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A conserved RNA polymerase III promoter required for gammaherpesvirus TMER transcription and microRNA processing

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Abstract

Canonical RNA polymerase III (pol III) type 2 promoters contain a single A and B box and are well documented for their role in tRNA and SINE transcription in eukaryotic cells. The genome of Murid herpesvirus 4 (MuHV-4) contains eight polycistronic **t**RNA-**m**icroRNA **e**ncoded **R**NA (TMER) genes that are transcribed from a RNA pol III type 2-like promoter containing triplicated A box elements. Here, we demonstrate that the triplicated A box sequences are required in their entirety to produce functional MuHV-4 miRNAs. We also identify that these RNA pol III type 2-like promoters are conserved in eukaryotic genomes. Human and mouse predicted tRNA genes containing these promoters also show enrichment of alternative RNA pol III transcription termination sequences and are predicted to give rise to longer tRNA primary transcripts.

Keywords

A box; Gammaherpesvirus 68; tRNA; type 2 promoter

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SUPPLEMENTARY DATA

Supplementary Data:

Supplementary Tables 1-2, Supplementary Figures 1-2, and Supplementary Figure Legends.

1. INTRODUCTION

In eukaryotic cells, RNA polymerase III (pol III) functions to generate a wide variety of essential, abundantly transcribed, small noncoding RNA transcripts. These include tRNAs, 5S rRNA, U6 snRNA, RNase P RNA, short interspersed repeated DNA elements-encoded RNAs (SINEs), and many others (Dieci et al., 2007). RNA pol III transcription utilizes a variety of promoter elements, often found intragenically, that can be categorized into three distinct promoter systems: type 1 (internal A and C boxes paired with an intermediate element), type 2 (internal A and B boxes), and type 3 (external, upstream elements consisting of the distal sequence element, the proximal sequence element, and the TATA box) (Dieci et al., 2007).

Transcription of eukaryotic tRNA genes uses the type 2 promoter system comprised of an A box (TRGYNNARNNG) and a B box (RGTTCRANTCC), variably spaced from one another by ~30-60bp in a gene-specific manner. This spacing variation reflects the inclusion of intron sequences found in several tRNA genes (Dieci et al., 2007; Hamada et al., 2001; Paule and White, 2000; Schramm and Hernandez, 2002). Transcription initiation in this promoter system begins with TFIIIC recognition of the A and B box followed by sequential accumulation of additional transcription factors, including the TFIIIB transcription factor complex to position RNA pol III at the start site of transcription. Initiation of transcription typically begins ~7-20bp upstream of the A box promoter element (Orioli et al., 2011a; Paule and White, 2000; Schramm and Hernandez, 2002). In mammals, canonical termination of RNA pol III transcribed genes occurs at a run of four or more thymidine residues in the coding DNA strand (Bogenhagen and Brown, 1981).

Of interest, there are several examples of viruses that utilize the RNA pol III type 2 promoter system in the production of small noncoding viral RNAs. These include the adenovirus VA transcripts, the Epstein-Barr virus (EBV) EBER transcripts, and the pol III transcripts of Murid herpesvirus 4 (MuHV-4), commonly referred to as Gammaherpesvirus 68 or γHV68. During adenovirus infection, the VA transcripts are abundantly expressed and serve to attenuate the interferon response by inhibiting the cellular PKR protein, allowing for efficient viral mRNA translation and high-titer virus production (Mathews and Shenk, 1991; Thimmappaya et al., 1982). The VA transcripts also interact with the miRNA biogenesis pathway to competitively inhibit the functions of both Exportin-5 and Dicer. Dicer processing of the VA transcripts yields miRNA-like, small RNA fragments ~22 nucleotides long that can be incorporated into RNA-induced silencing complexes (RISCs) (Andersson et al., 2005; Aparicio et al., 2006; Lu and Cullen, 2004; Sano et al., 2006). Currently, there are no known mRNA targets of the VA derived miRNAs. Additionally, the EBER transcripts of EBV accumulate to high levels in infected cells and interact with several cellular proteins including PKR, La, and ribosomal protein L22. However, the exact role(s) of the EBERs in the outcome of infection also remain unclear (Lerner et al., 1981; Steitz et al., 2011).

The abundant RNA pol III transcripts of γHV68, which we will refer to as TMERs (**t**RNA-**m**iRNA **e**ncoded **R**NAs), were originally identified through their sequence and predicted secondary structure homology to cellular tRNAs (Bowden et al., 1997). The γHV68 TMERs

represent the first reported examples of RNA pol III transcripts serving as primary-miRNA (pri-miRNA) molecules (Pfeffer et al., 2005). Little is known about the biological significance of the TMER genes. The tRNA-like portion of these transcripts is partially processed through the cellular tRNA maturation pathway by the cleavage of the TMER transcripts by RNaseZ^L and subsequent addition of nontemplated 3'CCA ends (Bogerd et al., 2010; Bowden et al., 1997). However, the tRNA-like molecules do not appear to be aminoacylated, and the incorporation of additional base modifications, common in cellular tRNA maturation, has not been reported. Processing of the miRNAs from the TMER transcripts has been shown to be dependent on Dicer cleavage and yields miRNAs that are functional in repressing gene expression during virus infection (Bogerd et al., 2010; Diebel et al., 2010).

γHV68 is a murid gammaherpesvirus widely used as a small animal model for the human gammaherpesvirus pathogens EBV and Kaposi's sarcoma-associated herpesvirus (KSHV) (Barton et al., 2011; Blaskovic et al., 1980; Simas and Efstathiou, 1998; Speck and Virgin IV, 1999). During the original identification and analysis of the γHV68 TMER genes it was shown that these genes contained the classical RNA pol III type 2 promoter system consisting of one A box coupled with one B box (Bowden et al., 1997). The TMER transcripts were observed to be abundant during both lytic and latent infections (Bowden et al., 1997; Ebrahimi et al., 2003; Martinez-Guzman et al., 2003; Simas et al., 1999). Upon closer examination of the γHV68 TMER genes we found that they contained a complex arrangement of three overlapping A box elements paired with a single B box (Diebel et al., 2010). We refer to this RNA pol III type 2-like promoter system as the "triple A box" or RNA pol III type $2_{(3A)}$ promoter system. To differentiate between the individual A box elements, we refer to the A boxes as either the A_1 box, A_2 box, or A_3 box, with the A_1 box positioned closest to the start site of transcription. The TMER genes of γHV68 are not the only known examples of noncanonical RNA pol III type 2-like promoters. The rat vault RNA gene contains multiple B boxes for optimal RNA pol III transcription while some yeast snoRNAs and tRNA genes have been documented to use solely an A box or B box (Guffanti et al., 2006; Vilalta et al., 1994). The TMER genes, however, are currently the only known examples of RNA pol III promoters that contain a set of three overlapping A boxes.

Our previous work showed that the complete removal of the entire RNA pol III type $2_{(3A)}$ promoter system resulted in the loss of transcription of the TMER genes (Diebel et al., 2010). However, the roles of the individual elements of the RNA pol III type $2_{(3A)}$ promoter system were unknown. To determine their contribution to TMER transcription, we introduced replacement mutations into the individual A box elements in the TMER-1 and the TMER-5 genes. We constructed each mutant promoter to retain at least one unaltered A box element that could potentially pair with the unaltered B box, and we predicted that this mutation strategy would leave a minimal RNA pol III type 2 promoter intact with each A box promoter mutation made. However, we found that mutating the individual A box elements often eliminated our ability to detect both the full-length TMER transcripts as well as mature processed miRNAs produced from those transcripts. This led us to speculate that the RNA pol III type $2_{(3A)}$ promoter may represent a previously unrecorded RNA pol III promoter system.

To test this theory we looked for examples of the RNA pol III type $2_{(3A)}$ promoter system in predicted tRNA transcripts in cellular genomes and in RNA pol III transcripts of viral genomes other than γHV68. By doing so, we found predicted tRNA genes in eukaryotic genomes that contained the same RNA pol III type $2_{(3A)}$ promoter arrangement found in the γHV68 TMER genes. Additionally, we found enrichment in alternative RNA pol III transcriptional stop sites in human tRNA genes containing the RNA pol III type $2_{(3A)}$ promoter. These same tRNA genes are predicted to produce slightly longer primary tRNA transcripts as compared with predicted tRNA genes that do not contain the RNA pol III type $2_{(3A)}$ promoter system. Interestingly, we did not detect the presence of the RNA pol III type $2_{(3A)}$ promoter associated with the transcription of the EBER genes of EBV or with the VA genes of adenoviruses. While these viral genes are known to be RNA pol III transcripts, they do not form a predicted tRNA-like fold in their structure. Together, these data argue for the existence of a previously unidentified RNA pol III type 2-like promoter system that contains a complex arrangement of three overlapping A box elements coupled with a single B box. This promoter is required for the production of mature processed γHV68 miRNAs and appears to be evolutionarily conserved among eukaryotic tRNAs.

2. MATERIAL AND METHODS

2.1. RNA folding prediction and RNA modelling software

RNA secondary structures were predicted using the software RNAstructure 5.3 (Mathews et al., 2004). Predicted RNA structures were modeled into jpeg format using the XRNA software found at http://rna.ucsc.edu/rnacenter/xrna/xrna.html.

