

The Structure and Function of the Rous Sarcoma virus RNA Stability Element

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ABSTRACT

For simple retroviruses, such as the Rous sarcoma virus (RSV), post-transcriptional control elements regulate viral RNA splicing, export, stability, and packaging into virions. These RNA sequences interact with cellular host proteins to regulate and facilitate productive viral infections. One such element, known as the RSV stability element (RSE), is required for maintaining stability of the full-length unspliced RNA. This viral RNA serves as the mRNA for the Gag and Pol proteins and also as the genome packaged in progeny virions. When the RSE is deleted from the viral RNA, the unspliced RNA becomes unstable and is degraded in a Upf1-dependent manner. Current evidence suggests that the RSE inhibits recognition of the viral *gag* termination codon by the nonsense-mediated mRNA decay (NMD) pathway. We believe that the RSE acts as an insulator to NMD, thereby preventing at least one of the required functional steps that target an mRNA for degradation. Here, we discuss the history of the RSE and the current model of how the RSE is interacting with cellular NMD factors. *J. Cell. Biochem.* 112: 3085–3092, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: RSE; ROUS SARCOMA VIRUS; NONSENSE-MEDIATED mRNA DECA; RNA STABILITY

Rous sarcoma virus (RSV) is an avian retrovirus originally discovered by Peyton Rous in 1911 as a filterable transmissible agent isolated from a sarcoma of the common fowl [Rous, 1911]. All retroviruses have two copies of a plus strand RNA genome, which is capped and polyadenylated [Beemon et al., 1974; Furuichi et al., 1975]. Upon entry into a receptive host cell, the genomic RNA is reverse transcribed into a proviral DNA copy. This proviral DNA is stably integrated into the host cell chromosomal DNA by the viral integrase. After integration, the provirus is treated as a cellular gene where subsequent steps of viral gene expression are completed by the machinery associated with cellular RNA polymerase II and ribosomes [Coffin et al., 1997].

RSV is a simple avian retrovirus with a genome containing four main open reading frames (ORFs) 5'-*gag-pol-env-src*-3' (Fig. 1). The first three genes (*gag*, *pol*, and *env*) are minimally required to generate a functional retrovirus. These proteins are expressed from three viral RNAs [Hayward, 1977]. The first is a full-length, unspliced RNA, which is the RNA genome for progeny virions and the mRNA for Gag and Pol proteins [Coffin et al., 1997]. Gag and Pol are expressed as polyproteins that are later cleaved by a viral protease into their individual functional polypeptides. In alpha retroviruses such as RSV, *gag* encodes the main structural components of the viral capsid and the viral protease. *Pol* encodes the reverse transcriptase and integrase. The *gag* and *pol* ORFs are

separated by a –1 frameshift that is facilitated by a pseudoknot that lies just downstream of the *gag* ORF [Coffin et al., 1997].

The first spliced RNA allows for expression of *env*, which encodes the glycoproteins that stud the outside of the retroviral lipid bilayer and interact with host cell surface receptors to facilitate cell entry. The second spliced RNA encodes for Src, which is the viral counterpart to the cellular proto-oncogene c-Src [Coffin et al., 1981; Martin, 2004]. Viral Src, contains several point mutations and a C-terminal substitution that results in its deregulation within host cells. In addition, RSV contains three short ORFs upstream of *gag*, of unknown function [Petersen et al., 1984; Petersen and Hackett, 1985]. Mutational studies suggest that these ORFs regulate the efficiency of translation at the *gag* start codon [Donze and Spahr, 1992; Donze et al., 1995]. Additionally, mutation of either the first or the third upstream ORF (uORFS) inhibits RNA encapsidation into the viral particle by up to 50-fold [Donze et al., 1995].

Upon infection of a receptive avian host cell, the integrated provirus is transcribed by cellular RNA polymerase II. Although this helps mask the viral RNA to make it resemble a cellular mRNA, in that it is capped and polyadenylated, there are many features of the unspliced viral mRNA that make it unique. First, the longest, most abundant viral RNA is unspliced. Typically, unspliced cellular pre-mRNAs are retained in the nucleus for degradation by the nuclear exosome [Bousquet-Antonelli et al., 2000]. Despite the presence of

