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Intronless β -Globin Reporter: A Tool for Studying Nuclear RNA Stability Elements

Jessica A. Brown and Joan A. Steitz

Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536 USA

Abstract

The intronless β -globin reporter, whose mRNA is intrinsically unstable due to the lack of introns, is a useful tool to study RNA stability elements in a heterologous transcript. Insertion of a stability element leads to the accumulation of intronless β -globin mRNA that can be visualized by conventional Northern blot analyses. In this chapter, we explain how to perform the β -globin reporter assay using the ENE, a triple-helix-forming RNA stability element that protects reporter mRNA from 3′-5′ decay. A list of considerations is included for the use of ENEs as a tool to stabilize other RNAs. In this chapter, we provide a brief description of how to insert an ENE sequence into the 3′-untranslated region of an intronless β -globin reporter plasmid using basic cloning technology. Then, we provide a detailed protocol for quantitative measurements of steady-state levels of β -globin mRNA. This entails the transient transfection of mammalian cells with β -globin reporter plasmids, isolation of total cellular RNA, and detection of reporter mRNA via Northern blot. This methodology can be applied for the study of any nuclear RNA stability element using the intronless β -globin reporter.

Keywords

β -globin; reporter assay; RNA stability; RNA decay; triple helix; ENE

1. INTRODUCTION

The β -globin reporter system has been adapted to study various facets of messenger RNA (mRNA) processing, particularly mRNA decay in eukaryotic cells. Historically, reporter plasmids encoding the human β -globin gene were instrumental in understanding nonsense-mediated mRNA decay because the β -globin gene from β -thalassemia patients contains naturally occurring nonsense mutations. These mutations create a premature termination codon (PTC) that renders the resulting β -globin mRNA susceptible, or sometimes resistant for rare mutations, to nonsense-mediated decay (Figure 1, β -PTC) (1,2). The β -globin reporter system has also been used to study RNA elements that stabilize or destabilize mRNA. Destabilizing RNA elements are typically studied in the context of a wild-type (WT), intron-containing β -globin reporter (Figure 1, β -WT), which generates a highly stable transcript that localizes predominantly to the cytoplasm (3,4). In contrast, RNA stability

elements are typically studied in the context of an intronless β -globin reporter (Figure 1, β 1,2), which accumulates to much lower levels than its intron-containing counterpart and exhibits predominantly nuclear localization (4,5).

Recently, a unique RNA stability element known as an ENE (expression and nuclear retention element) was discovered near the 3' end of viral and host long noncoding RNAs (lncRNAs), as well as in viral genomic RNAs (Table 1) (4,6,7). To stabilize RNA, the ENE folds into a stem-loop structure containing a U-rich internal loop, which sequesters the downstream 3'-poly(A) tail or a genomically encoded A-rich tract into a triple helix (ENE +A) composed of mostly U•A-U major-groove base triples flanked by A-minor base triples (Figure 2) (8,9). The formation of this RNA triple helix inhibits rapid 3'-5' RNA decay (9,10) and can stabilize β -globin, green fluorescence protein (GFP), and mCherry reporter transcripts (7,11). Insertion of viral or host ENEs into the 3'-untranslated region (UTR) of intronless β -globin reporter plasmids (Figure 1, β 1,2-ENE and β 1,2-ENE+A+“tRNA”) increases the accumulation of β -globin mRNA by 1.1- to 45-fold over an intronless reporter without an ENE in HEK293T cells (Table 1) (4,6,7).

Currently, two different classes of ENEs have been characterized: class I ENEs, which interact with a post-transcriptionally added 3'-poly(A) tail, and class II ENEs, which interact with a genomically encoded A-rich tract at the 3' end of transcripts (Figure 2). Class I ENEs have been discovered in viral lncRNAs as well as in viral single-stranded genomic RNA (4,6). The best characterized class I ENE is from polyadenylated nuclear (PAN) RNA produced by the Kaposi's sarcoma-associated herpesvirus (KSHV) (4,8,10,12). Class II ENEs have been found in cellular MALAT1 and MEN β lncRNAs whose 3' ends do not undergo canonical cleavage and polyadenylation (7,11). Rather, tRNA-like structures immediately downstream of a genomically encoded A-rich tract are endonucleolytically processed by RNase P to generate the 3' end of MALAT1 and MEN β (13,14). Although both class I and II ENE+A structures inhibit 3'-5' RNA decay, the decay mechanisms are expected to be different because class I ENE+A structures present a triple helix with a traditional poly(A) overhang whereas class II ENE+A structures present a triple helix with a blunt end (9).

