

Atypical transcription of microRNA gene fragments

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ABSTRACT

MicroRNAs (miRNAs) are short (~22 nt) RNAs that impact gene expression by sequence-specific interactions with messenger RNA or promoter sequences of genomic DNA. Ectopic expression of miRNAs can be accomplished by placing fragments of the corresponding miRNA precursor under the control of RNA polymerase II or III (RNAP II/III). Here, we report that, in the absence of exogenous promoters, DNA fragments incorporating miRNA precursors can be delivered directly into a variety of human cells and give rise to the corresponding mature miRNA. Notably, the transcription of these miRNA DNA fragments appears resistant to conventional inhibitors of RNAP I/II/III activity. Taken together, our findings suggest the existence of a previously unrecognized atypical transcription program for miRNA precursor sequences.

INTRODUCTION

Several hundred genes encoding miRNAs are currently known for the human genome (1,2). Genes encoding miRNAs are under the control of RNA polymerase II or III (RNAPII/III) (3,4). According to the current model of miRNA biogenesis, promoter occupancy leads to the generation of a long primary transcript (pri-miRNA) that is cleaved by the nuclear RNAase III enzyme Drosha into a precursor-miRNA (pre-miRNA) (5). The latter is exported to the cytoplasm where it is cleaved by the enzyme Dicer to yield the mature, single-stranded miRNA of 19–22 nt in length, the end effector of gene expression (6–9). Here, we report on the ability of a subgroup of miRNA precursors of 200–400 nt

in length to self-transcribe in the absence of exogenous promoters. In what follows, we examine the conditions under which this phenomenon occurs and present our findings from experiments with several miRNA/cell-line combinations.

MATERIALS AND METHODS

Nucleic acid constructs

Polymerase chain reaction (PCR) amplification for the creation of the various miRNA amplicons were performed in a 50- μ l reaction mixture containing forward and reverse primers at 2 μ M, 0.5 U of *pfu* DNA polymerase (Stratagene) and dNTP at 200 μ M. Constructs created in this manner are denoted as Amp^{miRNA-XX}. Human genomic DNA from HEK 293T cells (0.2 μ g) was used as the sole template for creation of Amp^{miRNA-XX} species. Primer sequences and the nucleotide length of the resulting amplicons are shown in Supplementary Table S1. The PCR reaction condition was 94°C for 3 min, 36 cycles of 94°C for 30 s, 60°C for 40 s and 72°C for 50 s and 72°C for 5 min. The PCR products were then separated by electrophoresis in a 1.5% Tris–acetate–EDTA (TAE) agarose gel, excised and gel-purified using a commercial kit (Qiagen). RNAP II- and RNAP III-promoter-driven miRNA-143 expression vectors were created by digesting the pri-miRNA-143 amplicon with BamHI and EcoR I prior to cloning into pcDNA (Invitrogen) and pSIREN-RetroQ (Clontech) vectors, respectively. The chimeric miRNA-143/125a amplicon was generated by placing miRNA-125a pri/pre sequence in a 3' PstI site in the backbone of miRNA-143. The backbone of miRNA-143 and 30a was altered such that mature and (*) species were replaced by those encoding the guide and passenger strands of siRNA targeting the

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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transactivator (tat) protein of HIV-1. These two latter constructs were directly synthesized as mini genes. Sensors for assessing miRNA/siRNA activity were created by inserting the respective antisense target sequences of small RNA species in the 3'-UTR of the *Renilla* gene of psichack-2 reporter vector (Promega) which harbors Firefly luciferase as an internal control. Amp^{miRNA-143} harboring a single nucleotide mutation in position 16 of the mature miRNA was created using the Quick-Change Site-Directed Mutagenesis kit (Stratagene). Constructs were verified by DNA sequencing. The Sanger miRBase Release 14.0 (September 2009) was used as a reference for all miRNA nomenclature/sequences. Short-hairpin RNA targeting RNAP I and mitochondrial spRNAP-IV was created by inserting the respective siRNA sequences into a generic shRNA expression vector. All siRNA sequences are shown in Supplementary Table S1. MiRNA amplicons were incubated at 37°C for 1 h with proteinase K (600 mAU/ml Qiagen) followed by enzymatic inactivation by incubation at 75°C for 20 min. Amplicons subsequently underwent agarose gel purification prior to cellular transfection. Biotinylated constructs were created by PCR by employing biotinylated primers. Amplicons were then gel purified and equal molar amounts were incubated with HEK 293T cell lysates. Streptavidin bead preparation, immobilization of amplicons and release of immobilized biotinylated molecules were performed according to the manufacturer's instructions (Dynabeads® MyOne™ Streptavidin T1). Antibody targeting RNAP II (Ab 8WG16) was used to visualize association with the respective biotinylated constructs.

Cell culture and transfections

Human cell lines used in this study included HEK 293T, Huh-7, HeLa, HCT116 and PBMC. Huh7, HEK 293T and HeLa cells were cultured in DMEM/EMEM media, respectively, supplemented with 10% (v/v) FBS and 2 mM L-glutamine. HCT116 cells were maintained in McCoy's 5A media supplemented with 10% (v/v) FBS. PBMC, obtained from an anonymous donor through the Rhode Island Blood Bank, were maintained in RPMI. Vector, PCR amplicon and siRNA transfection was performed by using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol for all cell lines. PBMC was transfected using the Amaxa system (Amaxa Biosystems). The absolute amount of DNA transfected was 2 µg/well (six-well plates) and 0.4 µg/well (24-well plates). The relative ratio of transfected product (ug) was 1:1:0.25 for vectors, siRNA and amplicons, respectively. POLR3A and irrelevant siRNA were obtained from Santa Cruz and were initially transfected into HEK 293T cells. Cells were subsequently re-seeded (at 24 h) and then transfected with the various products (at 48 h) prior to mature miRNA quantification by real-time (RT)-PCR at 72 h. Actinomycin D and α -amanitin (Sigma) were used at final concentrations of 2 µg/ml and 50 µg/ml, respectively. All experiments involving miRNA quantification were performed in duplicate, independent

experiments and two replicate measurements were associated with each experiment.

miRNA detection and quantification

Northern blot. Ten to twenty micrograms of total RNA extracted from HEK 293T cells using Trizol (Invitrogen) was resolved on 15% SequaGel (National Diagnostics) and transferred onto a GeneScreen Plus Hybridization Transfer Membrane (PerkinElmer) in 0.5 × TBE buffer. The membrane was hybridized with (γ -P³²) labeled miRNA-specific antisense LNA (Exiqon) probe overnight at 42°C in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion). The membrane was washed sequentially with 2% SSC containing 0.1% SDS three times and 0.1% SSC containing 0.1% SDS three times, 10 min for each time at room temperature, and then exposed to Kodak film overnight at -80°C. The same membrane was stripped with 0.1% SDS for 5 min by microwave oven and blocked with hybridization buffer and then hybridized with the U6 snRNA probe (TGTGCTGCCGAAGCGAG CAC) which served as a loading control.

