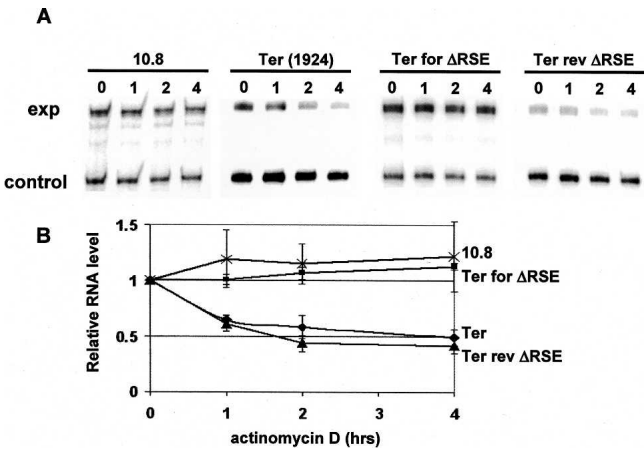


FIGURE 5. PTC-bearing RNA is stabilized by the RSE in the sense orientation. CEFs were transiently transfected with 10.8, Ter (Fig. 4A), Ter for ΔRSE and Ter rev ΔRSE (Fig. 4B) constructs. Two days post-transfection the cells were treated with 2 μg/mL actinomycin D, and RNA was harvested at 0-, 1-, 2-, and 4-h time points. Relative RNA levels were determined by RNase protection assay. A representative experiment is shown (A), and the relative stabilities from five experiments, normalized and quantified as in Figure 2, are plotted (B).

Ter rev ΔRSE (Fig. 4B). We then harvested RNA at 0-, 1-, 2-, and 4-h time points after addition of the drug. Representative gels are shown in Figure 5A. RNA levels at each time point were quantitated and plotted in Figure 5B, relative to the RNA level of each construct at zero time. We found that RNA bearing a PTC in *gag* followed by the RSE in the sense orientation (Ter for ΔRSE) was as stable as wild-type RNA. However, RNAs bearing the PTC alone (Ter) or with a PTC followed by the RSE in the reverse orientation (Ter rev ΔRSE) were both much less stable, with the RNA level dropping off rapidly during the first hour, and then stabilizing between 2 and 4 h. From this result we can conclude that the RSE is affecting RNA abundance at the level of RNA stability.

The RSE is a complex element

After observing that insertion of the 401-nt RSE after a PTC was sufficient to stabilize the RNA (Fig. 5), we asked whether the entire RSE region was necessary for this. To address this, we made truncations from the 3' end (construct A), the 5' end (construct C), and both ends of the RSE (constructs B and D) (Fig. 6A). These different truncated fragments were then inserted immediately downstream of a PTC at nt 1250 in the *gag* gene. We found that a sense insertion of the last 280 nt (C fragment) was sufficient to stabilize the RNA to 68% of the level of the full-length RSE insertion (Fig. 6B, lane 9), while the anti-sense insertion at the same site led to only 27% of full length (Fig. 6B, lane 10). An RNA bearing an insertion of the first 250 nt of the RSE (A fragment) was almost as stable as the full-length insertion (87%), but not significantly more stable

than the same insertion in the reverse orientation (55%) (Fig. 6B, lanes 5,6). It is possible that the reverse orientation of the A fragment also has some stabilizing ability. Interestingly, fragment B, the 140-nt common region shared by fragments A and C, was unable to stabilize the RNA to any extent (Fig. 6B, lane 7). A similar result was seen with fragment D (Fig. 6B, lane 11). The experiments with the truncated RSE fragments suggest that the element we are striving to characterize is complex and may have necessary, possibly redundant, features at both the 5' and 3' ends.