ENE+A structures can be used as a tool to stabilize the 3' ends of other RNAs *in vivo*. One successful example demonstrated that 5 copies of the KSHV PAN ENE increased primary microRNA transcripts for human let-7a-1 and *Caenorhabditis elegans* lin-4 by ~10-fold in HeLa cells (15). This application of ENE structures has not been tested extensively, but we can offer the following points of advice. 1) ENE+A structures preferentially stabilize intronless RNAs rather than spliced RNAs (4). 2) Class I ENEs are preferable when the 3' end of the RNA is generated via cleavage and polyadenylation. These ENEs appear to be most effective within 150 nucleotides (nts) upstream of the poly(A) tail; multiple copies can be inserted for enhanced stabilization (4,6). 3) Class II ENEs appear to be effective only when the A-rich tract is at the extreme 3' end of the transcript; the sequence of the tRNA-like structure must be included so that the 3' end can be generated via RNase P cleavage (Table 1 and Figure 1) (7). These ENEs are useful for studying non-polyadenylated RNAs (16). 4) Class II ENE+A structures enhance the translation of mRNAs in which they reside (11,17). 5) Avoid inserting the ENE adjacent to another highly structured region. 6)

Consider testing multiple ENEs in different cell types. We have tested the stabilization activity of only seven ENEs in the β -globin reporter introduced into HEK293T cells via transient transfection. Activity may vary in different sequence and structural contexts as well as cell types. 7) To block 5'-3' RNA decay, consider using XRN1-resistant RNA (xrRNA) structures derived from flaviviruses (18,19).

In this chapter, we describe how to insert an ENE sequence into an intronless β -globin reporter plasmid using standard cloning techniques. The β -globin reporter plasmids we describe use pcDNA3 as the vector; therefore, the β -globin gene is flanked upstream by a cytomegalovirus (CMV) immediate-early promoter and downstream by the bovine growth hormone polyadenylation (BGH pA) signal (Figure 1). Multiple copies of the class I ENE can be inserted, although reporter mRNA levels appear saturated at 3 or more copies in the case of KSHV PAN ENE (4). Reporters with ENEs inserted in the reverse orientation serve as negative controls. Lastly, we provide a detailed protocol to measure β -globin mRNA levels under steady-state conditions. Briefly, intronless β -globin reporter plasmids are introduced into HEK293T cells by transient transfection, total cellular RNA is harvested and mRNA levels are measured by quantitative Northern blot analysis.

2. MATERIALS

2.1 Construction of a β 1,2-ENE reporter plasmid

Because standard cloning techniques are used to create these plasmids, only the pertinent reagents are listed. For a complete description of all required materials, please refer to any edition of *Molecular Cloning: A Laboratory Manual* by J. Sambrook and others (20).

1. Reagents and equipment for performing PCR (e.g. Phusion® high-fidelity DNA polymerase), chemically synthesized PCR primers with an ApaI site, and genomic material. When the appropriate genomic material is not available, the ENE insert is created by annealing chemically synthesized oligonucleotides.
2. Intronless β -globin plasmid (β 1,2 in Figure 1, see Note 1).
3. ApaI.
4. Antarctic phosphatase (New England BioLabs).
5. T4 DNA ligase.

2.2 Transient transfection of mammalian cells with reporter plasmid

1. Complete DMEM media: high-glucose Dulbecco's Modified Eagle Medium, 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum, and 1% L-glutamine.
2. Low-passage HEK293T cells cultured at 37 °C and 5% CO₂

¹ β 1,2 and other plasmids can be obtained from the Steitz lab upon request. When preparing β 1,2 plasmids, yields are typically low, even though pcDNA3 (Invitrogen) is a high-copy number plasmid.