RT PCR. TaqMan real time RT-PCR detection kits were used to quantify mature miRNA levels in accordance with manufacturer's instructions (Applied Biosystems) and 18S RNA was used for normalization.

RNA cloning. The miRCat-33 kit (IDT) was used to clone mutated mature miRNA-143 from HEK 293T cells transfected with the miRNA-143 mutant amplicon. Briefly, total RNA was extracted from cells at 48 h after transfection using Trizol (Invitrogen). Fifty micrograms of total RNA was loaded on 12% denaturing PAGE gel (7 M Urea) running in 1 × TBE at 125 V for 1.5 h. Subsequently, the small RNA fraction was gel excised and ligated to a 3' linker, re-purified and finally ligated to a 5' linker. The ligated product was amplified, sub-cloned into TOPO TA Cloning vector (Invitrogen) and then sequenced.

Luciferase assays

Cells were seeded into 96-well plates 1 day before transfection. One-hundred nanograms of psichack reporter and 25 ng Amp^{miRNA-XX} or 100 ng plasmid were transiently transfected into Huh7 or HEK 293T cells. After 48 h, luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega). *Renilla* luciferase activity was normalized with firefly luciferase activity. All sensor assays were performed as three independent experiments. For each sensor experiment, a control employing an empty vector construct (\emptyset) was used and corrected luciferase values were averaged, arbitrarily set to a value of '1' and served as a reference for comparison of fold-differences in experimental values.

5' Rapid amplification of cDNA ends

5' rapid amplification of cDNA ends (RACE) was performed for identifying the primary transcripts of Amp^{miRNA-143-mut} using the protocol suggested by the manufacturer (Invitrogen). Briefly, 1 µg total RNA was

extracted from transfected HEK 293T cells and converted into cDNA using miRNA-143 specific reverse primer (5'-accagggaactgtgtagag-3') and then purified by S.N.A.P. Column. After addition of oligo-dC tail to 3' end of the purified cDNA with TdT (Terminal deoxynucleotidyl transferase), PCR was performed with a kit - provided forward primer and nested miRNA-143 specific primer (5'-cacaagtgctgatagatggagtc-3'). Using a 1:100 dilution of the primary PCR product as template, a second PCR was carried out with the same forward primer and another nested miRNA-143 specific primer (5'-acttaccactccaggctgatg-3'). The products were sub-cloned into TOPO TA Cloning vector (Invitrogen) and then sequenced.

Statistical analysis

Mixed linear models (proc mixed, SAS version 9.2, SAS Institute, Cary, NC, USA) were used to test for differences among the various experimental conditions while accounting for replicates from each sample as having correlated error. For example, nucleic acid species copy numbers were log transformed (base 2) to stabilize variance and thereby ensure that fold changes could be modeled as additive. If conditions still had unequal variances despite log transformation, the variance was modeled for each condition individually within the model (heterogeneous variance model). The choice between homogeneous and heterogeneous variance models was based on which had the lowest Bayesian information criteria (BIC) value. The denominator degrees of freedom for models was based on the Satterthwaite method (10). Parameters were estimated based on residual estimation of maximum likelihood (REML). The *P*-values for follow-up comparisons were adjusted for alpha inflation from multiple comparisons using the Bonferroni method to maintain family wise alpha at 0.05.

RESULTS

Transcription of miRNA-143 in the absence of an exogenous promoter

Originally, we set out to study miRNA-143. Our interest in this miRNA stemmed from its low expression in a number of cancers and a desire to better understand its transcriptional control (Gao, J.S., manuscript in preparation) (11). For our experiments, we created a panel of expression vectors by employing a previously described strategy whereby a 316-nt sequence fragment that included the full-length precursor for miRNA-143 was placed under the control of a RNAP II or III (CMV or U6) promoter in a plasmid (12). In parallel, we also created PCR amplicons encompassing the RNAP II (CMV) or RNAP III (U6) promoter and the 316-nt fragment, mentioned above (Figure 1a). As expected, both constructs led to mature miRNA-143 production upon transfection into HEK 293T cells as lipoplexes. As a control to the above experiments, we introduced the 316-nt long fragment as a PCR amplicon generated from human genomic DNA (Amp^{miRNA-143}). To our surprise, this amplicon, which contained no known exogenous

promoter element, led to the production of mature miRNA-143 as assessed by both northern blot and real time PCR, albeit at levels 100-fold lower than the RNAP II/III-driven constructs (Figure 1b and c).

In order to ensure that Amp^{miRNA-143} was indeed contributing to the increased levels of mature miRNA-143, we sought to distinguish the product derived from Amp^{miRNA-143} from any endogenous miRNA-143. To this end, we introduced a single-nucleotide mutation (T→C) at position 16 from the 5' end of the mature miRNA, a location that did not affect miRNA processing or production, as confirmed by northern blot (Supplementary Figure S1a and b). RNA was isolated from cells transfected with the mutated, promoter-less construct and small RNA species were gel-excised, ligated to 5' and 3' linker molecules, PCR-amplified and introduced into cloning vectors. DNA sequencing of clones confirmed the production of the designed, 'mutated' variant of mature miRNA-143 (Supplementary Figure S1c).

While northern blots revealed RNA production from our amplicons, we next sought to identify the sequence of the primary transcript of Amp^{miRNA-143}. Thus, we used RACE to analyze the 5'-end of the primary transcript in cells transfected with the amplicon. Importantly, for these experiments, we again worked with the mutated amplicon allowing us to differentiate RACE transcripts corresponding to endogenous miRNA-143 production from transcripts derived from Amp^{miRNA-143-MUT}. These experiments revealed a complicated picture whereby five transcripts were detected (Figure 1d). As expected, one of the five transcripts was identical to the known miRNA-143 precursor, whereas the others were slightly longer. Presently, it remains unclear whether the shorter RNA molecules detected in amplicon-transfected cells represent *bona fide* primary transcript variants or result from subsequent processing of the initial longer transcript. Nevertheless, the RACE results contributed further direct evidence that the miRNA amplicon was indeed transcribed into RNA.