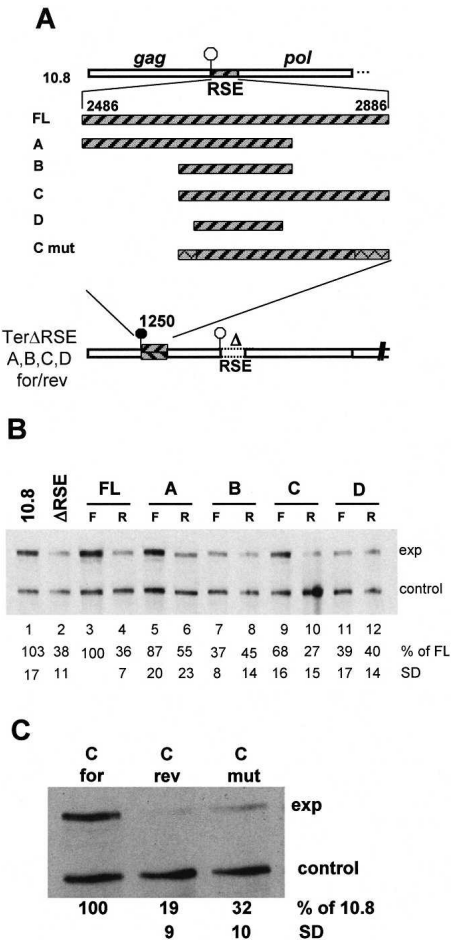


FIGURE 6. Two sub-fragments of the RSE can stabilize RNA. (A) To determine the minimal region that can stabilize a termination codon, various deletions were made at the ends of the RSE. Additionally, three groups of point mutations (14 nt mutated) were made in putative single stranded regions near the ends of the C fragment (C Mut). One group of five point mutations is located at the 5' end. One group of five and one group of four nt were mutated at the 3' end. These sub-fragments were inserted after a PTC at nt 1250. (FL) Full length. (B) A representative RNase protection assay is shown with average values from four experiments, and normalized to full-length insertion. (F) Forward; (R) reverse orientations of the RSE fragment. (C) The mutated fragment C of the RSE was inserted immediately after a PTC at nt 1250 in the ΔRSE construct. RNase protection assays were used to determine relative RNA levels. These were compared to those of constructs bearing the wild-type C fragment in the forward and reverse orientations. Average values from six experiments are indicated.

Although clearly not sufficient, the common region is thought to be necessary based on previous deletions in this area (Barker and Beemon 1994).

Since fragment C from Figure 6A induced the greatest stabilization relative to its insertion in the reverse orientation, we decided to use this fragment in future studies. A group of five point mutations was generated near the 5' end, and nine point mutations near the 3' end in regions that were predicted to be single stranded by MFOLD (Zuker 2003; data not shown). When this mutated fragment (C mut) was inserted after a termination codon at nt 1250 in the *gag* coding region, the resulting unspliced RNA was ~ 32% as abundant as the "C for" RNA (Fig. 6C). To further dissect the region containing these point mutations, we made constructs with mutations at only the 5' or the 3' end. Although both single end mutations had an intermediate effect, the 3' end appeared to be more crucial for accumulation of RNA (data not shown). This is consistent with the observation that the region shared by A and C is insufficient to promote a high level of RNA accumulation. We conclude that the RSE may have a necessary but not sufficient middle region, and a necessary but redundant feature at each end.

DISCUSSION

In this report we examined the mechanism by which termination codons are distinguished as natural or premature in RSV unspliced RNA expressed in chicken cells. Surprisingly, we observed that a 400-nt deletion downstream of the *gag* termination codon resulted in its recognition as a PTC, leading to NMD. When this 3' UTR region (which we call the RSE) was inserted downstream of a PTC in the *gag* gene, the RNA was stabilized. The anti-sense orientation, or a mutated RSE sequence, did not stabilize the RNA. These findings differ from previous observations in higher eukaryotic cells showing that unspliced RNA does not undergo NMD and that a downstream EJC triggers NMD (Le Hir et al. 2000a; Lykke-Andersen et al. 2000; Maquat and Li 2001; Brocke et al. 2002). We hypothesize that, in chicken cells, the RSE promotes proper translation termination of the viral RNA when next to a termination codon, and in its absence, NMD occurs. The RSE may recruit stabilization factors such as the eukaryotic release factors (eRFs), the Poly(A) binding protein, or cap binding proteins, in order to overcome such a long 3' UTR. In yeast, tethering of either the Poly(A) binding protein or eRF3 downstream of a coding region PTC stabilized the RNA (Amrani et al. 2004).

