Stability Determinants of Murine Cytomegalovirus Long Noncoding RNA7.2

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Cytomegalovirus is a ubiquitous herpesvirus that persistently replicates in glandular epithelial tissue. Murine cytomegalovirus expresses a 7.2-kb-long noncoding RNA (RNA7.2) that is a determinant of viral persistence in the salivary gland. RNA7.2 is an extremely long-lived intron, yet the basis of its stability is unknown. We present data that localize key sequence determinants of RNA stability to the 3' end of RNA7.2 and suggest that stability is a result of sustained lariat conformation.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that persistently replicates in glandular epithelial tissue and eventually establishes a lifelong latent infection of the host (1). We previously identified a conserved long noncoding RNA (lncRNA) of unknown function expressed by cytomegaloviruses (2, 3). HCMV expresses a 5-kb lncRNA (RNA5.0), and murine cytomegalovirus (MCMV) expresses a 7.2-kb ortholog (RNA7.2) that we showed to be an important determinant of viral persistence in the salivary gland. RNA7.2 accumulates in the nucleus of infected cells during infection and is an extremely long-lived intron, with a half-life ($t_{1/2}$) of ~29 h (4). In the current study, we sought to identify determinants of intron stability.

Since MCMV RNA7.2 accumulates as a consequence of a slow decay rate, we investigated if specific RNA sequence elements contribute to its stability. The sequence spanning the RNA7.2 locus was PCR amplified and cloned into a vector that allowed us to express the intron independent of virus infection and easily mutate sequence elements that might contribute to stability (pIntron) (Fig. 1A). The expression plasmids were transfected into mouse 10.1 fibroblasts, and at 24 h posttransfection, we quantified RNA stability as described previously (Fig. 1A) (4). The half-life of RNA7.2 from pIntron-transfected cells was 19.14 h, notably shorter than the reported RNA7.2 half-life from productively infected cells (4). Although the half-life of the transfected intron is still unusually long, several factors may account for the observed difference between virally infected cells and pIntron-transfected cells. For example, productive infection may promote RNA7.2 stability directly through biochemical interactions with viral proteins and RNAs or indirectly by altering cellular RNA decay pathways.

To determine if the internal sequence was critical for the stability of RNA7.2, the sequence was deleted by restriction digestion to create a smaller 3.58-kb RNA or a 1.8-kb RNA (pInt3.58 and pInt1.8). An additional construct was generated that reversed the orientation of an internal restriction fragment (pIntRVRev). The half-life measurements of pInt3.58 ($t_{1/2}$ = 14.43 h) and pInt1.8 ($t_{1/2}$ = 16.27 h) were only slightly reduced compared to that for wild-type RNA7.2 ($t_{1/2}$ = 19.14 h). The half-life of pIntRev was reduced to 8.69 h. Although still highly stable for an intron, this reduced half-life might result from structural instability introduced by inverting the nucleotide sequence. Regardless, we conclude that stability determinants of RNA7.2 are not internal and more likely reside near the ends of the intron.

We previously showed that a stem-loop structure located

near the 3' end of RNA7.2 contributes to intron accumulation (3). Although deletion of this structure does not affect expression and processing of the precursor transcript, accumulation of the intron during infection is dramatically impaired. To investigate this further, we quantified the stability of RNA7.2 in cells transfected with pIntron deleted for the hairpin (Fig. 2B). The half-life of the intron is reduced to 1.16 h. To determine if small modifications to the stem structure impact stability, mutations in the stem of the hairpin were generated in pIntron (Fig. 2C and D). The 5-bp upper stem substitution resulted in a rapid reduction in relative abundance of RNA7.2 to approximately half of its original concentration following actinomycin D treatment (Fig. 2C), whereas no further decay of RNA7.2 decay occurred at later time points posttreatment. Unlike the hairpin deletion mutant, the altered kinetics in RNA7.2 degradation due to the 5-bp substitution may reflect a heterogeneous population of stem-loop structures that either confer stability to RNA7.2 or cause it to be susceptible to cellular RNA decay machinery. A 4-bp substitution in the lower stem caused a modest reduction in RNA7.2 half-life ($t_{1/2}$ = 7.97 h). Our observations reinforce the importance of formation of this hairpin structure, especially for residues at the base of the stem, for RNA7.2 stability.

Introns are processed through the formation of a lariat structure via a 2'-to-5' phosphodiester bond linking the 5' splice donor (SD) site to a branch point 20 to 80 nucleotides (nt) upstream of the splice acceptor (SA) site (5). RNA7.2 contains a consensus branch point sequence 81 nt from the SA. To determine if the putative branch point is important in the generation of a stable RNA, we mutated it from CTAAC to CGTGC in pIntron (pBPM) (6). This substitution reduced the half-life of RNA7.2 to 6.05 h (Fig. 3A). As a control, we quantified relative RNA abundance for each pIntron construct and found little difference in RNA production for each mutant (Fig. 3B).