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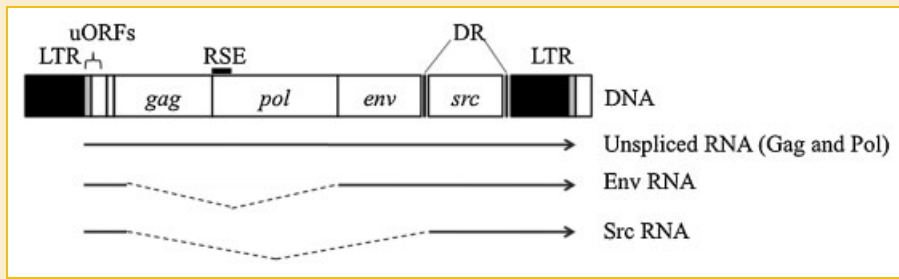


Fig. 1. The RSV genome and viral mRNAs. RSV contains three uORFs and four ORFs (*gag*, *pol*, *env*, and *src*) that are expressed from the proviral DNA in three RNA isoforms. The direct repeats (DRs) are required for export of the unspliced RNA. The long terminal repeats (LTRs) contain the sequences required for gene expression, including the promoter, enhancer elements and transcription start site.

splice donor and acceptor sequences within the viral RNA, the full-length RSV RNA is maintained due to weak 3' splice sites and a negative regulator of splicing (NRS)[Gontarek et al., 1993]. The RSV NRS forms a pre-splicing complex that is not competent to facilitate the first transesterification reaction, thereby preventing splicing [Giles and Beemon, 2005]. Export of this unspliced RNA is mediated in part by interactions between the RSV direct repeat (DR) sequences flanking the *src* gene and the cellular export factors Tap and Dbp5 [Ogert et al., 1996; Leblanc et al., 2007]. Second, the unspliced RNA is polycistronic. Normally mRNAs with multiple ORFs and consequently, multiple translation termination codons, are targets for nonsense-mediated mRNA decay (NMD). Third, the unspliced RNA primarily expresses the first long ORF (*gag*), in accordance with 5' loading of the eukaryotic ribosome. This means that the 6.9 kb after *gag* is presented to cellular factors as a 3' UTR. In aves, the average 3' UTR is 650 nts, with the longest observed 3' UTR being 3.3 kb [Pesole et al., 2001; Caldwell et al., 2005]. This means that the 3' UTR of RSV *gag* is abnormally large. Long 3' UTRs are also commonly targets of NMD [Hilleren and Parker, 1999].

Despite these unique attributes of RSV unspliced RNA, it still has a half-life of 10–20 h and during an infection can represent as much as 5% of the total cellular RNA [Baltimore, 1975; Stoltzfus et al., 1983; Weil and Beemon, 2006].

A HISTORY OF THE RSV STABILITY ELEMENT

In 1988, Arrigo and Beemon were studying the regulation of RSV gene expression and generated a mutant of RSV that contained a 465-nucleotide deletion from the *gag* ORF [Arrigo and Beemon, 1988]. This unintentionally generated a frameshift mutation that exhibited a >10-fold decrease in the level of unspliced RNA relative to wild-type, while the level of spliced *env* RNA remained unchanged. Further directed investigation indicated that decreased accumulation of unspliced RNA was restricted to mutants that caused premature termination codons within the *gag* ORF. This phenomenon was observed independently of deletions within the Gag proteins (p19, p2, p10, p27, p12, and p15) and the frameshift pseudoknot, suggesting that instability of the unspliced viral RNA was not due to loss of Gag protein components [Arrigo and Beemon, 1988; Barker and Beemon, 1991]. At this point in the literature, premature termination codons resulting in decreased RNA levels had

been observed for the cellular RNAs of beta-globin and triosephosphate isomerase; however, the mechanism of decay was not yet established [Chang and Kan, 1979; Baserga and Benz, 1988; Daar and Maquat, 1988].

A few years later, Barker and Beemon [1991] generated a series of mutations within *gag* that generated premature termination codons. They showed that unspliced, but not spliced, viral RNA levels were decreased to below 20% of wild-type levels. This was unlikely to be due to a transcription defect because spliced RNA levels were unchanged [Barker and Beemon, 1991]. Moreover, when RSV transfected cells were treated with the transcription inhibitor actinomycin D, the mutant RNAs decayed to <25% of their initial levels within 2 h, while wild-type viral RNAs and spliced viral RNAs remained unchanged over the course of the 6-h experiment [Barker and Beemon, 1991].