3. 100 × 20 mm tissue culture dishes treated by vacuum gas plasma and 6-well cell culture clusters.
4. Opti-MEM media (Gibco).
5. Mirus *TransIT*®-293 transfection reagent.
6. DNA plasmids: β -globin reporters (see 2.1 and 3.1), pBluescript, and pmaxGFP.
7. Fluorescence microscope.
8. TRIzol® reagent (Ambion).

2.3 Measurement of β -globin mRNA levels by Northern blot analysis

1. MOPS (4-Morpholinepropanesulfonic acid) buffer (10×): 200 mM MOPS pH 7.0, 5 mM sodium acetate, and 10 mM EDTA. Filter solution (0.22 μ m) and store in a light-protected bottle at room temperature (see Note 2).
2. A 1.2% agarose-formaldehyde gel: Weigh 3.6 g of agarose into a 500 ml Erlenmeyer flask and add 217.5 ml of distilled water. Dissolve agarose by heating in a microwave for ~3 min. Let solution cool for ~2–3 min and then add 30 ml of 10× MOPS buffer and 52.5 ml of 37% formaldehyde. Swirl to mix contents and pour agarose solution into a 24 × 20.5 cm gel casting tray (see Note 3). Use a pipet tip to remove any air bubbles. The gel surface should be bubble-free so that the blotting membrane is in direct contact with the gel during the transfer process. Allow the gel to set for 30 min at room temperature. Caution: Formaldehyde is toxic. Pour gel in a ventilated chemical fume hood and keep covered whenever possible.
3. Gel running buffer (1×): 1× MOPS buffer (see Note 4) and 0.925% formaldehyde. Prepare 2.5 L immediately before use.
4. Sample-loading dye (2×): 1× MOPS buffer, 5.6% formaldehyde, 50% formamide, 0.5% bromophenol blue, 0.5% xylene cyanol, and 10 mM EDTA. Prepare appropriate volume based on number of samples immediately before use (see Note 5).
5. 0.5–10 kb RNA ladder.
6. Horizontal gel electrophoresis apparatus.
7. Peristaltic pump and tubing to circulate gel running buffer.
8. Power supply.

²When preparing MOPS buffer, adjust pH with 10 M NaOH if using the free acid form (MW = 209.3 g/mol) or with concentrated HCl if using the sodium salt form (MW = 231.2 g/mol). MOPS buffer will color with age and light exposure. Straw-colored buffer works OK but anything darker does not.

³Quantities of gel running reagents are based on a gel system supporting a 24 × 20.5 cm gel, which provides the best resolving power for polyadenylated β -globin mRNA while avoiding overlap with 18s rRNA. Proportionally scale reagents for smaller or larger gel systems.

⁴MOPS running buffer as low as 0.1× can be used for gel runs.

⁵Load small sample volumes (<12 μ l) to maintain high resolution. Try not to exceed 20 μ l of total volume. Load 5–10 μ g of total RNA (~1–2 μ l) in 8 μ l sample-loading dye.