2.2. Plasmids

The construction of the pHV68-Left End Wildtype (pLE-WT) and pHV68-Left End miRNA KO (pLE-KO) plasmids were described previously (Diebel et al., 2010). Construction of the pLE-1ΔA_1, pLE-1ΔA_{2+3}, pLE-5ΔA_1, and pLE-5Δ$_{2+3}$ plasmids containing replacement mutations within the A_1 box or the A_2 and A_3 boxes from either the TMER-1 or TMER-5 gene of γHV68 and the pLE-WT.HS, pLE-1ΔA_1.HS, pLE-1ΔA_{2+3}.HS was performed using site-directed mutagenesis according to the manufacturer's protocol (Stratagene), using the primers listed in Supplemental Table 1. After the generation of each A box mutant plasmid or hard-stop mutant plasmid, the entire HV68 Left End insert was sequenced to confirm the presence of the desired replacement mutations without any off-target mutations.

The pRL-TK plasmid encodes the renilla luciferase gene under the control of the HSV thymidine kinase promoter and was used as a transfection control for dual luciferase assays. The pGL3-Control plasmid (Promega) encodes the firefly luciferase gene under the control of the SV40 promoter. All pGL3 target plasmids were constructed by inserting the appropriate miRNA target sequence into the XbaI site in the 3’ untranslated region between the stop codon of the firefly luciferase gene and the SV40 polyA tail sequence as previously described (Diebel et al., 2010). Oligos used in the construction of the pGL3 target plasmids were purchased from Integrated DNA Technologies (IDT) and are listed as XbaI M1-(X) Target Forward or Reverse in Supplemental Table 1.

2.3. Viruses and tissue culture

Mouse 3T3 fibroblasts (ATCC CRL-1658) were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS (Hyclone), 2mM L-glutamine, 10U/ml penicillin, and 10μg/ml streptomycin sulfate. Mouse 3T12 fibroblasts (ATCC CRL-164) and human 293 epithelial cells were cultured in DMEM supplemented with 5% FBS, 2mM L-glutamine, 10U/ml penicillin, and 10μg/ml streptomycin sulfate.

γHV68 strain WUMS (ATCC VR-1465) and γHV68Δ9473 were used for all infections (Clambey et al., 2002). Virus stocks used for infection were passaged, grown, and titer determined on 3T12 cells as previously described (Virgin et al., 1997). Infections used for total RNA isolation were conducted in 6 well plates. One day prior to infection, 2mls of 2×10^5 cells/ml were plated per well, and the following day, the cells were either mock infected or infected with γHV68 or γHV68Δ9473 at an MOI of 1. Cells were harvested for total RNA isolation at 24 hours post-infection.

2.4. Transfections

All transfections were performed using Lipofectamine 2000 (Invitrogen), per manufacturer's protocol. Transfections for the recovery of total RNA were conducted in 6 well plates. One day prior to transfection, 2mls of cells were plated at 2×10^5 cells/ml in antibiotic-free media. Each well was transfected with 2.2μg of each Left End plasmid and harvested for total RNA isolation at 48 hours post-transfection. Transfections for dual luciferase assays were conducted in triplicate in 24 well plates. One day prior to transfection, 500μl of cells were plated at 1.6×10^5 cells/ml in antibiotic-free media. Each well was transfected with 100ng of pRL-TK, 50ng of pGL3 (Control or target), and with 440ng of either the pLE-WT, pLE-1ΔA_1, pLE-1ΔA_{2+3}, pLE-5ΔA_1, pLE-5Δ$_{2+3}$, or the pLE-KO plasmid. Alternatively, 4 hours post-transfection with the pRL-TK and pGL3 plasmids, cells were infected at an MOI of 1 with either γHV68 or γHV68Δ9473 viruses. Forty-two to 48 hours post-transfection cells were harvested and cell lysates used for the dual luciferase assays.

2.5. Total RNA isolation

Total RNA was extracted from cells using the *mir*Vana miRNA Isolation kit (Applied Biosystems), using the manufacturer's recommended protocol.

2.6. Small RNA northern blot analysis

A 12% denaturing polyacrylamide gel containing 7M urea was prerun at 60mAmps for 45 minutes. Total RNA samples were prepared as follows: for RNA isolated from infected cells, 10μg of total RNA was combined with nuclease-free water (Fisher) and 2× gel loading buffer II (Applied Biosystems) to a total volume of 32μl; for RNA isolated from transfected cells, 15 – 20μg of total RNA was combined with nuclease-free water and 2× gel loading buffer II to a total volume of 90μl. All RNA samples were heated to 95°C for 5 minutes prior to gel loading and running at 30mAmps for 60-70 minutes. Gels were stained with 1μg/ml of ethidium bromide in 1× TBE for 5 minutes followed by destaining in 1× TBE for 2 minutes. Gel images were captured by a ChemiDoc XRS+ Molecular Imager (Bio-Rad) to confirm equivalent loading and integrity of the RNA samples. RNA was then transferred to

Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad) by semi-dry transfer at 500mAmps for 90 minutes in 1× TBE buffer followed by crosslinking of the membrane at 1,200 × 100μJ in a Stratalinker 1800 (Stratagene). The crosslinked membrane was prehybridized in 12mls of formamide hybridization buffer (KPL) containing 60μl of 20μg/μl sheared and denatured herring sperm DNA for 1 hour at 54 – 56°C. The 5'-biotinylated RNA probes were denatured for 10 minutes at 68°C prior to hybridization. Probe sequences are as follows: miR-M1-1 probe (5'-/biotin/AAAGGAAGUACGGCCAUUUCUA-3'), TMER-1 hard stop probe (5'-/dual biotin/AAAGUUGGACCCACUUCC-3'), and miR-M1-7-3p probe (5'-/biotin/AAUAAAGGUGGGCGCGAUAUC-3'). Following prehybridization, 12μl of 500ng/μl denatured RNA probe was added to the formamide hybridization buffer mixture and the membrane was hybridized overnight at 54°C for the miR-M1-1 probe, at 56°C for the miR-M1-7-3p probe, and at 58°C for the TMER-1 hard stop probe.

Following hybridization the membrane was washed as follows: two times with 2× SSPE / 0.5% SDS for 15 minutes at room temperature, two times with 2× SSPE / 0.5% SDS for 30 minutes at 47 – 48°C, and a final time with 1× SSPE for 5 minutes at room temperature. The hybridized probes were detected using the KPL blotting kit (no. 54-30-02) as in the manufacturer's protocol using the AP-SA conjugate at a 1:7,000 dilution. The membrane was exposed to Blue Lite Autorad Film (ISC Bioexpress) for 30 – 120 minutes.

2.7. Reverse ligation mediated RT-PCR

Ten micrograms of total RNA were combined with 50ng of either the RNA Linker Oligo or the 5'P-RNA Linker Oligo (Supplemental Table 1) for ligation and RT-PCR amplification of mature miRNA molecules as previously described, with minor modifications (Diebel et al., 2010). Briefly, the RNA-Oligo mixtures were combined with 50U of RNasin (New England BioLabs), 10U of T4 RNA Ligase 1 (New England Biolabs), 5μl of 10× T4 RNA Ligase 1 reaction buffer and brought up to 50μl total volume with nuclease-free water. The ligated RNA was then ethanol precipitated and resuspended in 100μl of nuclease-free water. 200ng of the ligated RNA was used as template for RT-PCR amplification with the following cycling conditions: 30 minutes at 50°C, 15 minutes at 95°C, 35 amplification cycles of 30 seconds at 94°C followed by 30 seconds at 48°C followed by 15 seconds at 72°C, and a single final incubation for 2 minutes and 30 seconds at 72°C. 18μl of the RT-PCR product was run on a 3% TAE-agarose gel and RT-PCR products were visualized by ethidium bromide staining. Primer sequences used in the RT-PCR amplifications are listed in Supplemental Table 1. Sequence confirmation of maturely processed miRNA products was conducted by cloning 4μl of the RT-PCR reaction using the TOPO TA Cloning Kit for Sequencing (Invitrogen).

2.8. Dual luciferase assays

All dual-luciferase assays were conducted using the Dual-Luciferase Reporter Assay System (Promega) as previously described (Diebel et al., 2010). Luciferase readings were collected using the Bio-Tek Synergy HT microplate reader (BioTek) with the photo multiplier tube (PMT) sensitivity set between 110 and 125. All data were analyzed by exporting the integral value of the reading taken from each well.

2.9. Determination of the 3A box promoter element consensus sequence in eukaryotes

All predicted tRNA sequences from the Genomic tRNA Database were downloaded as FASTA files (Lowe and Eddy, 1997). Each FASTA file was then loaded in the Geneious bioinformatics program and searched for the following 3A box motif (allowing for 2 mismatches): TRGYNNARNTGGTRGARNAGNNG (Drummond et al., 2012). A table of all of the tRNAs containing the 3A box promoter element motif with two or fewer mismatches were exported into an Excel file (Supplemental Table 2).

2.10. Best word frequency analysis

Based on a previously established protocol (Giuliodori et al., 2003), 150 bases up- and downstream from the predicted start site of transcription of all predicted human and mouse tRNA genes were analyzed for position-specific sequence enrichment. The background level of random sequence enrichment in the human genome was determined using sequence from chromosome 11 (build GRCh37) from position 118,391,300 to 118,744,099. This background level was then used to normalize the sequence enrichment values found within the human and mouse tRNA gene regions.

3. RESULTS

3.1. Features of the γHV68 RNA pol III coding locus

The γHV68 RNA pol III coding locus encompasses a region of the γHV68 genome that spans from nucleotide position 127 to nucleotide position 5,585. This region contains eight RNA pol III transcribed **t**RNA-**m**iRNA polycistronic **e**ncoded **R**NAs (TMERs) and two open-reading frames coding for the M1 and M2 proteins (Fig. 1A). Originally, each γHV68 TMER gene was predicted to be a viral tRNA-like (vtRNA) element (Bowden et al., 1997). Two of the viral tRNAs (vtRNA4 and vtRNA6) are capable of being partially processed into mature tRNA molecules through the identification of nontemplated 3'CCA additions (Bowden et al., 1997). However, these vtRNAs, as well as vtRNA3 and 5 are not aminoacylated, leaving their biological role in γHV68 infection unknown (Bowden et al., 1997). We later identified nine different pre-miRNAs encoded immediately downstream of the vtRNA genes (Pfeffer et al., 2005). Subsequent work on the γHV68 TMER genes has clearly established a relationship between RNA pol III transcription of the γHV68 vtRNAs and miRNAs as a single polycistronic RNA molecule (Bogerd et al., 2010; Diebel et al., 2010).