Termination codons inserted along the length of *gag* result in unstable full-length RNA until they occur within the final 100 nucleotides of the ORF [Barker and Beemon, 1994]. Within this range, there is a gradual increase in RNA stability as the distance between the premature and natural termination is reduced. When the premature termination codon is within 30 nts of the wild-type *gag* termination codon, the mutant RNA is present at 85% of wild-type RNA levels [Barker and Beemon, 1994]. This suggests a polarity effect such that a signal after the *gag* gene confers stability to the RNA so long as the termination codon is in close proximity to the aforementioned signal. To address this hypothesis, Barker and Beemon [1994] generated a series of mutations after the *gag* stop codon. They found that deletions between a stop codon in *gag* and the first 250 nucleotides of *pol* resulted in increased decay of the unspliced viral RNA levels. This supported the idea that an RNA element immediately after the *gag* termination codon regulated viral RNA stability.

Premature termination codons in unspliced RSV RNA had little effect on the nuclear metabolism of the RNA and appeared to reduce only the cytoplasmic RNA levels, with a parallel reduction in mutant protein production [Barker and Beemon, 1994]. It was hypothesized that this may be a mechanism for decreasing the production of defective progeny virions.

In 2006, Weil and Beemon designated RSV nucleotides 2,485–2,885, the 400 nt immediately following the *gag* natural termination codon, as the RSV stability element (RSE) [Weil and Beemon, 2006]. Deletion of the 400 nt RSE sequence results in reduction in the level

of unspliced RNA levels. There is clear redundancy within this region because either a 3' or a 5' fragment of approximately 300 nucleotides is functional, but a 135 nucleotide central fragment shared by the two fragments is not sufficient to confer stability to the unspliced RSV RNA [Weil and Beemon, 2006]. The 3' segment, known as the C fragment (Fig. 2: C_{frag}), was selected for studies of the RSE since its sequence excludes the frameshift pseudoknot and the reverse complement completely lacks function. Furthermore, the C fragment sequence can be moved downstream of a premature termination codon in *gag* to stabilize the RNA [Weil and Beemon,

2006]. This stabilization is sequence dependent; the reverse complement of the RSE sequence inserted after a premature termination codon did not protect the RNA from degradation.

DETERMINING FUNCTIONAL SEQUENCE ELEMENTS OF THE RSE

In vitro, the 400-nt RSE RNA is a structured element consisting of the frameshift pseudoknot, a long AU-rich single stranded region