Determining the minimal length of Amp^{miRNA-143} that preserves its ability to self-transcribe

The amplicon used in the above-described experiments was 316 nt in length, i.e. substantially larger than the length of the corresponding miRNA-143 precursor sequence. Thus, we investigated whether shorter amplicons also had the ability to self-transcribe and lead to the biosynthesis of mature miRNA. A series of progressively shorter, PCR-generated variants of Amp^{miRNA-143} with truncations on both the 5'- and 3'-ends revealed that a minimum length of 102 nt was sufficient for production of mature miRNA-143 following transfection into HEK 293T cells as assessed by RT-PCR (Figure 2b). Of the 26 nt preceding miRNA-143* in the known miRNA-143 precursor, a minimum of 12 nt directly adjacent to miRNA-143* was required in order for the (truncated) amplicon to be functional.

The functionality of the shorter molecules allowed us to determine whether synthetic oligonucleotides encoding

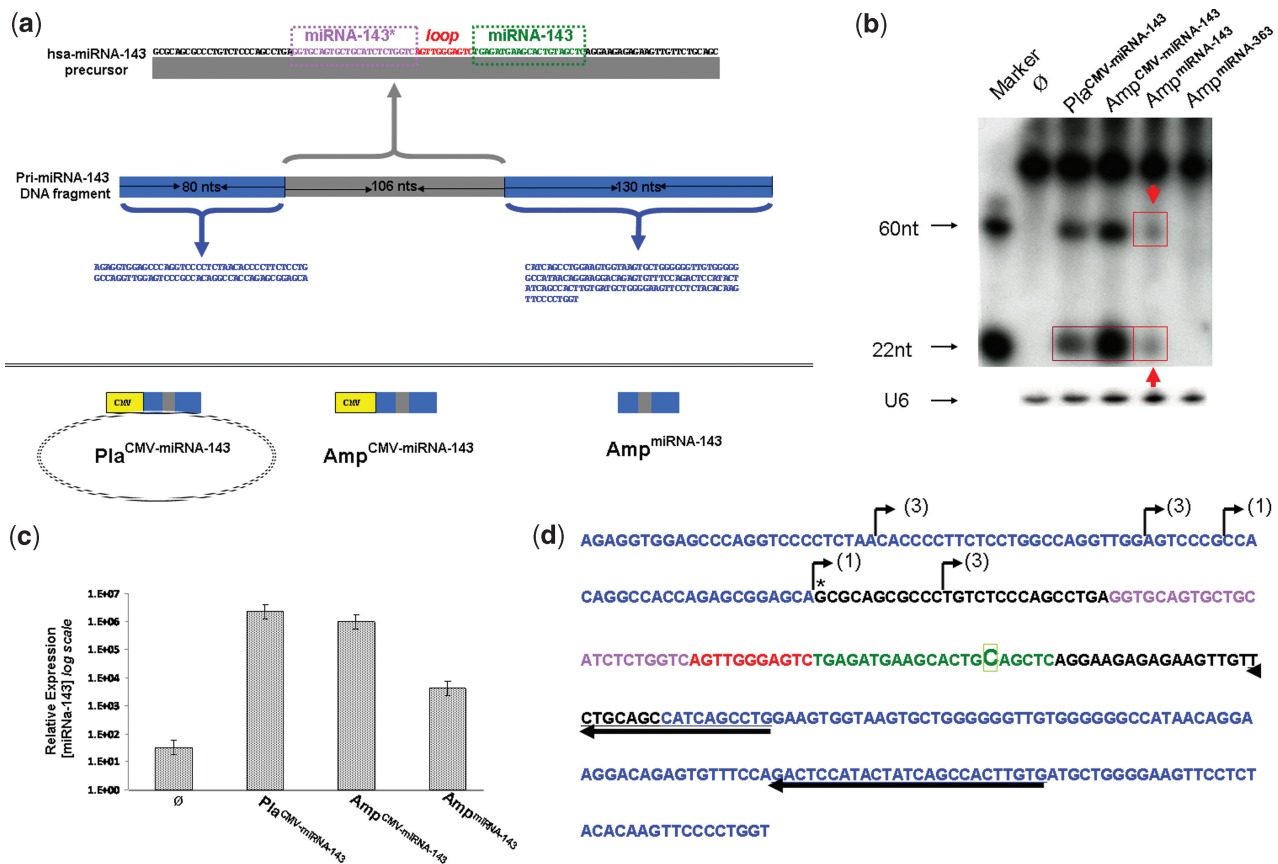


Figure 1. Detection and functional analysis of Amp^{miRNA-143}. (a) A color-coded schema of the 316-nt pri-miRNA-143 DNA fragment is shown. Blue shaded areas correspond to regions flanking the miRNA-143 precursor (gray shade). Nucleotide sequences as follows: miRNA-143 (green), miRNA-143* (purple), intervening loop (red), sequences of the precursor miRNA adjacent to the mature miRNA-143/143* (black) and sequences adjacent to miRNA-143 precursor (blue). Expression constructs were made using the pri-miRNA-143 DNA fragment. Pla^{CMV-miRNA-143} denotes a conventional plasmid in which 316 nt of pri-miRNA-143 was placed under the transcriptional control of RNAP II (CMV). Amp^{CMV-miRNA-143} refers to a PCR amplicon generated by selectively amplifying the CMV promoter and adjoining pri-miRNA-143 sequence from Pla^{CMV-miR-143}. Similar constructs were made using RNAP III (U6) promoter. Amp^{miRNA-143} was generated by selectively amplifying pri-miRNA-143 from human genomic DNA and thus did not include any exogenous promoter elements. (b, c) Mature miRNA-143 biogenesis by Pla^{CMV-miRNA-143}, Amp^{CMV-miRNA-143} and Amp^{miRNA-143} as assessed by northern blot (b) and real-time PCR (c) after introduction into HEK 293T cells. All constructs were transfected into cells as lipoplexes and RNA was harvested at 48 h. Northern blot utilized an LNA miRNA-143 probe and revealed no detectable endogenous mature miRNA-143 in cells transfected with an empty vector (∅), and variable levels produced by Pla^{CMV-miRNA-143}, Amp^{CMV-miRNA-143} and Amp^{miRNA-143}. Cells transfected with an irrelevant miRNA amplicon (Amp^{miRNA-363}) served as a negative control. M = markers corresponding to 20 and 60 nt. U6 RNA was used as a loading control. (c) Real-time PCR was used to quantify levels of mature miRNA-143. Introduction of Amp^{miRNA-143} into HEK 293T cells led to a statistically significant increase in mature miRNA levels compared to cells that had been transfected with an empty vector (∅) ($P < 0.005$) but at levels below that associated with Pla^{CMV-miRNA-143} and Amp^{CMV-miRNA-143}. All experiments were performed in duplicate and are shown as mean values and 95% CI. (d) 5' RACE results of primary miRNA-143 transcripts in amplicon transfected cells. We utilized the mutated miRNA-143 (Amp^{miRNA-143-MUT}) construct to allow differentiation from any endogenous miRNA-143 transcription. Arrows indicate the putative transcription start site(s) of Amp^{miRNA-143-MUT}. Primary sequence data revealed the presence of the single nucleotide mutation at 5' position 15 of the mature miRNA-143 (boxed) in analyzed clones. Asterisk indicates the start of pre-miRNA-143. Color scheme identical to that used in Figure 1a. Underlined sequence corresponds to reverse primers used in the nested PCR reaction. The number of clones associated with each sequence is noted in parenthesis.