9. SSC buffer (20×): 3 M sodium citrate pH 7.0 and 0.3 M sodium chloride. Prepare appropriate volume and store at room temperature.
10. Zeta-Probe® blotting membrane (Bio-Rad).
11. Whatman™ chromatography paper 3MM (Whatman).
12. Glass dish (23 × 33 cm), glass plate (30 × 45 cm), glass rod, parafilm, paper towels, plastic wrap, and weight (e.g. textbook).
13. UV Stratalinker 2400 (Stratagene).
14. Methylene blue staining solution (0.3%): 0.3 M sodium acetate pH 5.2 and 0.3 g methylene blue in water. Prepare 100 ml and store at room temperature. This staining solution can be reused.
15. ExpressHyb™ hybridization solution (Clontech).
16. Hybridization tubes and hybridization oven with a temperature range of 37–65 °C.
17. T4 polynucleotide kinase and [γ -³²P]ATP for 5′-[³²P]-labeling three DNA oligonucleotide probes against neomycin resistance (NeoR) mRNA: 5′-GCATCAGAGCAGCCGATTGTCTGTTG-3′, 5′-GCATCAGCCATGATGGATACTTTCTCGG-3′, and 5′-CGGCCATTTTCCACCATGATATTCGGCAAGC-3′.
18. SP6 RNA polymerase, DTT, ATP, CTP, GTP, [α -³²P]UTP, and NcoI-linearized β -globin plasmid for preparing RNA probe against β -globin mRNA.
19. Illustra MicroSpin G-25 columns (GE Healthcare) to remove unincorporated [³²P]NTPs from labeling reactions.
20. Wash buffer I: 2× SSC and 0.1% SDS in water. Prepare 1 L for each blot and store at room temperature.
21. Wash buffer II: 0.5× SSC and 0.1% SDS in water. Prepare 1 L for each blot and store at room temperature.
22. Wash buffer III: 0.1× SSC and 0.1% SDS in water. Prepare 1 L for each blot and store at room temperature.
23. Phosphorimager screen (Molecular Dynamics) and scanner (Storm 860).
24. Stripping buffer: 0.1% SDS in water. Prepare 1 L for each blot and store at room temperature.
25. Heated shaker at 65 °C.
26. Computer with appropriate software programs (e.g. ImageQuant, Microsoft Excel, GraphPad Prism, *etc.*) to quantitate mRNA levels.

3. METHODS

3.1 Construction of a β 1,2-ENE reporter plasmid

Standard cloning techniques are used to create plasmids; therefore, only the pertinent reagents and details are described. For a complete description of each step, please refer to any edition of *Molecular Cloning: A Laboratory Manual* by J. Sambrook and others (20).

PCR is used to create the insert containing the ENE region-of-interest flanked by *ApaI* sites. Phusion High-Fidelity DNA polymerase and the supplied GC buffer are used when genomic material is available. Otherwise, complementary oligonucleotides containing the ENE region-of-interest and *ApaI* sites are chemically synthesized and then annealed.

1. Digest the ENE insert and β 1,2 vector using *ApaI* according to the manufacturer's recommended protocol.
2. Because the β 1,2 vector is digested with only *ApaI*, it is subsequently treated with antarctic phosphatase per the manufacturer's recommended protocol. Removal of the 5'-phosphate minimizes the ligation of an empty vector.
3. Use an agarose gel to isolate the digested insert and the linearized, dephosphorylated β 1,2 vector.
4. Ligate the insert and β 1,2 vector using T4 DNA ligase per the manufacturer's recommended protocol. Be sure to include a vector-only control. If multiple copies of the ENE need to be inserted, then set up several ligation reactions, gradually increasing the insert:vector ratio.
5. Immediately after the ligation reaction, transform the reaction mixture into chemically competent DH5 α cells.
6. If colonies appear on the carbenicillin-treated LB agar plates, then prepare plasmids for DNA sequencing to determine the orientation of insert(s), the number of inserts, and if insert sequence is correct. The BGH reverse primer is used for DNA sequencing. Remember, β 1,2 vectors with inserts in both forward and reverse orientations are needed. Plasmids are prepared using any commercially available Maxi prep kit (see Note 1).

3.2 Transient transfection of mammalian cells with β 1,2 reporter plasmids

1. Grow low-passage HEK293T cells in complete DMEM until they are ~85–95% confluent. About 18–24 h before transfection, seed the HEK293T cells into 6-well plates. The total volume of each well is 2 ml with $\sim 6 \times 10^5$ cells/well. Distribute the cells uniformly by shaking the plate in a side-to-side and front-to-back (not circular) motion.
2. Before transfection, check the confluency of the HEK293T cells in each well. Confluency should be ~50–70%. Remove Opti-MEM media from the refrigerator and warm to room temperature.
3. Prepare plasmids for transfection by adding the following components to a sterile 1.5 ml tube immediately before transfection: 0.4 μ g β -globin reporter, 1.4 μ g

pBluescript, and 0.2 µg pmaxGFP. A total of 2 µg of plasmid DNA is transfected (see Note 6) and the total volume of plasmid should not exceed 10 µl.