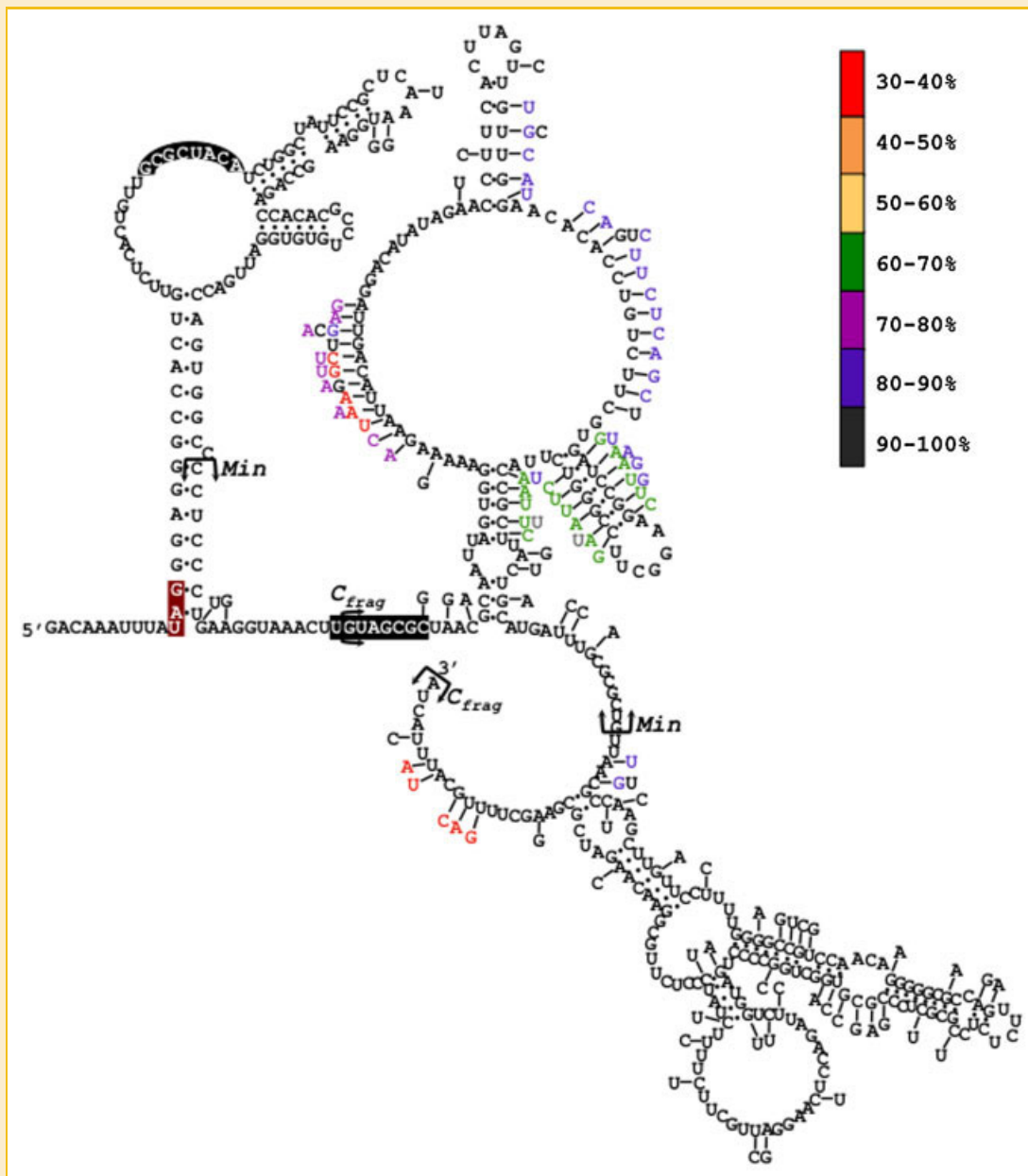


Fig. 2. Structure and summary of mutation studies of the RSE. Nucleotides on the main structure are according to accession number NC_001407. The structure shown was determined by S.H.A.P.E. chemistry and RNase digestion in vitro. The -1 frameshift pseudoknot was reprinted with permission [Marczinke et al., 1998]. The white letters on a black background represent the pseudoknot pairing. The gag stop codon is indicated in white letters on a red background. Arrow brackets indicate the 5' and 3' boundary of the minimal RSE (2,577–2,732) and the RSE C fragment (2,660–2,880). Mutations made to the RSE are indicated with the color of the letter representing the level of mutant RNA relative to wild-type RSV unspliced RNA levels.

and two small stem-loops, followed by a long GC-rich stem-loop (Fig. 2) [Weil and Beemon, 2006; Withers and Beemon, 2010]. To gain a better understanding of the function of the RSE, we conducted several mutational and phylogenetic studies [Weil and Beemon, 2006; Weil et al., 2009; Withers and Beemon, 2010]. Highlighted on the figure are residues where mutants retain function (black), display a partial loss of function (blue, purple, and green) or a complete loss of function (red). Nucleotide positions that differ in other avian retroviruses are considered to have no effect on RSE function and are also shown in black. The majority of the mutations generated resulted in a partial loss of function. This suggests that these nucleotide positions, when altered, can lead to a disruption of the in vivo structure of the RSE. Since RNA binding proteins commonly interact with single stranded loops, a disruption in the secondary structure may occlude a potential binding site. These mutations may manifest as a partial loss of function if the RSE RNA can fold into more than one stable structure, where only a subset of the potential conformations occupied by the RNA will allow the RSE to protect the unspliced viral RNA from Upf1-dependent degradation [LeBlanc and Beemon, 2004; Weil and Beemon, 2006]. The degree of observed stability may in part be determined by the time the RSE RNA may spend in each allowable structure, either as an individual mRNA or as a population of RNAs.

To date, a single mutant has been isolated that abolished RSE function, which would denote a region of the RSE that is absolutely required for function [Weil and Beemon, 2006]. As indicated on the structure, this mutant has 10 altered nucleotide positions that cluster in two locations (Fig. 2: red residues). However, truncation studies indicate that the minimal sequence required for RSE function is 155 nucleotides spanning 2,577 to 2,732 (Fig. 2: *Min*), suggesting that the mutations that lie within this region may be responsible for disrupting RSE function [Withers and Beemon, 2010]. Unfortunately, additional mutations studied within the minimal RSE surrounding position 2,630 have all either resulted in a partial loss of function, or a wild-type RSE. This again indicates that there is likely redundancy within the RSE [Withers and Beemon, 2010].