We previously identified a complex RNA pol III type 2-like promoter (RNA pol III type $2_{(3A)}$) consisting of a set of three overlapping A boxes coupled with a single B box associated with each TMER gene (Diebel et al., 2010). Transcription of the γHV68 TMER genes is predicted to initiate 7 nucleotides upstream from the left-most A box and terminates at both canonical and noncanonical RNA pol III transcriptional termination elements to generate transcripts approximately 100 – 200 nucleotides in length (Fig. 1B) (Bogerd et al., 2010; Diebel et al., 2010; Orioli et al., 2011b). The resulting γHV68 TMER transcripts consist of three structural domains. A 5' vtRNA followed by two stem-loop structures containing the miRNA sequences (Fig. 1,B, C). The 5' vtRNA portion of the molecule is cleaved from the stem-loops by the 3' tRNA processing enzyme, RNaseZ^L, liberating the

stem-loops for further processing by Dicer to generate mature miRNA molecules (Bogerd et al., 2010). The use of RNA pol III transcription to produce pri-miRNA transcripts containing a 5' tRNA-like domain and a requirement for RNaseZ^{L} to liberate pre-miRNA hairpins is unique to the miRNA maturation process of γHV68 (Bogerd et al., 2010). Here we focus on the role of the individual A box promoter elements within the RNA pol III type $2_{(3A)}$ system in the transcription and processing of two of the γHV68 TMER genes; TMER-1 and TMER-5, which give rise to the most biologically active γHV68 miRNAs during lytic infection and the most abundantly cloned miRNAs during latent infection *in vitro* (miR-M1-1, miR-M1-7-5p, and miR-M1-7-3p) (Diebel et al., 2010; Pfeffer et al., 2005).

3.2. Requirements of the RNA pol III type $2_{(3A)}$ promoters in the transcription of the γHV68 TMER-1 and TMER-5 genes

The RNA pol III type 2 promoter system is classically defined as two internal promoter elements; a single A box and a single B box (Paule and White, 2000; Schramm and Hernandez, 2002). Recently it was shown that positions T1, G3, A7, and G11 of the A box (TRGYNNARNNG) are fixed and invariant in highly transcribed tRNAs within the human genome (Fig. 2A) (Canella et al., 2010; Hamada et al., 2001). Scanning the γHV68 genome for A box consensus sequences in the γHV68 TMER genes demonstrated each TMER gene to contain a set of three overlapping A boxes with each individual A box containing no more than one mismatch from the A box consensus (Fig. 2A). Combining the overlapping A box promoter elements into a single 3A box consensus yields TRGYNNARNTGGTRGARNAGNNG, a sequence closely matching the consensus sequence of the A box region from the eight γHV68 TMER genes, TAGCTCAATTGGTAGAGCRNCAG (Fig. 2A and Supplemental Fig. 1). When comparing the 3A box consensus sequence of the γHV68 TMER genes to the constructed 3A box consensus sequence, only the A at position 19 and the G at position 20 are mismatched. These positions represent fixed bases within the A_3 box and A_2 box individual promoter elements, respectively. When looking at the individual γHV68 TMER genes, two of the TMER genes do contain both an A at position 19 and a G at position 20, while the other six TMER genes contain a single mismatch at either position 19 or 20. None of the TMER genes are mismatched at both positions. Assuming that the triplicated A boxes have the ability to function independently of one another, this demonstrates that each TMER gene has at minimum two completely functional A boxes at the sequence level.

To test whether the TMER genes of γHV68 can use their various A box promoter elements independently of one another, we generated A box replacement mutations consisting of an 8 nucleotide substituted stretch in the pLE-WT plasmid using site-directed mutagenesis to alter the A_1 box or the A_2 and A_3 boxes of the TMER-1 and TMER-5 genes. Each pair of replacement mutations eliminated either the consensus A_1 box, while leaving the A_2 and A_3 boxes intact, or vice versa, to generate plasmids pLE-1ΔA_1 and pLE-5ΔA_1 (A_1 box mutated in the TMER-1 and TMER-5 genes) and pLE-1ΔA_{2+3} and pLE-5ΔA_{2+3} (A_2 and A_3 boxes mutated in the TMER-1 and TMER-5 genes) (Fig. 2B). To test the capacity of the mutated promoters in transcription, we transfected these plasmids into 293 cells for total RNA isolation at 48 hours post-transfection followed by northern blot analysis, using probes

antisense to miRM1-1 or miR-M1-7-3p. As a control for TMER transcription, northern blots included total RNA from 293 cells 24 hours post-infection with either γHV68 or the viral mutant γHV68Δ9473, which lacks the section of the γHV68 genome that includes the TMER genes (Figs. 2C and 2D) (Clambey et al., 2002). Three bands were observed for both the TMER-1 and the TMER-5 transcripts. The top band represents the full-length ~200nt transcript product, the middle band at ~130nt represents the TMER transcripts ending at the alternative transcriptional stop site at the base of the first stem-loop, and the bottom band, at ~60nt, represents stem-loop 1 after being processed into pre-miRNA by RNaseZ^L. This same banding pattern is seen with the total RNA collected from 293 cells transfected with the pLE-WT plasmid (Figs. 2C and 2D). Mutation of either the A_1 box or the A_2 and A_3 boxes of the TMER-1 gene eliminated our ability to detect TMER-1 transcripts suggesting an intact RNA pol III type $2_{(3A)}$ promoter system is required for the transcription of TMER-1 (Fig. 2C). The northern blot probed with the miR-M1-7-3p probe revealed that the pLE-5ΔA_1 plasmid construct lost the ability to produce the TMER-5 transcript while the pLE-5ΔA_{2+3} plasmid construct did not. Therefore, the TMER-5 promoter system can function through the use of the A_1 box promoter alone, coupled with the B box. However, no processed stem-loop 1 band (~60 nucleotides) is detected in the cells transfected with the pLE-5ΔA_{2+3} plasmid, suggesting that the A_{2+3} box mutation in the TMER-5 gene inhibits the processing of the TMER-5 transcript, but not the transcription from the TMER-5 gene. Both the pLE-1ΔA_{2+3} and the pLE-5ΔA_{2+3} replacement mutations result in an alteration in the predicted secondary structure of their associated TMER transcripts (Supplemental Fig. 2). While we do not detect transcription of TMER-1 from the pLE-1ΔA_{2+3} plasmid, TMER-5 transcripts from the pLE-5ΔA plasmid have likely lost their ability to be recognized by RNaseZ^L $_{2+3}$ resulting in a failure to produce the pre-miR-M1-7 molecule.

Here we show that the A_1 box promoter element is essential in the production of both the TMER-1 and the TMER-5 genes while the A_2 and A_3 boxes are also necessary for the transcription of the TMER-1 gene but not the TMER-5 gene. It is worth noting that our A1 box substitution also introduces a single canonical RNA pol III stop sequence which may disrupt transcription following initiation. However, it has been demonstrated previously that the canonical run of Ts may not be sufficient for termination of RNA pol III transcription. In fact there are over 50 predicted human tRNAs that contain one or more runs of four or more Ts within the mature tRNA sequence with at least two of those tRNAs found in the “highly expressed” category of tRNAs (Canella et al., 2010). Further, it has been experimentally shown that tRNAK3 can be expressed despite containing a run of four Ts between the A and B box promoter elements (Scherer et al., 2007; Westaway et al., 1998). Therefore, termination may or may not be the explanation for why these A1 box substitution mutations fail to function in transcription. The A boxes between TMER-1 and TMER-5 are identical in the pLE-1ΔA_{2+3} and pLE-5ΔA_{2+3} plasmids, but only transcription of the TMER-5 gene is functional. This data suggests that sequence context surrounding the TMER genes influences RNA pol III type $2_{(3A)}$ promoter system function. Next, we sought to determine if there is a loss of miRNA production from the TMER-1 and the TMER-5 genes in the replacement mutation plasmids by measuring processed TMER-1 and TMER-5 associated miRNAs using biological function output and the RLM-RT-PCR assay.

3.3. A box promoter elements within the TMER-1 and TMER-5 genes are required for the production of biologically functional miRNAs

Northern blot analysis of RNA from pLE-1ΔA$_1$ and pLE-1ΔA$_{2+3}$ transfected cells revealed a loss of TMER-1 primary transcript products. Northern blot analysis of pLE-5ΔA$_1$ transfected cells also showed a loss of TMER-5 primary transcripts, while analysis of RNA harvested from pLE-5ΔA$_{2+3}$ transfected cells revealed a loss in the processing of the TMER-5 transcript, but no loss in the production of the TMER-5 primary transcript. To test for the presence TMER-1 and TMER-5 derived miRNAs, we performed RLM-RT-PCR analysis on the total RNA isolated from the 293 cells transfected with the mutant left end plasmids (Diebel et al., 2010).