4. For each sample, mix together 200 µl Opti-MEM and 6 µl Mirus *TransIT*-293 and incubate at room temperature for 15 min (see Note 7). If preparing a master mix, add an extra sample to allow for pipetting error.
5. Add 200 µl Opti-MEM-*TransIT*-293 to each DNA sample. Gently pipet up-and-down once to mix. Incubate DNA transfection complex at room temperature for 20 min.
6. Remove the 6-well plate from the incubator and add all of DNA transfection mixture dropwise to each well. Mix gently by slowly rocking the plate back-and-forth in the air, while holding rather than placing the plate on a surface, after *each* addition.
7. Incubate cells at 37 °C for 24–48 h. Examine the transfection efficiency by determining the percent of cells expressing GFP using a fluorescence microscope. The transfection efficiency of HEK293T cells is typically 80–100%.
8. Harvest cells 24–48 h after transfection. Remove medium and lyse cells by adding 1 ml of TRIzol® to each well. Isolate RNA according to the manufacturer's protocol for TRIzol®. Resuspend RNA in 10–12 µl RNase-free water and measure the A₂₆₀ value to calculate the amount of RNA in µg/µl. Yields are typically 50–70 µg of total RNA per well.

3.3 Detection of β-globin mRNA levels by Northern blot analysis

1. In a chemical fume hood, set up a horizontal gel electrophoresis apparatus by preparing a 1.2% agarose-formaldehyde gel and 1× gel running buffer (see Note 8).
2. Prepare RNA samples. Combine 10 µg of total RNA and sample-loading dye (see Note 5). For the RNA ladder, add 3 µl of 0.5–10 kb RNA ladder to sample-loading dye. Heat denature samples, including the ladder, at 95 °C for 5 min.
3. Load gel and resolve RNA at 100 V until the samples enter the gel. Meanwhile, prepare the peristaltic pump by installing the tubing and priming the tubing with leftover 1× gel running buffer. Once the samples have entered the gel, turn off the power supply and place tubing into buffer chambers so that the buffer can circulate. Buffer circulation is necessary to prevent formation of a pH gradient, whereby high pH would degrade RNA. Then, adjust the voltage for the appropriate length of run time; end the run when the bromophenol blue dye is about 1 inch from the bottom of the gel (see Note 9).

⁶All plasmid concentrations are checked by agarose gel electrophoresis prior to transfection to ensure that equal amounts of plasmid are transfected.

⁷The transfection procedure has been modified slightly from the manufacturer's protocol.

⁸Use a level to ensure that the gel is poured and run on an even surface; otherwise, RNA migrating in a thinner region of the gel will be lost.

⁹Gel run times will vary depending on the gel box; adjust accordingly. For daytime runs, increase the voltage to 140 V (~7–8 h). For nighttime runs, the voltage can be decreased to 70 V (~18 h).