miRNA-143 could also lead to mature miRNA synthesis upon cellular introduction. Oligonucleotides encoding truncated construct B (116 nt length) were commercially synthesized, annealed and transfected into HEK 293T cells. Surprisingly, Oligo^{miRNA-143-B} led to mature miRNA-143 levels comparable to those associated with the PCR generated Amp^{miRNA-143-B} (Figure 2c). All PCR-generated amplicons used human genomic DNA as a template and were gel excised and purified prior to transfection. To eliminate the theoretical possibility that bacterial proteins in the PCR reaction were transferred along with the resulting amplicons, we subjected

Amp^{miRNA-143-B} to proteinase K digestion prior to transfection. As seen in Figure 2c, such treatment had no effect upon its biosynthetic capacity.

A broad range of human cells support the atypical transcription of miRNA-143 and other miRNAs

We next sought to determine whether transcription of Amp^{miRNA-143} was restricted to certain cell types only (e.g. HEK 293T cells). Quantification of mature miRNA-143 in a variety of human cells with relatively low levels of endogenous expression (e.g. PBMC, Huh-7,

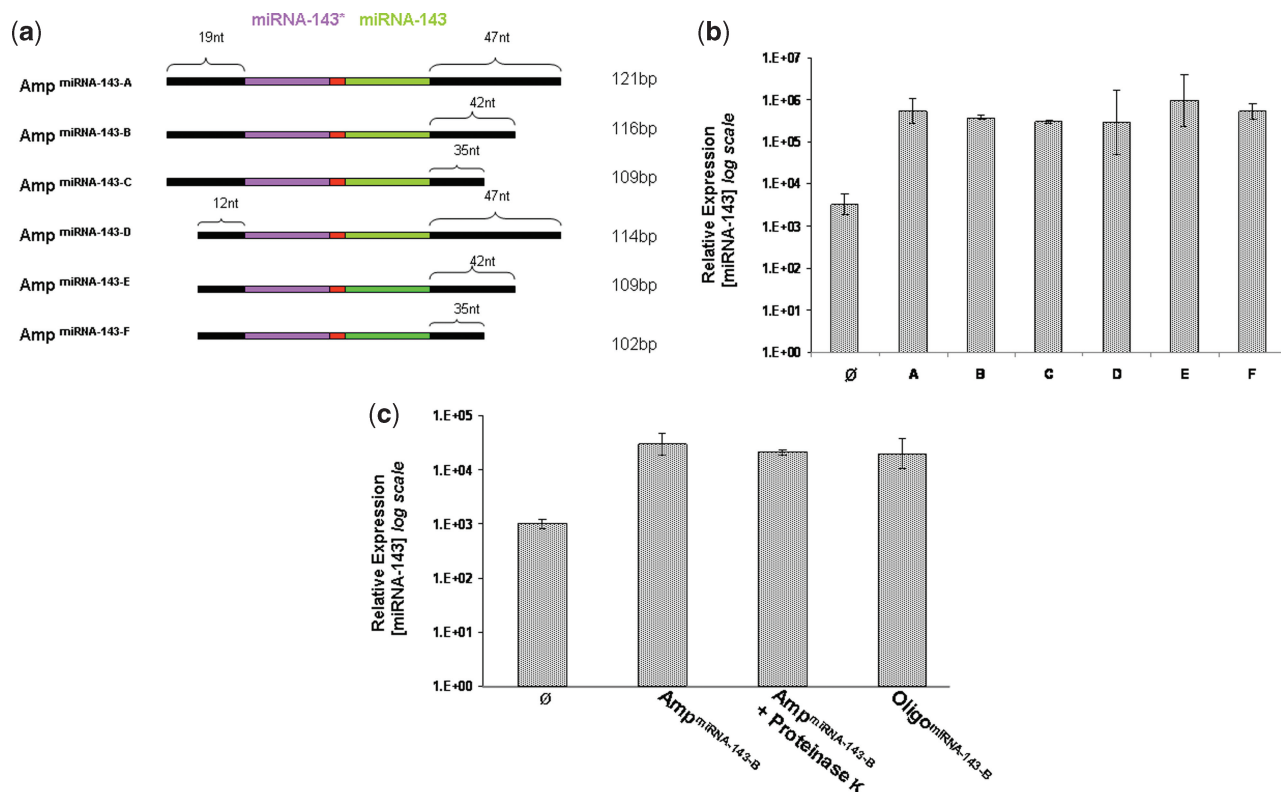


Figure 2. Transcriptional activity of shorter Amp^{miRNA-143} fragments. (a) Schema showing relative lengths of six (A–E) shorter versions of Amp^{miRNA-143} that were subsequently introduced into HEK 293T cells for real-time PCR quantification of mature miRNA-143 levels. Color scheme is the same as in Figure 1a. (b) Fragments as short as 102 nt were capable of producing mature miRNA but at levels less than the original 316-nt amplicon. All experiments were performed in duplicate and relative levels of mature miRNA-143 are shown compared to endogenous levels in cells transfected with an empty vector (Ø). Data are presented as mean values with 95% CI. All experimental conditions (A–E) were associated with a significantly higher level of mature miRNA-143 than Ø (all adjusted $P < 0.05$), and none were significantly different from one another after adjustment. (c) Oligonucleotides encoding the 116 bp of Amp^{miRNA-143-B} were commercially synthesized, annealed and introduced into HEK 293T cells. Oligo^{miRNA-143-B} retained similar biosynthetic capacity as compared to Amp^{miRNA-143-B}. Furthermore, the activity of Amp^{miRNA-143-B} was not affected by proteinase K digestion of the construct prior to cellular introduction. All experiments were performed in duplicate and mature miRNA-143 levels are shown as mean values + 95% CI, compared to cells transfected with an empty vector (Ø). All three miRNA constructs produced statistically significant levels of mature product compared to Ø (each adjusted $P < 0.001$); however, none of the three was significantly different from one another in terms of biosynthetic potential.