Using the same total RNA that was collected for use in the northern blot analyses, each total RNA sample was ligated to either a 5’ RNA linker or 3’ RNA linker molecule and used as template for a RTPCR reaction using primers to detect fully processed γHV68 miRNAs miR-M1-1 and miR-M1-10 (encoded by TMER-1), or miR-M1-7-3p and miR-M1-12 (encoded by TMER-5). Detection of the cellular miRNA, hsa-miR-15a, was used as an endogenous cellular control. Total RNA from γHV68 and γHV68Δ9473 infected 293 cells served as positive and negative controls for the production of viral miRNA. Only RNA samples from 293 cells infected with γHV68 or transfected with the pLE-WT plasmid contained detectable viral miRNA products. The cellular miRNA, hsa-miR-15a, was detected within all RNA samples tested (Fig. 3A).

We then tested γHV68 miRNA sensor plasmids of miR-M1-1, miR-M1-10, miR-M1-7-3p, and miR-M1-12 for evidence of miRNA mediated downregulation of firefly luciferase expression using “perfect target” firefly luciferase sensor constructs. These sensors each contain the complete complement miRNA binding site in the 3’UTR region of the firefly luciferase gene found in the pGL3-Control plasmid (Fig. 3B). All firefly luciferase values are normalized to the transfection control plasmid, pRL-TK, which expresses the renilla luciferase gene and is not targeted by the γHV68 miRNAs.

Dual luciferase assays were conducted in both 3T3 and 293 cells. Infection of cells transfected with the pGL3 sensor plasmids led to a downregulation of firefly luciferase expression ranging from 10% – 35% in 3T3 cells and 10% – 70% in 293 cells (Fig. 3C). miR-M1-7-3p conferred the greatest reduction and miR-M1-12, the least. This is consistent with the reported abundance of each miRNA detected during infection in previous studies (Reese et al., 2010; Zhu et al., 2010). Within the cells transfected with the pLE-WT plasmid, we saw a similar pattern of reduction. Overall, levels of downregulation during transfection were slightly higher than those seen during infection, similar to previous findings (Diebel et al., 2010). Interestingly, in both cell types tested, when either the A$_1$ box or both the A$_2$ and A$_3$ boxes were mutated in TMER-1 and TMER-5, biological activity of the corresponding downstream miRNAs was not detected. Therefore, even though each A box mutation retains at least the minimal RNA pol III type 2 promoter element in both the TMER-1 and TMER-5 genes, the corresponding miRNAs were not detected. This is likely due to a lack of pri-miRNA transcription for the pLE-1ΔA$_1$, pLE-1ΔA$_{2+3}$, and the pLE-5ΔA$_1$ plasmids and to a lack of processing of the pri-miRNA transcript from the pLE-5ΔA$_{2+3}$ plasmid.

After establishing the requirement of the fully intact RNA pol III type $2_{(3A)}$ promoter system in the production of miRNAs from the TMER genes, we searched for the RNA pol III type $2_{(3A)}$ promoter system within predicted tRNA genes from the mouse and human genomes to analyze the evolutionary origin of this promoter system outside of the γHV68 genome.

3.4. The RNA pol III type $2_{(3A)}$ promoter system is highly conserved and is associated with noncanonical transcriptional stop elements and longer transcripts in eukaryotic genomes

To detect whether organisms other than γHV68 harbor the RNA pol III type $2_{(3A)}$ promoter system, we searched the sequences of all the predicted tRNAs from the Genomic tRNA Database using the Geneious bioinformatics program (Drummond et al., 2012; Lowe and Eddy, 1997). Analyzing only the tRNAs from the mouse genome, we found that 45 of the 433 predicted mouse tRNAs contained 3A box promoter elements with two or fewer mismatches when compared to the constructed 3A box promoter consensus sequence (Fig. 2A). This represents 10.4% of all of the predicted mouse tRNAs. Interestingly, a similar frequency of predicted tRNAs containing the RNA pol III type $2_{(3A)}$ box consensus sequence was apparent in the human genome. Here, 124 tRNAs out of a total of 1,256 predicted tRNAs (9.9%) had two or less mismatches compared to the constructed 3A box consensus sequence (summarized in Supplemental Table 2). Alignment of all the eukaryotic tRNAs that contained the 3A box promoter element revealed a highly conserved consensus (Supplemental Figure 1B).

To determine whether the presence of the 3A box promoter element is associated with previously undefined sequence context characteristics of tRNA genes, we employed a best word frequency analysis on the sequence surrounding all predicted human and mouse tRNAs. This analysis was based on a previous study which showed that there is sequence conservation among yeast tRNAs upstream from the transcriptional start site in the form of T-rich elements and TATA-like sequences (Giuliodori et al., 2003). Because there may be sequence context requirements in the function of TMER gene promoters, we sought to determine whether sequences surrounding 3A box containing tRNAs were distinct from the combined average sequence of all the human or mouse tRNAs combined. To do this, we downloaded the predicted human and mouse tRNA sequences from the UCSC Genome Browser, adding 150 additional bases up and downstream from the predicted start site of transcription of each tRNA gene. We then searched these sequences using a 9 base sliding window to determine the most common 6 base word within each 9 base window. After determining the most common 6 base word per 9 base window, we tabulated the occurrence of this word and any 6 base words containing one mismatch to the most common word per position and divided this sum by the total number of words per each window position to generate the “Best Word Frequency”. To accurately reflect the amount of sequence enrichment per position, we randomly picked 352,800 bases of sequence from human chromosome 11 for best word frequency analysis. This region of human chromosome 11 was chosen to set a background best word frequency level because it is not enriched for any particular type of genetic element. We found that the average background 6 base best word frequency per 9 base window in this region of the human genome is 0.024025298. We subtracted this value from each position of the best word frequency analysis on the human and mouse tRNA genes generated above and displayed the results in Figure 4.

As displayed in Figure 4, peaks representing the highest frequency correspond to the A box, B box, and the termination sequence regions of the mouse and human tRNAs. Interestingly, the 3A box subset of tRNAs contain a much broader and more highly enriched sequence near the A box promoter and a narrower and more distal peak for the termination sequences. The conservation of the B box promoter element is nearly identical between the two data sets. In regards to the termination sequences, this data suggests that the 3A box containing tRNA genes have a greater likelihood to produce longer primary tRNA transcripts. Specifically, when looking at all of the human tRNAs together, the position with the highest best word frequency beyond the B box region lies at base 80 downstream from the predicted start site of transcription which represents a sequence of “TTTTTT” with a score of 0.0712 (Fig. 4). Considering only the human tRNAs that have the RNA pol III type $2_{(3A)}$ promoter system, the best word frequency at base 80 is only 0.0326 and represents a sequence of “TTATTT”, a RNA pol III alternative transcriptional stop site (Orioli et al., 2011b). An additional peak at base position 104 is visible in human tRNAs containing the RNA pol III type $2_{(3A)}$ promoter system which has a score of 0.0468 and represents a canonical termination sequence of “TTTTTT” (Fig. 4). Similar results can be found in the predicted tRNAs from the mouse genome. When comparing all of the mouse tRNAs together, the position with the highest best word frequency beyond the B box region lies at base 81 downstream from the predicted start site of transcription and represents the canonical termination sequence of “TTTTTT” with a score of 0.1763 (Fig. 4). Considering only the mouse tRNAs that have the RNA pol III type $2_{(3A)}$ promoter system, there still remains a strong peak at base position 81, with another strong peak appearing at base position 93. Both positions represent canonical termination sequences of “TTTTTT” and have scores of 0.1038 and 0.0760, respectively.

The negative correlation between canonical transcriptional stop elements and 3A box containing tRNAs is intriguing due to the similarity with the γHV68 TMER genes. These features may serve as a hallmark for TMER-like transcripts in cellular genomes and could aid in identification of TMER-like transcripts within genomes other than γHV68. To determine whether a canonical termination sequence nearer to the start site of transcription could negatively impact transcription of the γHV68 TMER genes, we constructed a version of the TMER-1 transcript containing a “hard stop” run of Ts in place of the alternate stop sequence 92 bases downstream from the transcriptional start site of the TMER-1 gene. We then tested the ability of this construct to transcribe a truncated version of the TMER-1 transcript.

3.5. Incorporation of canonical transcriptional stop sites near the tRNA domain of TMER-1 does not alter the function of the RNA pol III type $2_{(3A)}$ promoter system

Based on the observation that there are conserved sequence context variations in tRNAs containing the 3A box promoter element, particularly in the RNA pol III transcriptional termination sequences of both human and mouse tRNAs, we sought to determine the role of the termination sequences on transcription of γHV68 TMER-1. To do this, we inserted point mutations into the γHV68 TMER-1 gene to convert the alternative transcriptional stop site (position 222 in the γHV68 genome, Fig. 5A) to a series of canonical transcriptional stop elements (AAC**TTTAT**CCCTATAGTG to AAC**TTTTT**CC**TTTTT**GTG). We created this

mutation in the pLE-WT, pLE-1ΔA$_1$, and the pLE-1ΔA$_{2+3}$ plasmid constructs, generating the "hard stop" (HS) mutant plasmids pLE-WT.HS, pLE-1ΔA$_1$.HS, and pLE-1ΔA$_{2+3}$.HS. Using these plasmids we investigated the mutant TMER-1 genes for their ability to generate primary transcripts.