4. Remove gel in casting tray and place in a clean dish. For steps 4–6, always keep the gel in the casting tray so that the large gel does not break. Add ~800 ml distilled water to the dish and place it on a shaker at room temperature for 5 min to gently wash away formaldehyde in the gel running buffer.
5. Decant water and add ~600 ml of 20× SSC. Return gel to the shaker to allow it to equilibrate for 45 min. The gel will float because of the high-salt buffer. To keep the gel submerged during equilibration, adjust the shaker RPM so that it is strong enough to keep the gel submerged but gentle enough to keep the gel intact. If gel tearing is a problem, tape the ends of the gel casting tray to restrict mobility of the gel.
6. During the equilibration step, begin to set up the capillary transfer system (Figure 3). Fill an RNase-free 23 × 33 cm glass dish with ~800 ml of 20× SSC. Set an RNase-free 30 × 45 cm glass plate on top of the glass dish so that its position is perpendicular to the glass dish (see Figure 3). Wet 2 pieces of 9 × 19 inch Whatman paper by passing the sheets under the glass plate and pulling them over to rest atop the glass plate. Both ends of the Whatman paper should be submerged in the buffer. Smooth the Whatman paper surface by rolling a glass rod over any air pockets. Fold a roll of paper towels accordion style and set aside until ready for use. Cut Zeta-Probe® blotting membrane and 4 pieces of Whatman paper.
7. After the 45-min equilibration period, decant buffer off the gel and use a razor blade to trim away excess gel. The bromophenol blue defines the lower boundary; ~2 inches above the xylene cyanol band is the upper boundary. Cut one corner of the gel to track gel orientation. Carefully transfer the gel to the center of the wetted Whatman paper (see Note 10). Outline, but do not cover, the gel with strips of parafilm so that the surface of the wetted Whatman paper is completely covered. Importantly, there should be no direct contact between the SSC-wetted Whatman paper and the stack atop the membrane. Otherwise, transfer of RNA from the gel to the membrane will be inefficient due to a short-circuiting effect.
8. Wet the Zeta-Probe® blotting membrane, whose dimensions are cut so that it is ~1 cm wider than the gel, in distilled water. Carefully, center the membrane and place it on top of the gel so that the membrane completely covers the gel. Do not move the membrane once it is in contact with the gel. Cut the membrane in the same corner as the gel to track orientation. Wet 2 pieces of Whatman paper, whose dimensions are ~1 cm wider than the membrane, in distilled water. Carefully center the wetted papers and place them on top to completely cover the membrane. Roll a glass rod over the wetted papers and membrane to remove any air pockets so that the membrane surface is in direct contact with the gel surface.

¹⁰Because the RNA sample sinks to the bottom upon loading a horizontally run gel, the gel is inverted so that the RNA has a shorter distance to travel to the membrane. To invert gel, several gloved fingers are placed on top of both sides of the gel and the casting tray is flipped over onto the wetted Whatman paper. The gel should remain adhered to the casting tray. To peel it off, use one finger to pry away one corner until the gel falls from the casting tray onto the Whatman paper.

Carefully center 2 pieces of dry Whatman paper and place on top of the smooth, wetted Whatman paper. Center the stack of paper towels and place on top of the dry Whatman paper. Drape plastic wrap over the paper towels and glass tray. Place a weight (*e.g.* textbook) on top of the plastic wrap-covered paper towels. The plastic wrap will keep the weight from absorbing the 20× SSC buffer and minimize evaporation. Allow the transfer to proceed overnight (or at least 8 h).

9. Disassemble the transfer stack by removing each layer until the membrane is exposed. Place the membrane face up in an RNase-free dish and wash the membrane gently with distilled water to remove any salt or gel deposits. Then, transfer the membrane face up to a clean, dry piece of Whatman paper, whose dimensions are ~2 cm wider than the membrane. Allow the membrane to air dry.
10. Once the membrane is dry, UV crosslink RNA to the membrane using a Stratalinker 2400 set to 1200 $\mu\text{J}\times 100$.
11. Transfer the membrane face up to an RNase-free glass dish and cover the membrane surface with 0.3% methylene blue stain until the entire surface is stained blue. Then, decant the stain into the original bottle for re-use (see Note 11). Destain the membrane by rinsing it with distilled water several times. Destain until the ribosomal RNA (rRNA) bands (18s at 1.9 kb and 28s at 5 kb) are visible but the blue background is washed away. If rRNA bands are not visible, then the RNA has become degraded and the experiment will need to be repeated, beginning with step 1 in Section 3.2. If rRNA bands are visible, then an image of the blot is captured using a GBox or any digital camera device. Loading can also be examined. Use an ethanol-resistant marker to write the name of the experiment on the blot as well as to mark the 0.5–10 kb ladder bands, rRNA bands, and xylene cyanol and bromophenol blue dyes.
12. Place the blot in a hybridization tube (see Note 12) and add ~20 ml of ExpressHyb (see Note 13). Rotate the blot in a hybridization oven at 37 °C for at least 1 h.
13. Prepare radiolabeled probes against NeoR and β -globin mRNAs. 5'-[^{32}P]-label three DNA oligonucleotide probes (1 μM each) against NeoR mRNA using T4 polynucleotide kinase and [γ - ^{32}P]ATP according to the manufacturer's protocol. Use SP6 RNA polymerase to synthesize an antisense β -globin ribonucleotide probe in the presence of 20 mM DTT, 3.3 mM ATP, 3.3 mM CTP, 3.3 mM GTP, 5.75 μM [α - ^{32}P]UTP, and 100 ng/ μl NcoI-linearized β -globin plasmid. Illustra MicroSpin G-25 columns are used to remove unincorporated [^{32}P]NTPs according to the manufacturer's protocol.