HeLa) that had been treated with Amp^{miRNA-143} readily confirmed its biosynthetic capacity in these cell types as well (Figure 3a). In each case, Amp^{miRNA-143} introduction was associated with a statistically significant increase in mature miRNA-143 levels compared to cells that had been transfected with an empty vector.

To determine whether atypical transcription was limited to miRNA-143, we generated and tested additional PCR amplicons comprising miRNAs with relatively low endogenous expression in HEK 293T cells: Amp^{miRNA-363}, Amp^{miRNA-145} and Amp^{miRNA-517a}. In each case, the respective amplicons produced the corresponding mature miRNA in a statistically significant fashion just like Amp^{miRNA-143} as assessed by RT-PCR (Figure 3b).

We next created a PCR amplicon for miRNA-125a, which is expressed at relatively ‘high’ levels in HEK 293T cells. Here, RT-PCR confirmed that, as expected, amplicon transfection did not lead to any appreciable increase in the already high endogenous mature miRNA levels. However, when we introduced Amp^{miRNA-125a} into

Huh-7 cells that are characterized by low endogenous levels of miRNA-125a, we observed a significant (>1000-fold) increase in its expression (Figure 3c). We also made a similar observation for the endogenously abundant, liver-specific miRNA-122. Indeed, introduction of Amp^{miRNA-122} into hepatic cell lines did not lead to any appreciable increase in the levels of mature miRNA-122. However, introduction into HEK 293T cells again led to a >1000-fold increase in the measured levels of mature miRNA-122 (Figure 3c).

Given that Amp^{miRNA-143} exhibited transcriptional ability, we reasoned that we might be able to exploit the phenomenon and allow the transcription, in tandem, of other mature miRNAs. With that in mind, we introduced the precursor sequence of miRNA-125a at the 3'-end of the original Amp^{miRNA-143} and transfected the resulting construct into Huh-7 cells. Cellular introduction of this chimeric amplicon resulted in the synthesis of mature miRNA-143 as well as of mature miRNA-125a at nearly equivalent levels (Figure 3d). Finally, northern blot was used to verify mature miRNA production in cells