The impact on transcription imposed by the insertion of the hard stop sequences in TMER-1 was investigated by northern blot analysis of total RNA isolated from 293 cells transfected with the various left end plasmids. To conduct these northern blots we used a probe designed to anneal to the region between the tRNA domain and the first alternate stop or hard stop sequences of the TMER-1 transcript (TMER-1 hard stop probe) (Fig. 5A). Northern blot analysis revealed that the hard stop sequences were efficient in terminating the transcription of the TMER-1 gene in the pLE-WT.HS plasmid construct (Fig. 5B). However, TMER-1 transcription was undetectable in the pLE-1ΔA$_1$.HS lane and scarce in the pLE-1Δ$_{2+3}$.HS lane, which suggests that the hard stop insertion in these constructs is not sufficient to rescue TMER transcription in the context of the mutated A boxes. To confirm the functioning of the hard stop mutation within the left end plasmid constructs we performed a RLM-RT-PCR assay to detect the presence of mature processed miRNAs miR-M1-1 and miR-M1-10 using the same total RNA used for the northern blot analysis as the starting material for these assays. RLM-RT-PCR analysis revealed that only the pLE-WT plasmid had the ability to produce miRNAs from the TMER-1 gene, as expected (Fig. 5C). Taken together, these data suggest that the function of the RNA pol III promoter system and the location of the transcriptional stop sequences appear to function independently of each other in the γHV68 TMER-1 gene.

4. DISCUSSION

Here we show that the RNA pol III type 2$_{(3A)}$ promoter system is required for the production of miRNAs from the γHV68 TMER genes. This is due, in part, to the dual roles of the promoter sequences in the production of tRNA transcripts. First, at the level of transcription, the promoters are required for the recruitment of the transcription factor TFIIIC, while second, at the level of the tRNA molecule itself, their sequence makes up the conserved D- and T-loops universally conserved within tRNA structures (Orioli et al., 2011a). Therefore, even though transcription was detected from the TMER-5 gene when the A$_2$ and A$_3$ boxes were mutated, no biologically functional miRNAs were detected. This may be due to the structural change to the tRNA-like domain of TMER-5, which could interfere with TMER-5 processing by RNaseZ^L. However, the same A box mutations in TMER-1 results in the complete loss of detectable transcription, despite a similar predicted secondary structure (Supplemental Figure 2). This data suggests that the regulation of TMER transcription may go beyond both the promoter sequence requirements and the potential TMER transcript structure making it possible that these RNA pol III transcription units may be subject to regulation not yet appreciated, including at the level of primary transcript stability. However, similar A box mutations in the other 6 TMER transcripts of γHV68 are predicted to result in either a complete loss of transcription of the TMER gene or a lack or processing of the TMER gene as shown here for TMER-1 and TMER-5.

The impact of positive selection on genetic information is important in conservation through evolution. Here we found that the RNA pol III type $2_{(3A)}$ promoter system can be found in beyond the murine gammaherpesvirus genome. One reason this may be is that this promoter may be required, or may enhance, the transcription of its associated tRNA gene. However, a general role for the requirement of the RNA pol III type $2_{(3A)}$ promoter system in transcription of human tRNA genes does not appear to exist. A recent study identifying RNA pol III transcription factors and the RNA pol III transcription machinery on human tRNA genes revealed that not all human tRNA genes appear to be transcribed at the same rate (Canella et al., 2010). In this work, Canella et al., categorized human tRNAs into four groups: tRNAs with a high transcription level, moderate transcription level, low transcription level, and then those tRNAs not predicted to be transcribed at all based on RNA pol III and RNA pol III transcription factor occupancy at these genes. Using this data, we searched for human tRNAs that contained the RNA pol III type $2_{(3A)}$ promoter system to test for enrichment of tRNA genes with the RNA pol III type $2_{(3A)}$ promoter that would correlate with one of the above categories. We found that human tRNAs containing the RNA pol III type $2_{(3A)}$ promoter system included 17 in the highly transcribed, 7 in the moderately transcribed, 15 in the lowly transcribed, and 14 in the group of tRNAs not predicted to be transcribed. Thus the transcription potential of human tRNA genes is not linked to the presence of the RNA pol III type $2_{(3A)}$ promoter system. This also suggests that the RNA pol III type $2_{(3A)}$ promoter system may be conserved in a biological role beyond functioning solely as a promoter for transcription.

We also found that human and mouse tRNAs are embedded in a very similar sequence context. That is, the 3A box promoter element containing tRNAs from the human and mouse genome are similar in context to one another, but different from the rest of the tRNA genes in these genomes. Most intriguing is that fact that the transcriptional termination sequences are diminished in the tRNAs that contain the 3A box promoter elements. This would result in tRNA transcripts with similar properties to the TMER genes of γHV68. Namely, a tRNA leader sequence followed by a longer 3' tail. Finally, human 3A box promoter element containing tRNAs also appear to be enriched in alternative transcriptional stop elements, similar to that of the γHV68 TMER genes.

When we investigated the consequence of an enhanced transcriptional stop (hard stop) in the γHV68 TMER-1 gene, we found no impact on the previously established transcription conditions of these genes. The wildtype promoters still functioned, while the mutant A box promoters did not. Therefore, the combination of alternative stop sequences or longer tRNA transcripts with the RNA pol III type $2_{(3A)}$ promoter system is not required for promoter function. While there is an association between alternative transcriptional stops and tRNA genes containing the RNA pol III type $2_{(3A)}$ promoter, it is not yet clear whether this motif will facilitate the identification of cellular TMER genes predicted to be present in cellular genomes.

Previous research has shown that the γHV68 miRNAs are transcribed and processed without the use of viral specific enzymes. This raises the significant possibility that cellular genes may also be transcribed and processed to produce functional miRNA molecules from tRNA-miRNA discistronic (TMER) RNA pol III transcripts (Bogerd et al., 2010; Diebel et al.,

2010). Beyond the processing of the γHV68 miRNAs, there is a growing body of evidence that small RNAs are derived from tRNA-like sequences and that the production of these small RNAs is dependent on cleavage of the tRNA-like molecule by the tRNA processing enzyme RNaseZ^{L}. These small RNAs, known as tRFs or tsRNAs, have recently been shown to have the ability to function as bona fide miRNA molecules, knocking down the expression of target mRNA molecules (Maute et al., 2013). These RNAs can also control translation indirectly through the apparent selective loading of RISC complexes that control the expression of target mRNAs directly (Haussecker et al., 2010; Lee et al., 2009). Importantly, these RNAs appear to play key roles in the progression of the cell cycle (Haussecker et al., 2010; Lee et al., 2009). Other evidence directly links the processing of tRNA by RNaseZ^{L} to result in RNaseZ^{L} mediated cleavage of the target mRNA using processed tRNA fragments (Elbarbary et al., 2009a, 2009b). Initially, it was shown that pol III transcription of tRNA stemloop constructs could generate synthetic siRNAs to target a gene of interest and processing of the siRNA was hypothesized to use the RNaseZ^{L} tRNA processing enzyme (Scherer et al., 2007).

Together these data show that the γHV68 TMER genes serve as alternative pri-miRNA transcripts with features that are evolutionarily conserved. These genes can be transcribed and processed using conserved cellular tRNA processing machinery independent of viral infection to produce mature, biologically functional miRNAs. These observations suggest that endogenous cellular tRNA-miRNA dicistronic transcripts may also exist and may also rely on the transcription and processing features described for γHV68 miRNA biogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5'P	5' monophosphate
AP-SA	Alkaline phosphatase conjugated streptavidin
Δ	deletion or change from wildtype
DMEM	Dulbecco's Modified Eagle Medium
EBER	Epstein-Barr virus encoded RNA

EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
FBS	fetal bovine serum
γHV68	Gammaherpesvirus 68
hsa	Homo sapiens
HS	hard-stop
IDT	Integrated DNA Technologies
jpeg	Joint Photographic Experts Group
KO	knockout
KPL	Kirkegaard & Perry Laboratories, Inc.
KSHV	Kaposi's sarcoma-associated virus
MOI	multiplicity of infection
mRNA	message RNA
miRNA	microRNA
MuHV-4	Murid herpesvirus 4
NTC	non-template control
pLE-KO	pHV68-Left End miRNA KO plasmid
pLEWT	pHV68-Left End Wildtype plasmid
PMT	photo multiplier tube
polyA	poly-adenosine
pre-miRNA	precursor-microRNA
pri-miRNA	primary-microRNA
pRL-TK	plasmid containing the renilla luciferase gene whose transcription is driven by the HSV thymidine kinase promoter
PKR	protein kinase R
pol	polymerase
RISC	RNA-induced silencing complex
RLM-RTPCR	reverse ligation-mediated – reverse transcription – polymerase chain reaction
RNase P	ribonuclease P
RNase Z^L	ribonuclease Z (long form)
rRNA	ribosomal RNA
RT-PCR	Reverse transcription – polymerase chain reaction

SDS	sodium dodecyl sulfate
SINE	short interspersed repeated DNA elements-encoded RNAs
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
SSPE (1x)	150mM sodium chloride, 10mM monosodium phosphate, 1.25mM EDTA, pH 7.4
SV40	simian virus 40
TAE	40mM Tris base, 20mM acetic acid, and 1mM EDTA
TBE	89mM Tris base, 89mM Boric acid, 2mM EDTA, pH 8.5
TFIIIB	transcription factor III B
TFIIIC	transcription factor III C
TMER	tRNA-microRNA encoded RNA
tRF	tRNA-derived RNA fragment
tRNA	transfer RNA
tRNAK3	transfer RNA for lysine (#3)
tsRNA	tRNA-derived small RNA
UCSC	University of California, Santa Cruz
UTR	untranslated region
VA	viral associated
vtRNA	viral tRNA-like
WT	wildtype
WUMS	Washington University Medical School

REFERENCES

Andersson MG, Haasnoot PCJ, Xu N, Berenjian S, Berkhout B, Akusjärvi G. Suppression of RNA Interference by Adenovirus Virus-Associated RNA. J. Virol. 2005; 79:9556–9565. [PubMed: 16014917]

Aparicio O, Razquin N, Zaratiegui M, Narvaiza I, Fortes P. Adenovirus Virus-Associated RNA Is Processed to Functional Interfering RNAs Involved in Virus Production. J. Virol. 2006; 80:1376–1384. [PubMed: 16415015]