Different organisms have developed alternative mechanisms of NMD

NMD was first observed in yeast (Losson and Lacroute 1979), where it requires both translation and the Upf proteins, just as it does in eukaryotic cells. In yeast, only about 4% of genes are spliced (Lopez and Seraphin 2000), and the identification of

PTCs does not require splicing (Gonzalez et al. 2001). Recent work in yeast suggests that this organism differentiates premature from normal termination events by a faux UTR model (Amrani et al. 2004). This model posits that the natural termination codon is identified by its proximity to an appropriate 3' UTR and termination stabilizing factors. Conversely, the region downstream of a PTC within the coding region is a "faux" (or false) UTR, which is unfit to direct proper termination (Hilleren and Parker 1999). Jacobson and coworkers (Amrani et al. 2004) have shown that translation termination at a PTC is aberrant, and the ribosome does not properly release from the RNA. A natural 3' UTR can promote proper termination, as can tethered poly(A) binding protein. This challenges a previous model in yeast that suggests the existence of a *cis*-acting exonic downstream sequence element (DSE) (Ruiz-Echevarria et al. 1998; Gonzalez et al. 2000).

Some notable exceptions to the generally accepted NMD model exist (Enssle et al. 1993; Barker and Beemon 1994; Carter et al. 1996; Zhang et al. 1998b; Gudikote and Wilkinson 2002; Wang et al. 2002; LeBlanc and Beemon 2004). In mammalian cells, a mark deposited by a prior splicing event downstream of a termination codon appears to signal premature termination. Thus, an RNA without an intron downstream of a PTC should be immune to NMD (Maquat and Li 2001; Brocke et al. 2002). However, a loosely characterized "failsafe signal" in globin mRNA acts to direct NMD even in the absence of a downstream intron (Zhang et al. 1998b). Similarly, we have observed that a viral mRNA undergoes NMD in chicken cells in the absence of splicing, thus it does not conform to the rules established for NMD in mammalian and, presumably, other higher eukaryotic cells. Izaurrealde and coworkers (Gatfield et al. 2003) first highlighted differences between mammals and other metazoans by showing that knockdown of several key EJC components had no effect on NMD, and PTC discrimination appears to be independent of exon-exon boundaries in *Drosophila*. Our observations are similar to this work in *Drosophila* in that we observe splicing-independent NMD in chicken cells. Like mammals, chickens utilize extensive mRNA splicing (Hillier et al. 2004).

Possible NMD mechanisms for RSV RNA

We have observed that a PTC in the *pol* gene elicits decay only when translated efficiently, suggesting that PTC recognition during any round of translation does not induce NMD. Similarly, it has been proposed that in mammalian cells, NMD occurs only during a pioneer round of translation (Lejeune et al. 2004). In contrast, studies in both yeast and rats have shown a decrease in RNA levels due to an infrequently translated PTC (Buzina and Shulman 1999; Plant et al. 2004). This is not surprising since RNAs in yeast can undergo NMD during any round of translation (Maderazo et al. 2003). Further, the RNA tested in rats was Ig- μ (Buzina and Shulman 1999), a transcript that seems to require special NMD rules (Buhler et al. 2004, 2005).

Similar to experiments in yeast where a 3' UTR was used to stabilize a PTC (Amrani et al. 2004), we used various deletions and insertions to position the RSE immediately downstream of a PTC in the *gag* gene. In all cases, the RSE showed sequence-specific stabilization of the RNA. In yeast, poly(A) binding protein tethered downstream of a PTC caused stabilization. It therefore seems plausible that a feature of the correct 3' UTR may involve proximity to the poly(A) tail or its associated factors such as the eRFs. However, when we moved the proper *gag* 3' UTR (the RSE) immediately after a PTC, the poly(A) tail was still about 7 kb away. It is conceivable that the RSE itself interacts with factors that recruit the eRFs. Alternatively, this region may help the viral unspliced RNA overcome an extremely long 3' UTR, possibly by promoting long-range interactions with distant parts of the transcript. Without this stability element, the untranslated region following a termination codon is seen as aberrant by the cell and undergoes decay.