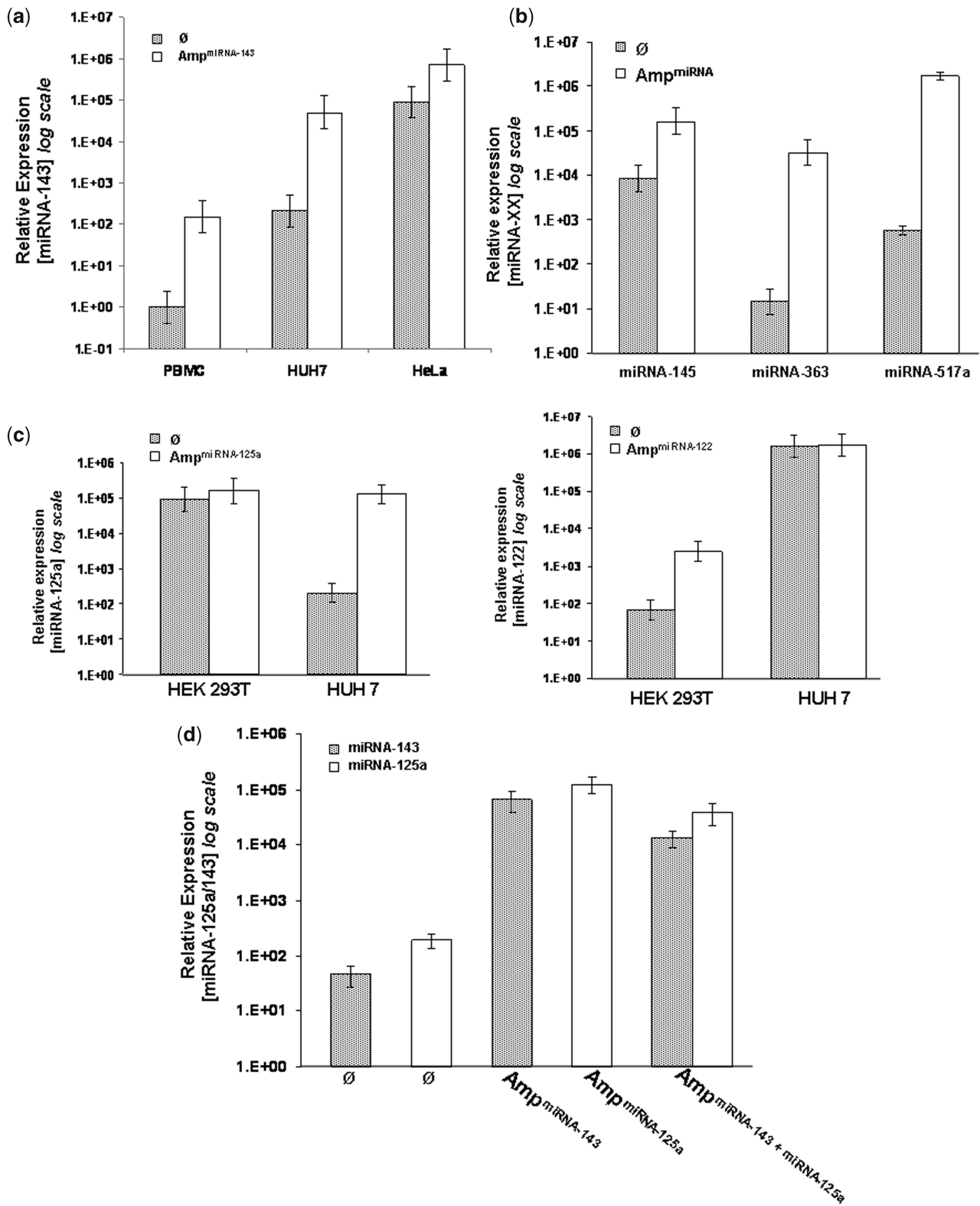


Figure 3. (a) Biosynthetic activity of Amp^{miRNA-143} in different human cells. Mature miRNA-143 levels were quantified in amplicon transfected cells and are shown relative to endogenous levels in cells transfected with an empty vector (∅) with associated 95% CI. All experiments were performed in duplicate, independent experiments. In each cell type, Amp^{miRNA-143} increased mature miRNA-143 levels in a statistically significant manner ($P < 0.05$). (b) Varied cellular activity of amplicons encoding pri-miRNA fragments of miRNA-145, 363 and 517a. All amplicons (252–339 nt) were generated from human genomic DNA and transfected into HEK 293T cells in duplicate, independent experiments. Levels of mature miRNA were quantified by real-time PCR and are expressed relative to background levels in cells transfected with an empty vector (∅) and 95% CI. Each Amp species led to a statistically significant increase in the levels of the respective miRNA compared to ∅ ($P < 0.0001$). (c) The biosynthetic potential of a given amplicon was quantifiable in cells with relatively low endogenous levels of the miRNA studied. For example,

(continued)

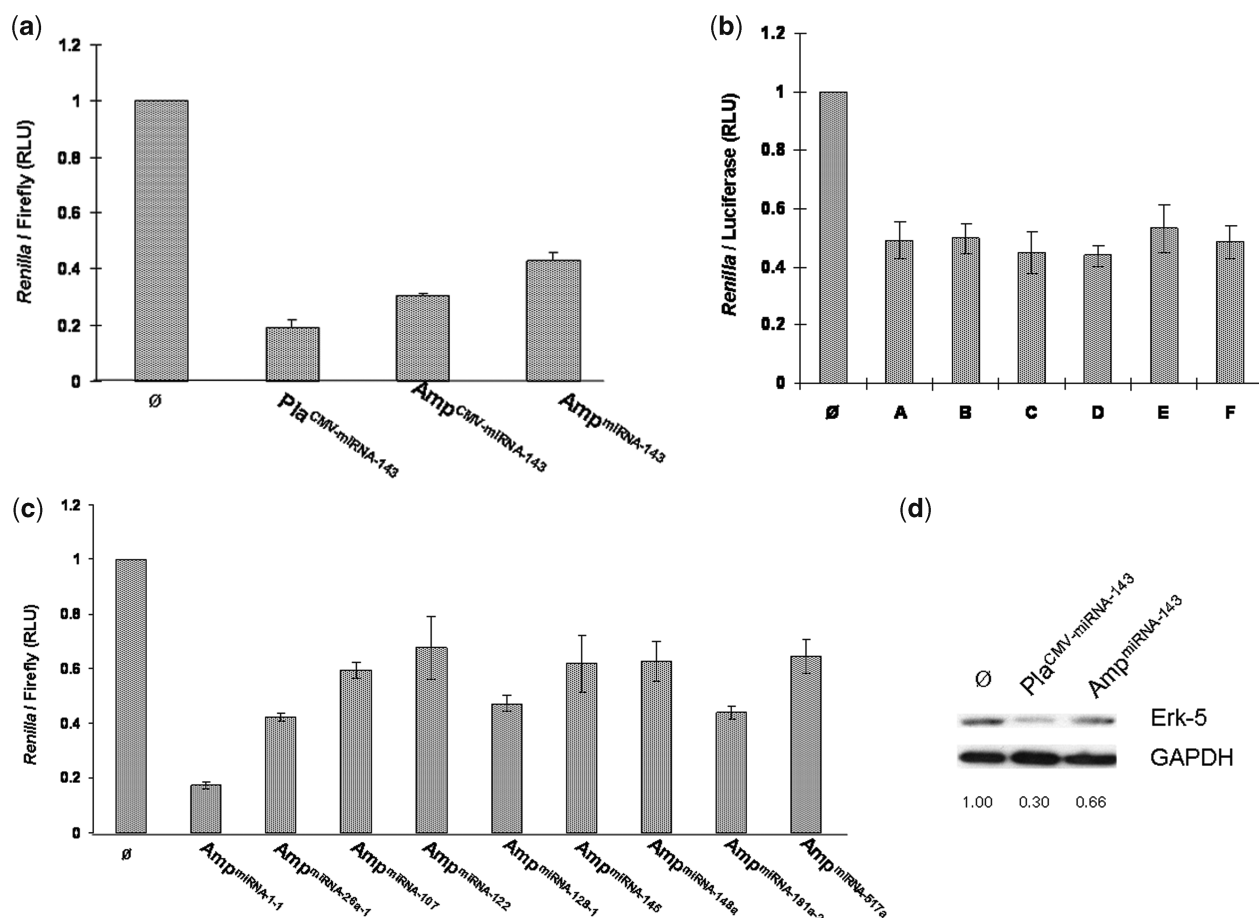


Figure 4. Functional activity of Amp^{miRNA-XX}. Luciferase-based miRNA sensor assays were used to compare the functional activity of various miRNA expression units. We created reporter constructs, in which the exact target sequence of a given miRNA was introduced into the 3'UTR of the gene encoding *Renilla* luciferase and quantified the relative reduction in luciferase levels compared to control experiments involving the same sensor but an empty vector (Ø). An internal firefly luciferase gene served to normalize data. All sensor assays were performed as three independent experiments and data are shown as mean reduction \pm SD compared to control conditions. (a) Functional activity of Amp^{miRNA-143}, Amp^{CMV-miRNA-143} and Plac^{CMV-miRNA-143}. Co-transfection of the miRNA-143 sensor and Amp^{miRNA-143} led to 57% decrease in luciferase activity, a statistically significant level ($P = 0.02$) approaching that achieved in transfections using a CMV-driven miRNA-143 expression plasmid (81% reduction) or amplicon (70% reduction) in HEK 293T cells. (b) Functional activity of shorter amplicons Amp^{miRNA-143-A/B/C/D/E/F}. All constructs retained functionality as assessed by sensor assays. Shown are statistically significant (adjusted $P < 0.05$) reductions in normalized relative light units (RLU) in cells transfected with the amplicons (A–F) in comparison to cells transfected with an empty vector (Ø). (c) Sensor assays were performed for an additional nine Amp^{miRNA-XX}. In each case, sensor activity was decreased in a statistically significant manner (adjusted $P < 0.05$) compared to experiments involving an empty vector (Ø). (d). The ERK5 protein is a target of miRNA-143. Introduction of Amp^{miRNA-143} into HEK 293T cells was associated with a reduction in ERK5 protein levels as determined by western blot. RNAP II-driven plasmid and an empty vector (Ø) served as positive and negative controls, respectively.

transfected with other amplicons including those encoding miRNA-363 and 181a-2 (Supplementary Figure S2).