Barton E, Mandal P, Speck S. Pathogenesis and host control of gammaherpesviruses: lessons from the mouse. Annu. Rev. Immunol. 2011; 29:351–397. [PubMed: 21219186]

Blaskovic D, Stanceková M, Svobodová J, Mistríková J. Isolation of five strains of herpesviruses from two species of free living small rodents. Acta Virol. 1980; 24:468. [PubMed: 6111210]

Bogenhagen DF, Brown DD. Nucleotide sequences in Xenopus 5S DNA required for transcription termination. Cell. 1981; 24:261–270. [PubMed: 6263489]

Bogerd HP, Karnowski HW, Cai X, Shin J, Pohlers M, Cullen BR. A Mammalian Herpesvirus Uses Noncanonical Expression and Processing Mechanisms to Generate Viral MicroRNAs. Mol. Cell. 2010; 37:135–142. [PubMed: 20129062]

Bowden RJ, Simas JP, Davis AJ, Efstathiou S. Murine gammaherpesvirus 68 encodes tRNA-like sequences which are expressed during latency. J. Gen. Virol. 1997; 78:1675–1687. [PubMed: 9225045]

Canella D, Praz V, Reina JH, Cousin P, Hernandez N. Defining the RNA polymerase III transcriptome: Genome-wide localization of the RNA polymerase III transcription machinery in human cells. Genome Res. 2010; 20:710–721. [PubMed: 20413673]

Clambey ET Iv, H.W.V. Speck SH, Virgin HW. Characterization of a Spontaneous 9.5-Kilobase-Deletion Mutant of Murine Gammaherpesvirus 68 Reveals Tissue-Specific Genetic Requirements for Latency. J. Virol. 2002; 76:6532–6544. [PubMed: 12050366]

Diebel KW, Smith AL, van Dyk LF. Mature and functional viral miRNAs transcribed from novel RNA polymerase III promoters. RNA. 2010; 16:170–85. [PubMed: 19948768]

Dieci G, Fiorino G, Castelnuovo M, Teichmann M, Pagano A. The expanding RNA polymerase III transcriptome. Trends Genet. 2007; 23:614–622. [PubMed: 17977614]

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A. Geneious v5.6. 2012

Ebrahimi B, Dutia BM, Roberts KL, Garcia-Ramirez JJ, Dickinson P, Stewart JP, Ghazal P, Roy DJ, Nash AA. Transcriptome profile of murine gammaherpesvirus-68 lytic infection. J. Gen. Virol. 2003; 84:99–109. [PubMed: 12533705]

Elbarbary RA, Takaku H, Uchiumi N, Tamiya H, Abe M, Nishida H, Nashimoto M. Human cytosolic tRNase ZL can downregulate gene expression through miRNA. FEBS Lett. 2009a; 583:3241–3246. [PubMed: 19751732]

Elbarbary RA, Takaku H, Uchiumi N, Tamiya H, Abe M, Takahashi M, Nishida H, Nashimoto M. Modulation of gene expression by human cytosolic tRNase Z(L) through 5′-half-tRNA. PLoS One. 2009b; 4:e5908. [PubMed: 19526060]

Giuliodori S, Percudani R, Braglia P, Ferrari R, Guffanti E, Ottonello S, Dieci G. A Composite Upstream Sequence Motif Potentiates tRNA Gene Transcription in Yeast. J. Mol. Biol. 2003; 333:1–20. [PubMed: 14516739]

Guffanti E, Ferrari R, Preti M, Forloni M, Harismendy O, Lefebvre O, Dieci G. A Minimal Promoter for TFIIIC-dependent in Vitro Transcription of snoRNA and tRNA Genes by RNA Polymerase III. J. Biol. Chem. 2006; 281:23945–23957. [PubMed: 16787917]

Hamada M, Huang Y, Lowe TM, Maraia RJ. Widespread use of TATA elements in the core promoters for RNA polymerases III, II, and I in fission yeast. Mol. Cell. Biol. 2001; 21:6870–81. [PubMed: 11564871]

Haussecker D, Huang Y, Lau A, Parameswaran P, Fire AZ, Kay MA. Human tRNA-derived small RNAs in the global regulation of RNA silencing. RNA. 2010; 16:673–695. [PubMed: 20181738]

Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 2011; 39:D152–D157. [PubMed: 21037258]

Lee YS, Shibata Y, Malhotra A, Dutta A. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). Genes Dev. 2009; 23:2639–49. [PubMed: 19933153]

Lerner MR, Andrews NC, Miller G, Steitz JA. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. U. S. A. 1981; 78:805–809. [PubMed: 6262773]

Lowe TM, Eddy SR. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 1997; 25:955–964. [PubMed: 9023104]

Lu S, Cullen BR. Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and MicroRNA biogenesis. J. Virol. 2004; 78:12868–76. [PubMed: 15542639]

Martinez-Guzman D, Rickabaugh T, Wu T-T, Brown H, Cole S, Song MJ, Tong L, Sun R. Transcription Program of Murine Gammaherpesvirus 68. J. Virol. 2003; 77:10488–10503. [PubMed: 12970434]

Mathews DH, Disney MD, Childs JL, Schroeder SJ, Zuker M, Turner DH. Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc. Natl. Acad. Sci. U. S. A. 2004; 101:7287–92. [PubMed: 15123812]

Mathews MBMB, Shenk T. Adenovirus Virus-Associated RNA and Translation Control. J. Virol. 1991; 65:5657–5662. [PubMed: 1920611]

Maute RL, Schneider C, Sumazin P, Holmes A, Califano A, Basso K, Dalla-Favera R. tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. Proc. Natl. Acad. Sci. U. S. A. 2013:2–7.

Orioli A, Pascali C, Pagano A, Teichmann M, Dieci G. RNA polymerase III transcription control elements: Themes and variations. Gene. 2011a

Orioli A, Pascali C, Quartararo J, Diebel KW, Praz V, Romascano D, Percudani R, van Dyk LF, Hernandez N, Teichmann M, Dieci G. Widespread occurrence of non-canonical transcription termination by human RNA polymerase III. Nucleic Acids Res. 2011b; 39:5499–5512. [PubMed: 21421562]

Paule MR, White RJ. Survey and Summary: Transcription by RNA polymerases I and III. Nucleic Acids Res. 2000; 28:1283–1298. [PubMed: 10684922]

Pfeffer SS, Sewer A, Lagos-Quintana M, Sheridan R, Sander C, Grässer FA, van Dyk LF, Ho CK, Shuman S, Chien M, Russo JJ, Ju J, Randall G, Lindenbach BD, Rice CM, Simon V, Ho DD, Zavolan M, Tuschl T. Identification of microRNAs of the herpesvirus family. Nat. Methods. 2005; 2:269–276. [PubMed: 15782219]

Reese TA, Xia J, Johnson LS, Zhou X, Zhang W, Virgin HW. Identification of Novel MicroRNA-Like Molecules Generated from Herpesvirus and Host tRNA Transcripts. J. Virol. 2010; 84:10344–10353. [PubMed: 20660200]

Sano M, Kato Y, Taira K. Sequence-specific interference by small RNAs derived from adenovirus VAI RNA. FEBS Lett. 2006; 580:1553–1564. [PubMed: 16472808]

Scherer LJ, Frank R, Rossi JJ. Optimization and characterization of tRNA-shRNA expression constructs. Nucleic Acids Res. 2007; 35:2620–2628. [PubMed: 17426139]

Schramm L, Hernandez N. Recruitment of RNA polymerase III to its target promoters. Genes Dev. 2002; 16:2593–2620. [PubMed: 12381659]

Simas JP, Efstathiou S. Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol. 1998; 6:276–282. [PubMed: 9717216]

Simas JP, Swann D, Bowden R, Efstathiou S. Analysis of murine gammaherpesvirus-68 transcription during lytic and latent infection. J.Gen.Virol. 1999; 80(Pt 1):75–82. [PubMed: 9934687]

Speck SH, Virgin HW IV. Host and viral genetics of chronic infection: a mouse model of gamma-herpesvirus pathogenesis. Curr. Opin. Microbiol. 1999; 2:403–409. [PubMed: 10458986]

Steitz, J.; Borah, S.; Cazalla, D.; Fok, V.; Lytle, R.; Mitton-Fry, R.; Riley, K.; Samji, T. Perspect. Biol. Vol. 3. Cold Spring Harb; 2011. Noncoding RNPs of viral origin..

Thimmappaya B, Weinberger C, Schneider RJ, Shenk T. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell. 1982; 31:543–551. [PubMed: 6297772]

Vilalta A, Kickhoefer VA, Rome LH, Johnson DL. The rat vault RNA gene contains a unique RNA polymerase III promoter composed of both external and internal elements that function synergistically. J. Biol. Chem. 1994; 269:29752–29759. [PubMed: 7525587]

Virgin HW, Latreille P, Wamsley P, Hallsworth K, Weck KE, Dal Canto AJ, Speck SH. Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. 1997; 71:5894–904. [PubMed: 9223479]

Westaway SK, Cagnon L, Chang Z, Li S, Li H, Larson GP, Zaia JA, Rossi JJ. Virion encapsidation of tRNA(3Lys)-ribozyme chimeric RNAs inhibits HIV infection. Antisense Nucleic Acid Drug Dev. 1998; 8:185–97. [PubMed: 9669656]

Zhu JY, Strehle M, Frohn A, Kremmer E, Höfig KP, Meister G, Adler H. Identification and Analysis of Expression of Novel MicroRNAs of Murine Gammaherpesvirus 68. J. Virol. 2010; 84:10266–10275. [PubMed: 20668074]

Highlights

- Novel RNA polymerase III promoters are required for the production of viral miRNAs.
- These promoters are conserved within tRNA genes of eukaryotic genomes.
- These promoters are overrepresented in tRNA genes lacking canonical terminators.
- These promoters may identify longer tRNA transcripts with regulatory potential.