ROLE OF NMD IN DECAY OF RSV RNA

NMD is a quality control mechanism that identifies mRNAs that contain premature termination codons and targets them for degradation by cytoplasmic RNases. The central NMD proteins are known as the Upf proteins (Up-frameshift). These were originally identified in a *Saccharomyces cerevisiae* genetic screen for proteins that increased frameshifting efficiencies or suppressed upstream initiation codons [Culbertson et al., 1980; Leeds et al., 1992; Lee et al., 1995]. At the same time that we observed that premature termination codons in RSV resulted in reduced RNA stability, other groups were describing NMD in cellular RNAs.

In spliced mammalian mRNAs, NMD is believed to rely on the exon-junction complex (EJC) as a signal to identify premature termination codons. The EJC is a multi-protein complex that is deposited 20–24 nucleotides upstream of exon–exon junctions during splicing [Le Hir et al., 2000]. The EJC is a mark signaling a

history of splicing that can travel into the cytoplasm with the mRNA. The natural termination codon is found in the final exon of almost all mRNAs, meaning that splicing, and consequently deposition of an EJC, rarely occurs downstream of a natural termination codon [Hawkins, 1988]. As a result, NMD identifies premature termination by the presence of a downstream exon junction [Zhang et al., 1998]. Briefly, NMD is triggered when an EJC downstream of the translation termination codon interacts with the central NMD proteins Upf1, Upf2, and Upf3, and the release factors of the terminating ribosome [Kashima et al., 2006]. This is the initial signal to identify premature translation termination. This is followed by a series of steps, including a phosphorylation cycle of Upf1 by the Smg proteins, cleavage of the RNA by Smg6, and recruitment of cytoplasmic RNA decay factors [Yamashita et al., 2001; Ohnishi et al., 2003; Huntzinger et al., 2008; Eberle et al., 2009]. Finally, Upf1-catalyzed ATP hydrolysis results in NMD mRNP remodeling and release from the RNA [Franks et al., 2010].

One model suggests that NMD may be present to dampen the noise of transcription. In global expression studies where one or more of the NMD components have been depleted, many normal cellular RNAs are up-regulated [He et al., 2003; Mendell et al., 2004]. These cellular NMD targets could be categorized as possessing one of the following RNA features: upstream ORF (uORF), PTC due to alternative splicing, retained introns, 3' UTR intron, site of selenocysteine insertion, long 3' UTRs or the product of a pseudogene, endogenous transposon, or retrovirus. Interestingly, RSV unspliced RNA contains four of these features, and yet is not a natural target of NMD.

Nonetheless, RSV RNA does not appear to be immune to NMD, as has been reported for a few other unspliced eukaryotic RNAs [Maquat and Li, 2001; Brocke et al., 2002]. The loss of stability in RSV RNA containing a premature termination codon, or lacking the 400-nt RSE is dependent upon translation of *gag* and the NMD factor Upf1 [LeBlanc and Beemon, 2004; Weil and Beemon, 2006]. However, the RSV RNA is an *unspliced* RNA so the viral RNA should be unoccupied by EJCs, which appear to act as the strongest signal to trigger NMD recognition in mammals.

Alternative models of NMD have proposed mechanisms of NMD recognition that do not rely upon splicing. *S. cerevisiae* transcripts are predominantly intronless, and yet some of the earliest observations of NMD come from studies in yeast. Original models focused on the presence of a downstream element (DSE; TGYGATGYYYY), that in a similar fashion to the EJC, would stimulate NMD when present 3' of a terminating ribosome [Ruiz-Echevarria et al., 1998]. Certain transcripts studied also contained AU-rich stabilizer elements (STES; GCN4, and YAP1) that bind to Pub1p and dominantly interfere with NMD and protect a termination codon from detection [Ruiz-Echevarria et al., 1998; Ruiz-Echevarria and Peltz, 2000]. These are devoid of any extensive sequence similarity to the minimal RSE.