Functionality of Amp^{miRNA-XX}

While quantitative assays revealed the biosynthetic activity of Amp^{miRNA-XX}, we next sought to assess functional capacity. We created miRNA sensors in which the antisense sequence of a particular mature miRNA was

placed in the 3'-UTR of the gene encoding *Renilla* luciferase. In the presence of mature miRNA, levels of *Renilla* activity decrease through the classical RNAi pathway. Co-transfection of Amp^{miRNA-143} with its sensor led to a statistically significant reduction in *Renilla* activity, albeit at levels below those attained with CMV-driven miRNA-143 expression vector (Figure 4a). Similar experiments employing the shorter variants Amp^{miRNA-143-A/B/C/D/E/F} revealed their functional

Figure 3. Continued

Amp^{miRNA-125a} significantly ($P < 0.0001$) increased levels of mature product in Huh-7 cells (miRNA-125a^{low}) but not in HEK 293T cells (miRNA-125a^{high}). Conversely, Amp^{miRNA-122} produced significantly ($P = 0.0008$) increased levels of mature product only in HEK 293T cells (miRNA-122^{low}) but not in Huh-7 cells (miRNA-122^{high}). (d) A hybrid amplicon consisting of miRNA-143 and miRNA-125a (Amp^{miRNA-125a + 143}) was capable of producing both miRNAs at levels comparable to individual expression units after introduction into Huh-7 cells. Relative levels of mature miRNA were quantified by real-time PCR in duplicate experiments and are shown as mean values and 95% CI. All Amp^{miRNA-XX} constructs including the hybrid molecule were associated with a statistically significant increase (adj $P < 0.0001$) in the respective mature miRNA levels compared to cells treated with an empty vector (Ø).

activity as well (Figure 4b). Finally, we used the sensor assay to examine the activity of nine additional Amp^{miRNA-XX}, all of which were expressed at relatively low levels in HEK 293T cells. In each case, transfection of Amp^{miRNA-XX} with its sensor led to a statistically significant reduction in Renilla activity (Figure 4c). Next, we asked whether cellular protein levels could be downregulated by exogenously introduced Amp^{miRNA-XX}. Previous work has identified ERK5 as a target of miRNA-143 (13). Treatment of HEK 293T cells with Amp^{miRNA-143} led to the relative reduction of ERK5 protein levels as seen in Figure 4d.

Of the 12 miRNA with functional Amp constructs, we noted that four have had previously detailed characterization of their promoter regions (miRNA-26a1,107,122,517a) (14, 15). Significantly, in each case, the corresponding Amp^{miRNA-XX} species did not include these promoter motifs yet still led to mature miRNA production/function upon cellular introduction.

On the atypical transcriptional nature of Amp^{miRNA-143}

To date, RNAP II/III promoters have been implicated in the transcriptional control of miRNA genes, with the majority of the studied genes being under the control of RNAP II (4,11,14–16). In an attempt to gain a better understanding of the transcriptional machinery involved in the processing of Amp^{miRNA-143}, we first sought to identify which of the currently known transcriptional programs mediated endogenous miRNA-143 production in human cells.

We used chromatin precipitation (ChIP) to look for the physical presence of RNAP II on the 2.5-kb genomic region immediately upstream of miRNA-143*. These experiments utilized HCT116 cells that produce high levels of mature miRNA-143. PCR primers amplifying ~100–200-nt fragments of the putative promoter region were immunoprecipitated with IgG against RNAP II (anti-pol II:8WG16) and revealed RNAP II localization at ~1 kb upstream relative to the pre-miRNA-143, i.e. in an area well outside of the sequence captured by Amp^{miRNA-143} (Figure 5a).

Next, we made use of the limited number of drugs that are available for differentiating between the RNAP II and III mammalian transcriptional programs. The mushroom toxin α -amanitin is known to adversely affect RNAP II/III activity in a dose-dependent manner with RNAP II activity being inhibited at far lower concentrations of drug. We thus treated HEK 293T cells with α -amanitin and transfected, in turn, with either the RNAP II/III-driven miRNA-expressing plasmids or Amp^{miRNA-143}. In the case of the plasmids, quantification of mature miRNA-143 levels 8 h post-transfection revealed differential sensitivity to α -amanitin, with RNAP II activity being inhibited by >90% and RNAP III activity being inhibited by 40%. However, and importantly, when we transfected with Amp^{miRNA-143}, α -amanitin had no effect on the amplicon's biosynthetic activity and the production of mature miRNA-143 (Figure 5b). We next assessed the effect of α -amanitin on endogenous miRNA-143

expression. Treatment of HCT116 cells with drug led to a >90% reduction in pri-miRNA-143 levels compared to control cells that had been treated with vehicle alone, as expected from our ChIP experiments that localized RNAP II to the miRNA-143 gene locus. These data in sum raised the intriguing possibility that RNAP II was not associated with the transcription of Amp^{miRNA-143}. To formally investigate this possibility, we created biotinylated versions of Amp^{miRNA-143} with and without RNAP II/III promoter sequences and incubated them in HEK 293T cell lysate. Streptavidin coated bead based retrieval of the molecules allowed us to localize RNAP II to Amp^{RNAP II-miRNA-143} but not to Amp^{RNAP III-miRNA-143} or Amp^{miRNA-143} (Figure 5c).

Next, we used actinomycin, a general and more potent inhibitor of RNA transcription that acts by intercalating between successive GC base pairs and preventing RNAP mediated elongation of the nascent transcript (17). In this case, both RNAP II- and RNAP III-driven plasmids were exquisitely sensitive to actinomycin with >97% reduction in their respective biosynthetic function. However, Amp^{miRNA-143} was less sensitive exhibiting only 50% reduction in function (Figure 5d).

The extremely compact nature of Amp^{miRNA-143} and its products led us to hypothesize that components of RNAP III machinery may be involved in its transcription (18). Furthermore, RNAP III has been recently implicated in the cytosolic transcription of a poly(dA–dT) template into 5'-ppp RNA (19). Thus, we treated cells with short interfering RNA (siRNA) targeting the largest subunit of RNAP III (POLR3A) and measured the effect on the output of our RNAP III-driven plasmid and Amp^{miRNA-143}, respectively (20). As seen in Figure 5e, siRNA-mediated silencing of POLR3A inhibited the RNAP III-driven plasmid but had no effect on Amp^{miRNA-143} in two independent experiments.