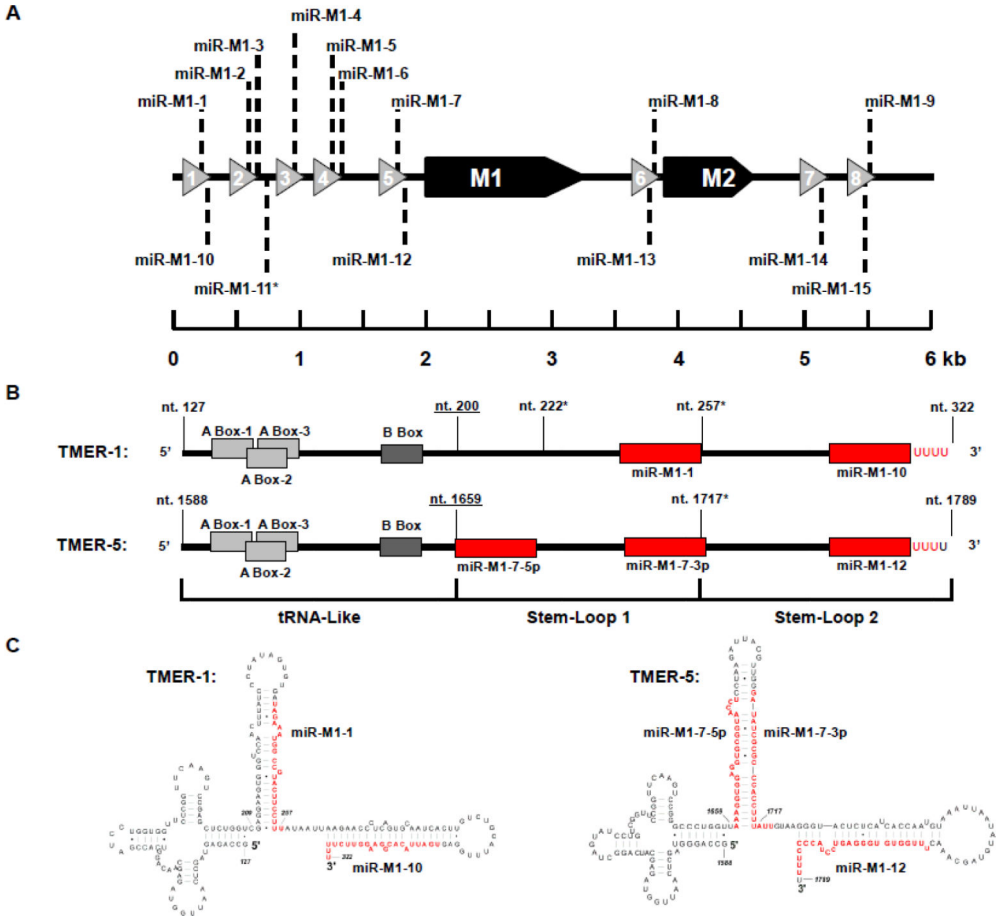

Figure 1. Genomic organization and predicted secondary structures of the γHV68 TMER genes

(A) Schematic of the left end of the γHV68 genome containing all eight of the RNA pol III transcribed TMER genes. The TMER genes are represented as grey arrows labeled 1 through 8. The protein coding transcripts M1 and M2 are shown as black arrows. Locations of the γHV68 encoded miRNA genes are shown as dashed lines and labeled miR-M1-(1-15). *miR-M1-11 is the only γHV68 miRNA that is not directly embedded within a predicted TMER primary transcript (Reese et al., 2010; Zhu et al., 2010). The miRNA genes are labeled here as found on the miRBase website (Kozomara and Griffiths-Jones, 2011). (B) Detailed schematics of the TMER-1 and TMER-5 transcripts. Shown are the predicted start and stop sites of transcription along with the predicted RNaseZ^L cut site (underlined) and alternative transcriptional stop sites (marked with an asterisk). Positions of the A box promoter elements are shown in light gray and the B box promoter elements in dark gray. miRNA locations associated with these TMER transcripts are shown by red boxes and nucleotides. The boundaries of the three structural domains of each transcript are detailed below the schematics. (C) Predicted secondary structures of the TMER-1 and the TMER-5 transcripts. The positions of the TMER associated miRNAs are highlighted in red.

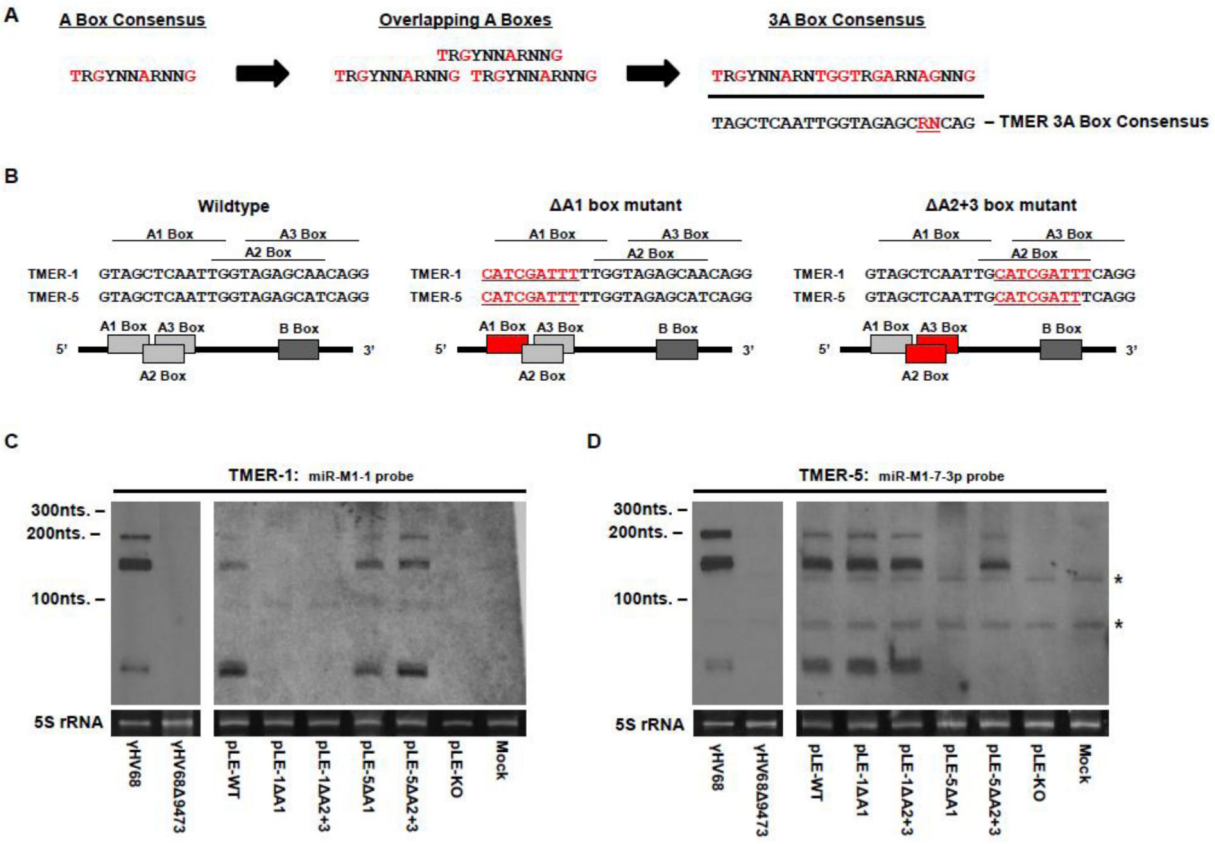

Figure 2. RNA pol III type $2_{(3A)}$ A box promoter element requirements in the transcription of the γHV68 TMER-1 and TMER-5 genes

(A) The A box promoter element consensus sequence, the overlapping A box configuration, and the 3A box consensus sequence and its comparison to the γHV68 TMER gene A box consensus sequence is shown. Bases within the A box promoter elements that are highlighted red are invariant in highly expressed human tRNAs (Canella et al., 2010). The underlined R and N bases in the TMER 3A box consensus sequence represent differences between this consensus sequence and the constructed 3A box consensus sequence above it at positions where the bases are predicted to be invariant. (B) Schematic detailing the wildtype and mutant sequences of the RNA pol III type $2_{(3A)}$ promoter sequences found within the TMER-1 and the TMER-5 genes. Red underlined bases represent replacement mutations created within each TMER gene. Below each sequence is a schematic of the resulting potential RNA pol III promoter. Light grey boxes represent wildtype A box promoter elements while red boxes represent mutated A box promoter elements. Dark grey boxes represent wildtype B box promoter elements. (C, D) Northern blot analysis of γHV68 TMER-1 and TMER-5. (C) Blot probed using a 5'-biotinylated probe designed to hybridize antisense to the miR-M1-1 sequence. (D) Blot probed using a 5'-biotinylated probe designed to hybridize antisense to the miR-M1-7-3p sequence. Two non-specific bands in the miR-M1-7-3p blot are starred to the right of the blot. Below each blot is the ethidium bromide stained 5S rRNA band from each gel photographed to gauge both RNA quality and loading prior to transfer. Labeled below each gel is the infection or transfection condition from which the total RNA was isolated.