An alternative hypothesis is that there is a DSE in the coding region of RSV, as proposed in yeast (Zhang et al. 1995). A DSE-like sequence might be found in the RSV coding region, potentially binding EJC proteins, SR proteins, or other potential RNA decay triggering factors such as the recently identified protein Stauf1 (Lykke-Andersen et al. 2000; Zhang and Krainer 2004; Kim et al. 2005). Also, it is arguable that the deletion analysis we carried out (Fig. 4A) may be changing the distance between the termination codon and a downstream DSE. However, we observed stabilization of the PTC-bearing RNA only by a sense insertion of the RSE (Fig. 4B). If we were simply moving a DSE too far away, then the anti-sense insertion would be predicted to stabilize the RNA in the same fashion. Thus, it appears that the stabilization is due to specific sequences within the RSE, which may recruit positive stability factors or repel negative decay factors.

Conserved 3' UTR motifs may play a role in stabilizing natural termination events

A recent report aligned untranslated portions of many annotated genes from human, rat, mouse, and dog (Xie et al. 2005). Several conserved motifs were found in both the promoter regions of genes and the 3' UTRs of these genes. In addition to an enriched pool of predicted eight-mer miRNA target sites, conserved motifs were found in the 3' UTR. Although several of these motifs have a known function (poly(A) site, AU rich elements), most of the 3' UTR motifs have no identified function. Perhaps some of these unknown motifs are involved in stabilization of a natural termination codon. The RSE sequence contains similarities to some of the 3' UTR motifs, which overlap our 3' set of mutations. Future experiments will be designed to dissect the region containing these point mutations and to ask whether they disrupt common termination-stabilizing motifs.

Implications

A recent report describes up-regulation of human endogenous retroviral RNAs when Upf1 is knocked down by RNAi in human cells (Mendell et al. 2004). It appears that the NMD pathway may be keeping these viral RNAs in check, even though most of the coding regions lack downstream introns (Bohne et al. 2005). The observation that inactivation of NMD affects transcripts from human endogenous retroviruses suggests that they may undergo NMD in human cells in a manner similar to the decay of avian retroviral transcripts in chicken cells.

Perhaps as higher organisms evolved the formation of an EJC, this became the primary signal that a termination codon was a PTC. Chicken and *Drosophila*, although possessing EJC protein genes, may not require the EJC to promote NMD. Thus far all of the work probing the composition of the EJC has been done in human cells. These other organisms may be relying on an ancient, alternate mechanism to direct NMD. It has recently been suggested that different organisms have evolved mechanisms of PTC determination best suited for the amount and type of pre-mRNA processing that each one utilizes (Tange et al. 2004; Conti and Izaurralde 2005; Lejeune and Maquat 2005). Our identification of the *cis*-acting RSE sequence helps to close the gap in the understanding of how NMD works in different organisms. Due to its important role in viral replication, the full-length RSV RNA is unspliced, exported from the nucleus, and translated, while bearing a 7-kb 3' UTR. The RSE may have evolved in order to help the virus overcome the RNA destabilization from such a long 3' UTR. Understanding how this works may help us realize why transcripts with extended 3' UTRs are NMD substrates (Hilgren and Parker 1999) and eventually develop a better understanding of the mechanism of NMD.

MATERIALS AND METHODS

Plasmid construction

The 10.8 plasmid is a permuted copy of the Prague C strain of RSV (Accession number: NC_001407), containing a deletion in the nucleocapsid region of the *gag* gene (Meric et al. 1988). The 10.8 +1 construct was generated with the Quickchange site directed mutagenesis kit (Statagene), using the primer 5'-GGGCTCCGCTTGACAAATTTAATAGGGAGGGCC-3', and its reverse complement. The underlined A is the inserted nt, immediately upstream of the *gag* termination codon. The 10.8 *pol* Ter and 10.8 +1 *pol* Ter constructs were also made by Quickchange, generating the following point mutations which changed normal codons into termination codons: A2533T, T3005A, A3739T, and G4620A.

The Δ RSE construct contains a deletion of nt 2486–2886. This was generated by inverse PCR from 10.8, using the following primers; Δ 401F — 5'-CTCCCCCTCTGTGAATAACAGGCCCGCC-3' and Δ 401R — 5'-CTATAAATTTGTCAAGCGGAGCCCTAGCC-3'. The primers were kinased using T4 polynucleotide kinase (NEB) and used in a touchdown PCR reaction.