Currently, the favored model for splicing-independent NMD recognition suggests that the distance between the termination codon and the polyA binding protein distinguishes premature termination from natural termination. Initially, observations in yeast and human clinical isolates identified mutations that resulted in longer 3' UTRs and a corresponding reduction in mRNA levels

compared to wild-type transcripts [Losson and Lacroute, 1979; Higgs et al., 1983; Zaret and Sherman, 1984]. The first observation that correlated long 3' UTRs to NMD came from *Caenorhabditis elegans* [Pulak and Anderson, 1993]. The mutant worm known as *unc54-r293* has a mutation in the polyA signal of the *unc54* gene, which generates a 3' UTR that is approximately 2 kb longer than wild-type. Mutant *unc54-r293* mRNA levels are elevated in the Smg mutants relative to wild-type. The Smg proteins in worms are key NMD regulatory proteins (Smg1, Smg 5/6/7), including the central Upf proteins (Smg 2,3,4). The authors of this work noticed that mRNAs containing PTCs are also elevated in Smg mutants and hypothesized that mRNAs containing a long 3' UTR or a PTC may be recognized by the same mechanism of mRNA decay.

Ten years later, studies in yeast demonstrated that PABP tethered to mRNAs lacking a polyA tail are stabilized, but only if the mRNA is translated [Coller et al., 1998]. Then the Parker group suggested that the "terminal mRNP" is the signal that determines whether an mRNA will undergo decay [Hilleren and Parker, 1999]. Finally in 2003, NMD in *Drosophila melanogaster* was shown to be EJC-independent, and the signal to evade NMD "a generic feature, such as the poly(A) tail or a mark deposited during cleavage and polyadenylation" [Gatfield et al., 2003]. This idea was followed up 4 years later to show it was in fact the polyA binding protein [Behm-Ansmant et al., 2007]. By this point, the plant community had shown that both downstream introns and long 3' UTRs can trigger NMD [Kertesz et al., 2006]. In 2008, this result was reiterated in mammalian culture cells using reporter constructs [Eberle et al., 2008, 2009; Singh et al., 2008].

A model summarizing all of these results suggests that PABP may directly compete with the NMD factors for interaction with the terminating ribosome. Depending on the mRNA environment at the termination codon, PABP may protect the mRNA from recognition by NMD. PABP need only be close to the termination codon in spatial distance because even in the context of a long 3' UTR, secondary structure or RNA-RNA interactions may bring the polyA tail adjacent to the termination codon [Stalder and Muhlemann, 2008].

MODELS OF RSE FUNCTION

We hypothesize that the RSE functions as an NMD insulator to protect the *gag* termination codon from recognition by NMD proteins. It is believed that during translation termination, eRF1 and eRF3 are recruited, along with Upf1, to the ribosome, regardless of whether or not translation termination is premature [Kashima et al., 2006]. In order to prevent NMD recognition of the *gag* termination codon, it is most likely that the RSE recruits an inhibitor of Upf1 (Fig. 3).

Upf1 regulation is central to all steps of NMD. During NMD recognition, a structural rearrangement occurs that allows Upf1 bound to the eRFs to associate with the other NMD factors, such as Upf2 and Upf3 [Franks et al., 2010]. This is coordinated with a phosphorylation-dephosphorylation cycle of Upf1 and ATP hydrolysis by Upf1. Over-expression of ATPase-deficient Upf1 leads to accumulation of hyperphosphorylated Upf1, which can prevent further rounds of translation initiation, so it is unlikely that the RSE is preventing dephosphorylation of Upf1 [Isken et al., 2008]. At this point, we have not observed Smg6-dependent cleavage of PTC-containing RSV RNA [Huntzinger et al., 2008; Eberle et al., 2009]. However, it is clear that RSE inhibition of Upf1 must occur before the NMD cleavage step to protect the RSV unspliced RNA from degradation.

A recent study of Upf1 activity suggests that ATPase activity may be the rate-limiting step for mRNA remodeling [Franks et al., 2010]. In the presence of an ATPase-deficient Upf1, the authors observed accumulation of full-length mRNA and the 3' mRNA fragment resulting from Smg6 cleavage. The authors hypothesized that the NMD factors or ribosomal subunits may be responsible for blocking the decay of the NMD substrate. The factors present at this step are likely candidates for RSE-interaction partners.

The RSE could interfere with Upf1 function at many different steps, including its binding to eRF3, ATPase activity, or NMD RNP remodeling. These steps may all be inhibited either by direct interaction of the RSE with the NMD factors themselves or through recruitment of a secondary, yet unidentified protein.