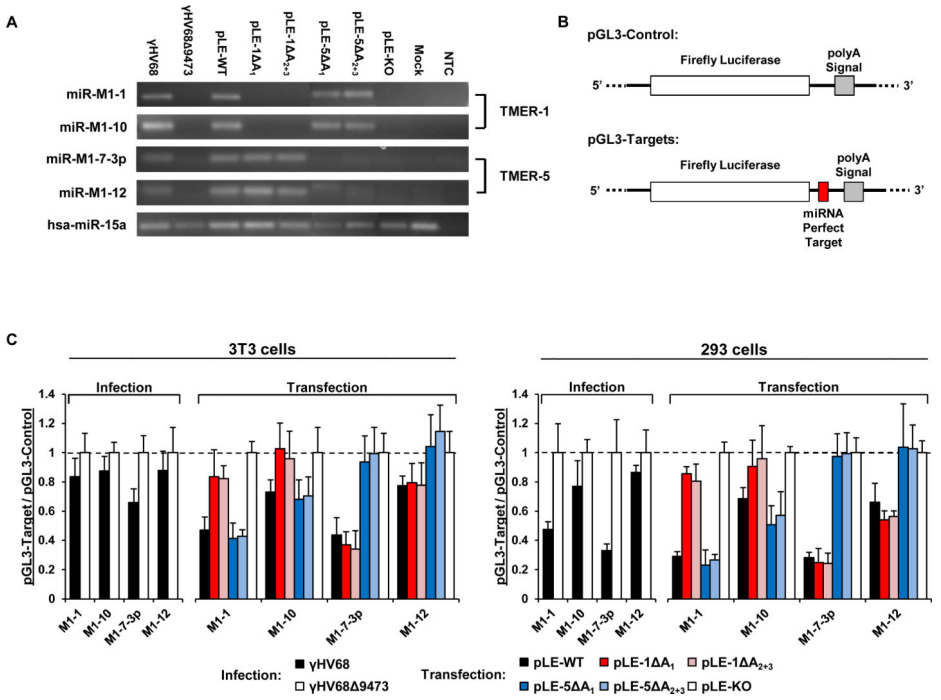

Figure 3. Mutation of the 3A box promoter elements inhibits the production of mature miRNAs

(A) RLM-RT-PCR of the viral miRNAs of TMER-1 and TMER-5. 293 cells were either infected with γHV68 or γHV68Δ9473 or transfected with the various Left End plasmid constructs followed by total RNA isolation and RLM-RT-PCR analysis. The human miRNA hsa-miR-15a is an endogenous cellular control for the RLM-RT-PCR assay. NTC – non-template control. (B) Schematics of the pGL3 firefly luciferase reporter system. A single copy of the miR-M1-1, miR-M1-10, miR-M1-7-3p, and miR-M1-12 were each individually cloned into a XbaI cut site located between the firefly luciferase coding region and the polyA signal in the pGL3-Control plasmid. (C) Dual luciferase reporter analysis with the A box mutant plasmids in 3T3 and 293 cells. The pGL3 luciferase reporter target is labeled below each graph. In infected samples, results are shown as a ratio of firefly luciferase expression in γHV68 infected cells divided by firefly luciferase expression in γHV68Δ9473 infected cells. In transfected samples, results are shown as a ratio of firefly luciferase expression in pLE-WT or A box mutant plasmids divided by the firefly luciferase expression in the pLE-KO transfected cells. All firefly luciferase values were normalized to the renilla luciferase transfection control readings prior to the comparative analysis shown on the graphs. Readings below the dashed line represent translational repression by the corresponding viral miRNA. Data represents the average of two experiments with each experiment conducted in triplicate. Error bars represent one standard of deviation.

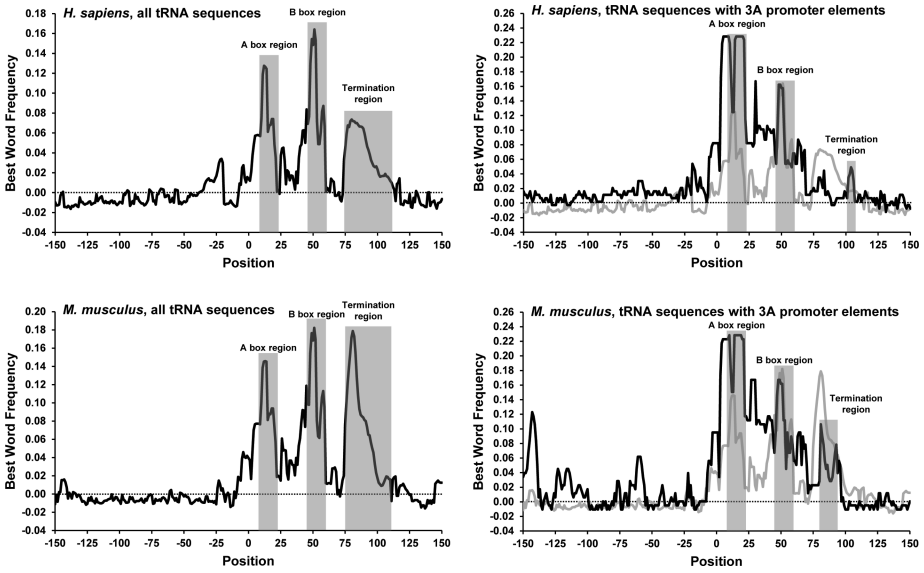

Figure 4. Sequence context comparison of human and mouse tRNAs with and without the 3A box promoter element

Histograms represent the most common 6 base word within a 9 base sliding window (Best Word Frequency) starting at 150 bases upstream from the predicted transcriptional start site and ending at 150 bases downstream from the predicted transcriptional start site. Any point above the dotted line at zero is considered to have sequence enrichment at that position. The A box and B box promoter regions and the termination regions are highlighted in light grey. The left histograms represent the best word frequency of all human and mouse tRNAs. The right histograms represent the best word frequency of human and mouse tRNAs containing the type $2_{(3A)}$ A box promoter element (black line) overlaid on top of the best word frequency of all human or mouse tRNAs (grey line).

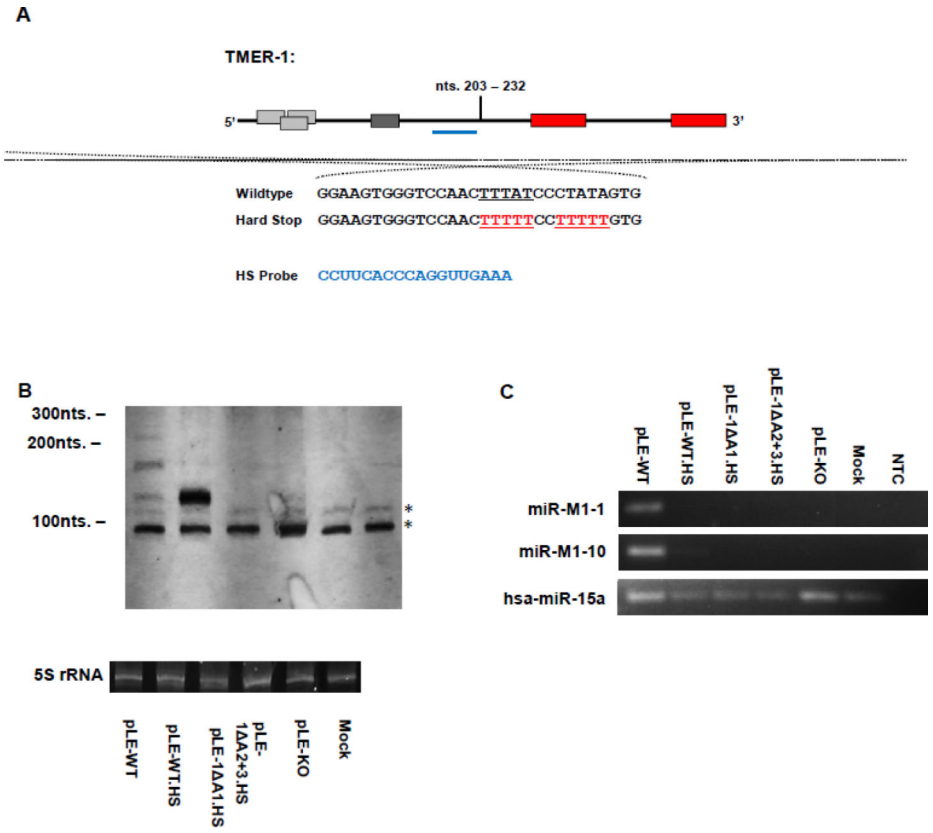

Figure 5. The impact of canonical RNA pol III termination signals near the predicted tRNA domain of TMER-1 on transcription and miRNA production from the TMER-1 gene

(A) Schematic of TMER-1. Shown is the location and sequence of the hard stop mutation. The alternative transcriptional stop location in wildtype TMER-1 is underlined in the wildtype sequence. The hard stop sequences and locations generated in the hard stop mutant plasmids are underlined and in red below the wildtype sequence. The location of the hard stop probe use for northern analysis of TMER-1 in this figure is shown as a blue line below the TMER-1 diagram. The sequence of the TMER-1 hard stop probe in shown in blue from 3' to 5'. (B) Northern blot for TMER-1 using a probe immediately downstream from the tRNA region of the TMER-1 transcript. Non-specific bands are indicated with asterisks. Below each blot is the ethidum bromide stained 5S rRNA band from each gel photographed to gauge RNA quality and loading prior to transfer. Labeled below each gel is the transfection condition from which the total RNA was isolated. (C) RLM-RT-PCR for viral miRNAs associated with the transcription of γHV68 TMER-1. The human miRNA, hsa-miR-15a is included as a cellular control for the RLM-RT-PCR assay. NTC – non-template control.