The Ter Δ *gag* construct was created using the plasmid 1924T described previously (Barker and Beemon 1994). 1924T contains a

linker, with stop codons in all three reading frames, inserted at the SmaI site at nt 1924. Nucleotides 1934 (immediately after the in frame PTC) through 2485 (up to and including the gag stop codon) were deleted by inverse PCR as described above, using the following primers: 2486for — 5'-GGGAGGGCCACTGTTCTCTGTTGCGC-3', and KTreverse — 5'-CTAGACTAGCGGGGATCCACAAGTGTAGCAGAGCCC-3'. Construct Ter Δ gag Δ RSE was made by inverse PCR using primers KTreverse and Δ 401F described above. This construct has a deletion extending from nt 1934–2887. Construct 1547C (loading control) is a similar construct with a control insertion (no PTCs) at nt 1547 (Barker and Beemon 1994). The different sites of linker insertion led to two different products upon RNase digestion of the complementary riboprobe (LeBlanc and Beemon 2004).

To make the constructs Ter for/rev, the RSE sequence (nt 2483–2887) was amplified by PCR from the 10.8 plasmid template, using primers possessing AatII sites at their 5' ends. These PCR fragments were cloned into the unique AatII site at nt 1250 in both the forward and reverse orientations. The natural gag termination codon is present at the 5' end of the RSE forward fragment. A termination codon was also introduced at the 5' end of the RSE in the reverse orientation. Ter Δ RSE for/rev were made in the same fashion and cloned into the Δ RSE background. Fragments A, B, C, or D were made by PCR, and inserted at the same site. Each fragment begins with a TAG stop codon and then encompasses the following sequences: (A) 2486–2735, (B) 2600–2735, (C) 2600–2880, (D) 2617–2706. Note, fragment C ends 7 nt before the end of the full-length RSE.

The construct C mutant contains the same insertion as C above, with a series of point mutations. The mutagenic primers used to make this fragment are: MutCfor 5'-GCGCGGACGTCTAGCGCTAACGCAATTAGTGGAAAAAGATAAAGCGTTAGGACATATAGAAC-3', and MutCrev 5'-GCGGACGTCTAGTAATAGGTCACACCTTGAGGTCTAAG-3', with the underlined nt mutated from the wild-type sequence. The 3' end of this fragment ends at the same nt as the 3' end of the full-length RSE.

RNA isolation and RNase protection assays

In vitro transcription of the RSV gag probe was carried out as described in LeBlanc and Beemon (2004). RNA isolation and RNase protection assays were done as described previously (LeBlanc and Beemon 2004). In brief, total cellular RNA was harvested 40–48 h after transfection, using RNA-Bee (Tel-Test) per the manufacturer's instructions. After hybridization with 250,000 cpm of probe, RNA was digested with 10 U/mL RNase T1 (Calbiochem) and 5 μ g/mL RNase A at 34°C. After 45 min the digestion was stopped by addition of sodium dodecyl sulfate and Proteinase K (Roche) and incubated at 37°C for 15 min. RNA was extracted with phenol:chloroform:isoamyl alcohol, precipitated with ethanol, and resuspended in 8 M urea loading buffer, and denatured for 5 min at 95°C. Samples were loaded onto a 6% acrylamide, 8 M urea gel, and electrophoresed at 60W for 2–4 h. RNA levels were quantified using either an Instant Imager (Packard), or a Typhoon 9410 PhosphorImager (Amersham).

Cell culture and transfection

Cell culture and transfection of secondary CEF cells were carried out as described in LeBlanc and Beemon (2004). Transfections were carried out in 60-mm plates that were 90%–95% confluent. Aniso-

mycin (Calbiochem) was used at a final concentration of 25 μ g/mL to inhibit cellular translation. Actinomycin D (Calbiochem) was used at a final concentration of 2 μ g/mL to inhibit cellular transcription.

Western blot analysis

CEF cells were removed from the plates at 39°C with 0.05% trypsin-EDTA (Gibco) and pelleted. Western blotting was carried out as described in LeBlanc and Beemon (2004). RSV Gag and Gag-Pol proteins were detected using polyclonal rabbit anti-avian myeloblastosis virus (AMV) 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).

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