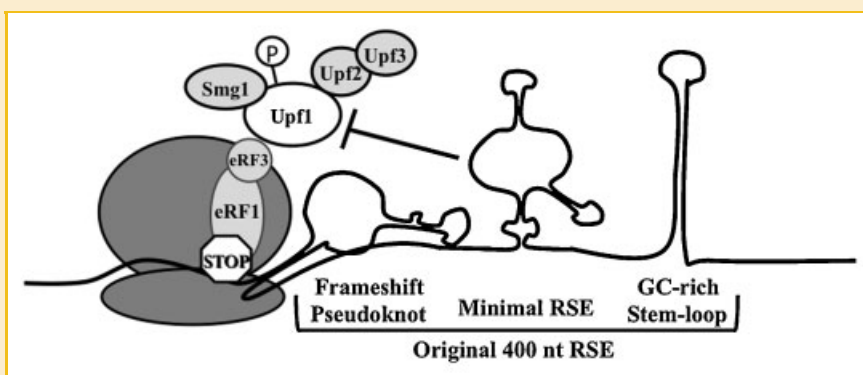


Fig. 3. The RSE inhibits Upf1 to prevent NMD recognition of the *gag* termination codon. The RSE interferes with Upf1 binding to eRF3, ATPase activity or NMD RNP remodeling. These steps may be inhibited by direct interaction of the RSE with the NMD factors or through recruitment of a secondary, yet unidentified protein.

RNA STABILITY AND PREMATURE TERMINATION CODONS IN HIV-1

A human relative of RSV is the human immunodeficiency virus-1 (HIV-1). Like RSV, HIV-1 full-length viral RNA contains multiple ORFs, long apparent 3' UTRs, and is incompletely spliced. However, the effect of Upf1 on the HIV-1 life cycle appears to be different from that of RSV. Knockdown of Upf1 in HIV-1 infected cells causes a reduction in the wild-type unspliced RNA levels, and a corresponding reduction in Gag protein expression [Mouland et al., 2000]. Conversely, when Upf1 is over-expressed, HIV-1 Gag production is further elevated above wild-type levels. This phenotype is separate from the NMD functions of Upf1 because over-expression of Upf1 variants which are mutant for helicase activity, Upf2 binding, or ATPase activities are still capable of enhancing HIV-1 production. Many of these mutants are well characterized for their ability to abrogate NMD and act as dominant negative inhibitors of the pathway.

During HIV-1 infection, Upf1 may recruit the full-length unspliced viral RNA from a non-translating pool to polysomes to enhance Gag expression. Alternatively, Upf1 may associate with Staufen to provide a direct enhancement for translational efficiency. Both Staufen and Upf1 are packaged into HIV-1 virions and are required for generation of infectious virus [Chatel-Chaix et al., 2004, 2008].

In direct contrast, a more recent study isolated Upf1 as an interaction partner of HIV-1 3' UTR RNA [Hogg and Goff, 2010]. This study proposed that Upf1 associates in a sequence-nonspecific manner with long 3' UTRs to promote mRNA decay. This greater association is independent of the translation state of the mRNA. The effect of Upf1 on decay of the population of target mRNA could be drastically altered by allowing rare (<1%) translational read-through of the termination codon. Although the association of Upf1 with a long 3' UTR was initially made with bait RNA from HIV-1, it remains to be seen if the extensive Upf1 association holds true for full-length HIV-1 RNAs.

Although the RSV RSE is located just downstream of the *gag-pol* junction, it is distinct from sequences involved in frame-shifting that allow translational read-through in RSV. The minimal RSE element (nts 2,577–2,732) and the C fragment (2,600–2,885) do not include sequences needed for frameshifting [Weil and Beemon, 2006; Withers and Beemon, 2010]. Furthermore, mutation of sequences required for frame-shifting do not affect levels of Gag protein product produced [Jacks et al., 1988; Barker and Beemon, 1994; Marczinke et al., 1998]. Thus, we do not think that RSE functions by allowing read-through.

FUTURE DIRECTIONS AND OUTSTANDING QUESTIONS

Future work is required to address many of the outstanding questions regarding the function of the RSE. We would like to know what proteins are involved, and to determine which NMD factors are required. Furthermore, we would like to investigate whether the RSE is conserved among other retroviruses, and what role it may play

during viral infection. Perhaps the mechanism of NMD inhibition by the RSE may be conserved in other cellular messages that possess traits of NMD targets yet are immune to decay, such as those with long 3' UTRs.

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