¹¹The 0.3% methylene blue stain can be re-used until the stain becomes too weak to visualize rRNA.

¹²To put the membrane in a hybridization tube, roll up the blot so that face-up side faces the interior of the tube and adheres flat against the tube walls.

¹³A "homemade" hybridization solution can be used rather than ExpressHyb and results in lower background for the β -globin ribonucleotide probe. Components of the homemade hybridization solution are 50% formamide, 25 mM sodium phosphate pH 6.5, 6× SSC, 5× Denhardt's solution, 0.5% SDS, and 0.3 mg/ml carrier RNA.

14. Heat denature the pooled α -NeoR oligonucleotide probes at 95 °C for 5 min and then add probe to hybridization tube (see Note 14). Allow probes to hybridize to the membrane overnight at 37 °C.
15. On the following day, wash the membrane with wash buffer I three times for 5 min each at room temperature. After each wash, use a Geiger counter to make sure the radioactive signal is detectable. A stronger signal should be detected in the 1–1.5 kb range.
16. Next, wash the membrane with wash buffer II three times for 5 min each at room temperature. After each wash, use a Geiger counter to make sure the radioactive signal is detectable.
17. If the background is still high, the membrane can be washed with wash buffer III three times for 5 min each at room temperature. Expose the membrane to a Phosphorimager screen for 24–48 h depending on signal strength and then scan the screen using a Storm 860.
18. To remove the α -NeoR probe, wash the membrane in boiling-hot stripping buffer three times for 15 min each in a heated shaker at 65 °C. After each wash, decant the stripping buffer and check the radioactive signal using a Geiger counter. The radioactive signal should be at or below background after 3 washes.
19. Repeat steps 12 and 14–17 to probe for β -globin mRNA. However, since this probe is RNA, the prehybridization and hybridization steps are performed at 65 °C. Also, the washing procedure is modified for wash buffer III (step 17). First, wash the blot at room temperature for 15 min in 400 ml of wash buffer III. Then, wash the blot in a heated shaker at 65 °C for 15 min using 600 ml of wash buffer III that has been pre-warmed in a microwave for 3 min.
20. Expose the membrane to a Phosphorimager screen for 12–24 h depending on signal strength. Scan the screen using a Storm 860.

3.4 Quantitation of β -globin mRNA levels

1. Use ImageQuant (or another comparable program) to measure the NeoR and β -globin mRNA levels by volumetric densitometry analysis. A box of equal size is placed around the signal in each lane. Then, a box of equal size is dragged to a position below each sample; this box provides the background signal.
2. After subtracting the background signal, the β -globin signal is divided by the signal for NeoR mRNA. NeoR serves as a loading and transfection control as it is transcribed from the same plasmid as the β -globin reporter.
3. All values are normalized relative to β 1,2 (or the corresponding WT counterpart if investigating mutants), which is set to an arbitrary value of 1.

¹⁴The blot must be probed for α -NeoR mRNA first, then for the reporter mRNA because the antisense β -globin ribonucleotide probe cannot be stripped from the blot.

4. The assay is repeated at least two more times so that the mean and standard deviation can be calculated for each sample. Neighboring samples can affect relative accumulation levels. For example, a strong signal in one lane may artificially increase the signal in adjacent lanes because of signal diffusion. This problem can be minimized by skipping a lane when loading samples.