As additional confirmation of its nonlinear form, we tested

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FIG 1 Deletion of RNA7.2 internal sequence does not alter its stability. (A) A schematic representation of sequence spanning the RNA7.2 locus that was inserted into the pCDNA3.1+ vector is shown (pIntron). The sequence cloned into pCDNA3.1+ includes 201 bp upstream of the RNA7.2 splice donor site (SD) spanning the transcriptional start site (+1) in addition to 306 bp downstream of the splice acceptor site (SA), totaling 7,764 bp. This sequence was sequentially cloned as two fragments (BamHI-NotI followed by NotI) into the pCDNA3.1+ plasmid after amplification using Phusion polymerase (Finnzymes) and subsequent restriction digestion. Splicing of pIntron in transfected cells produces a truncated, spliced mRNA in addition to RNA7.2 as an intron detected by primer probe sets that target either RNA7.2 or the spliced mRNA (data not shown) (4). Sequence was excised from pIntron using either EcoRV or the combination of PstI-EcoRV restriction sites to generate either a 3.58-kb RNA (pInt3.58) or a 1.8-kb RNA (pInt1.8) following splicing of the primary transcript. The EcoRV fragment was reinserted in the reverse orientation to create pIntRVrev. A total of 2.0×10^{17} copies of each plasmid were transfected into mouse fibroblasts using a 6:1 ratio of PEI to DNA. Twenty-four hours posttransfection, cells were treated with 4 μ g/ml actinomycin D. Total RNA was harvested over a time course starting at time zero posttreatment. RNA abundance over time was measured relative to time zero by reverse transcription-quantitative PCR (qRT-PCR) for RNA7.2 and normalized by 18S rRNA (4). RNA half-life ($t_{1/2}$) was calculated by the best-fit curve to the data points. Half-life decay curves for pIntron (B), pInt3.58 (C), pInt1.8 (D), and pIntRVrev (E) are indicated. Dots represent the means; error bars represent standard errors of the means (SEM).

the susceptibility of RNA7.2 to degradation by a 5'-to-3' exonuclease known as Terminator (Epicentre). If RNA7.2 is a lariat, it will be protected from digestion since Terminator digests RNA containing 5' monophosphate (7–9). 18S rRNA serves as a positive control for Terminator degradation since it lacks a 5' m7G cap. As a negative control for Terminator digestion, we used a pCDNA3 minigene construct of the herpes simplex virus 1 (HSV-1) latency-associated transcript (LAT) consisting of a 2.8-kb fragment of the LAT primary transcript that spans the 2.0-kb LAT intron (pLAT) (10). Although RNA7.2 and LAT do not share sequence homology, both lncRNAs have conserved

splice donor and acceptor sequences, are processed as introns, and have a stem-loop structure within their 3' regions that is critical for their stable retention. Previous work demonstrated LAT is nonlinear and therefore should be resistant to Terminator digestion (10–13). As shown in Fig. 4A, RNA7.2 was highly resistant to Terminator digestion. As expected, HSV-1 LAT was also resistant to Terminator digestion (Fig. 4B), while 18S rRNA was completely degraded (Fig. 4C). These data are additional evidence that the 5' end of RNA7.2 is protected from degradation. RNA7.2 may remain in a lariat because RNA7.2 appears to be resistant to Terminator degradation following

FIG 2 Disruption of the RNA7.2 stem-loop sequence reduces RNA7.2 stability. (A) Schematic representation of the predicted hairpin loop sequence that is located between a putative RNA7.2 branch point sequence and polypyrimidine tract. pIntHPM was generated by amplifying the second, NotI fragment of pIntron from a preparation of MCMV *delHP* BAC DNA for insertion into the pCDNA3.1+ vector. The 5-bp and 4-bp substitution mutations were generated by site-directed mutagenesis (Stratagene) on pIntron. Predicted free energies (calculated in mFold) are indicated below each stem-loop structure. Transfection and half-life analysis were performed as described for Fig. 1. Half-life decay curves for pIntHPM (B), 5-bp substitution (C), 4-bp substitution (D). Dots represent the means; error bars = SEM.

treatment with the cellular debranching enzyme (Dbr1) (preliminary data not shown). Future experiments will be needed to determine whether a stable lariat persists and contributes to RNA stability.

Our results indicate that stability of the MCMV RNA7.2 is due primarily to sequences in the 3' region of the intron. A strong, secondary structure is critical for accumulation and stability of

this RNA, and we hypothesize that it is important for preventing debranching and decay. The predicted branch point sequence also confers stability to RNA7.2 likely due to its consensus sequence that is easily recognized by splicesomal machinery as well as its location within the 3' region. Future studies will determine if the sequence mutations analyzed in this report alter cellular location and functionality of RNA7.2. Although the function of the RNA

FIG 3 Mutation of a putative branch point sequence reduces RNA7.2 stability. (A) Sequence map demonstrating the 3-bp substitution generated within pIntron for a putative branch point sequence. Transfection and half-life analysis were performed as described for Fig. 1. (B) Transcriptional output for each plasmid was examined 24 h posttransfection in mouse fibroblasts. Total RNA was harvested and RNA7.2 accumulation was examined for the indicated plasmids relative to empty pCDNA3.1+. All samples were normalized to 18S rRNA. Bars represent the means; error bars = SEM.

FIG 4 RNA7.2 is protected from 5'-to-3' exonucleolytic decay. Total RNA was isolated 24 h posttransfection from fibroblasts transfected with either pIntron or pLAT. Three micrograms of total RNA was treated with Terminator (1U/ μ g RNA) for either 30 min or 1 h at 30°C. Following Terminator treatment, RNA was purified using an RNeasy minikit and analyzed by qRT-PCR for RNA7.2 degradation (A), 18S rRNA degradation (representative 18S rRNA degradation from pIntron-transfected cells) (B), or pLAT (C). RNA was normalized to GAPDH and compared relative to untreated RNA. RNA was normalized to GAPDH and compared relative to untreated RNA. Bars on all graphs represent the means; error bars = SEM.

remains unknown, its long life is likely to reflect a key role in establishing persistent infection of the host.

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