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Figure 1. Schematic of β -globin reporter plasmids

For all constructs, the backbone vector is pcDNA3; it also contains neomycin resistance and ampicillin resistance genes (not shown). Each β -globin sequence is flanked by a human CMV immediate-early promoter at the 5' end and a bovine growth hormone polyadenylation (BGH pA) signal at the 3' end. There is a FLAG tag (not shown) at the N-terminus of the β -globin sequence. Introns are represented by black lines, exons are represented by boxes, PTC denotes the premature termination codon, and the RNase P cleavage site is denoted by an arrowhead. Lengths (in nts) of introns and exons are given below. All ENEs (green boxes) are inserted into the *Apa*I site in the 3'-UTR. Downstream of class II ENEs are a genomically encoded A-rich tract (purple box) and a tRNA-like sequence (orange box). All plasmids have been described in detail previously (2,4,6,7).

Figure 2. Schematic of ENE+A structures

Triple-helical ENE+A structures are schematized for representative examples of class I and class II ENEs: KSHV PAN ENE bound to the 3'-poly(A) tail and MALAT1 ENE bound to its A-rich tract. The U-rich internal loops are in green font, while the poly(A) tail and A-rich tract are in purple font. Base pair and base triple interactions are defined in the legend. Nucleotide numbers at the 5' end are from the native transcripts.

Figure 3. Schematic of capillary transfer of RNA from agarose gel to blotting membrane
RNA migrates from the agarose gel to the blotting membrane by a moving phase of 20×
SSC buffer. Refer to Section 3.3 steps 6–8 for a detailed description of how to assemble this
apparatus.

Table 1

ENE sequences tested in the β 1,2 reporter and fold increase in RNA levels.

<i>Class I: ENE + poly(A) tail</i>			
ENE ¹	Relative RNA Levels ²	Sequence of ENE Insert (5'-3') ³	Ref.
KSHV PAN	9 ± 2	TGTTTTGGCTGGGTTTTCTTGTTCGCACCGGACACCTCCAGTGACC AGACGGCAAGGTTTTATCCCAGTGTATATT	(6)
RRV PAN	3 ± 1	AGACGTTTGTGTGGTTTTATGACCAGCTTGGTACAAAACCTGCTG GTGATTTTTACCCAACAATAATT	(6)
EHV2 PAN	2.0 ± 0.4	AAAGAATATTTTAAAGACTTTTTCCCCAACCTCTGGGTGGGTTT TTCTCTTTAAATATTCAATC	(6)
<i>C.c. bracovirus</i>	1.7 ± 0.2	TTCATCAAGGAGGTTTTCCCAGCCTAGCTGGGTCTCTCTTTGG GG	(6)
PSIV	1.10 ± 0.03	CATTGGCAGTAGAGTTTTCCCAGGGAGCTTCACTGTCTGGGTTTC TCTACTTATCAC	(6)
<i>Class II: ENE + A-rich tract</i>			
Human MALAT1	45 ± 7	TAGGGTCATGAAGGTTTTCTTTCTTCTGAGAAAACAACGTATTGTT TTCTCAGGTTTGCTTTTGGCCCTTTTCTAGCTTAAAAAAAAAAAA GCAAAAGATGCTGGTGGTTGGCACTCCTGGTTCCAGGACGGGGTTCA AATCCCTGCGGCGTCTTTGCTTTGACT	(7)
Human MEN β	6 ± 1	GCCGCCGCAGGTGTTCTTTTACTGAGTGCAGCCCATGGCCGCACTCAGGTT TTGCTTTTACCTTCCCATCTGTGAAAGAGTGAGCAGGAAAAAGCAAAA GGCGCTGGTGGTGGCAGTCCAGCACGGCTGGGCCGGGGTTTCAGTCCCCGC AGTGTGCTGCTTCCTT	(7)

¹RRV is rhesus rhadinovirus; EHV2 is equine herpesvirus 2; *C.c.* is *Contesia congregata*; PSIV is *Plautia stali* intestine virus.

²Relative RNA levels are the fold increase in RNA levels relative to WT β 1,2. All RNA levels were determined in HEK293T cells. All values are for a single ENE insert.

³The U-rich internal loops of ENEs are shown in green; A-rich tracts are purple; and tRNA-like sequences are orange.