Further support for the lack of involvement of RNAP III in Amp^{miRNA-XX} transcription derived from more detailed experiments involving miRNA-517a. As mentioned, Amp^{miRNA-517a} was associated with mature miRNA production after introduction into HEK 293T cells. The genetic organization of miRNA-517a is somewhat unique in that it is a substrate for RNAP III with a concise organization including the traditional A and B boxes required for RNAP III docking (Figure 6a) (4). Importantly, our initial Amp^{miRNA-517a} was 262 nt in length and did not contain either of the known A or B boxes located upstream of its pre-miRNA sequence. We created a series of progressively longer variants of the original Amp^{miRNA-517a}, the longest of which contained both the A and B boxes. Interestingly, segmental truncations of Amp^{miRNA-517a}, with partial or complete removal of the A and B box motifs, also led to detectable levels of functional mature miRNA-517a, albeit at levels far lower than the two amplicon variants (#3 and #4) that included both boxes (Figure 6b and c). Not surprisingly, cells treated with a siRNA targeting POLR3A exhibited a severely compromised ability to transcribe the miRNA-517a variants that contained either or both of the A and B boxes. However, in the presence of the

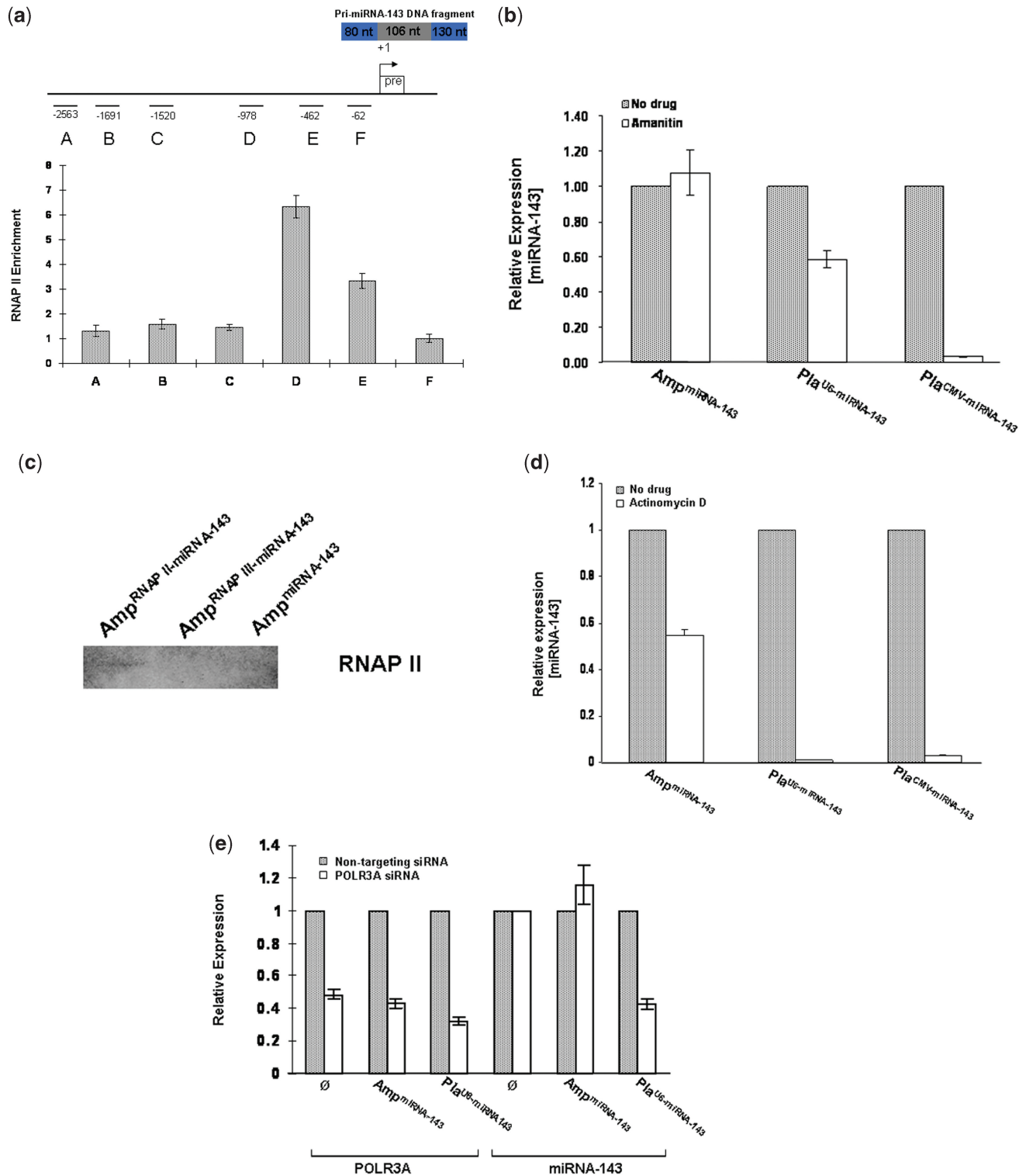


Figure 5. Deciphering the transcriptional network of Amp^{miRNA-143}. (a) We assigned RNAP II promoter occupancy to the miRNA-143 gene in HCT116 cells by chromatin immunoprecipitation using antibodies specific to RNAP II. Sheared chromatin from HCT116 cells that had been cross-linked with formaldehyde was immunoprecipitated with anti-RNAP II antibodies. Cross-links were removed and the DNA was purified. The promoter region of miRNA-143 was arbitrarily deconstructed into six segments of ~100–200 nt each. Specific PCR primers were designed to amplify each component and revealed relative enrichment in sector D, corresponding to nucleotide position –978 with respect to the pre-miRNA start site. All values are relative to nonimmune IgG and normalized to an intergenic control region. Antibodies to GAPDH served as a positive control and revealed ~30-fold enrichment in the respective promoter region. The corresponding location of Amp^{miRNA-143} is shown and lies outside the region of RNAP II enrichment. (b) We used various inhibitors of RNAP to test their effect on the biosynthetic activity of Amp^{miRNA-143} as well as RNAP II/III-driven expression constructs. Cells were treated with α -amanitin (50 μ g/ml) and actinomycin (2 μ g/ml) and transfected with the various constructs prior to harvesting and mature miRNA-143 quantification by real-time PCR. Data are shown as fold reduction in mature miRNA

(continued)

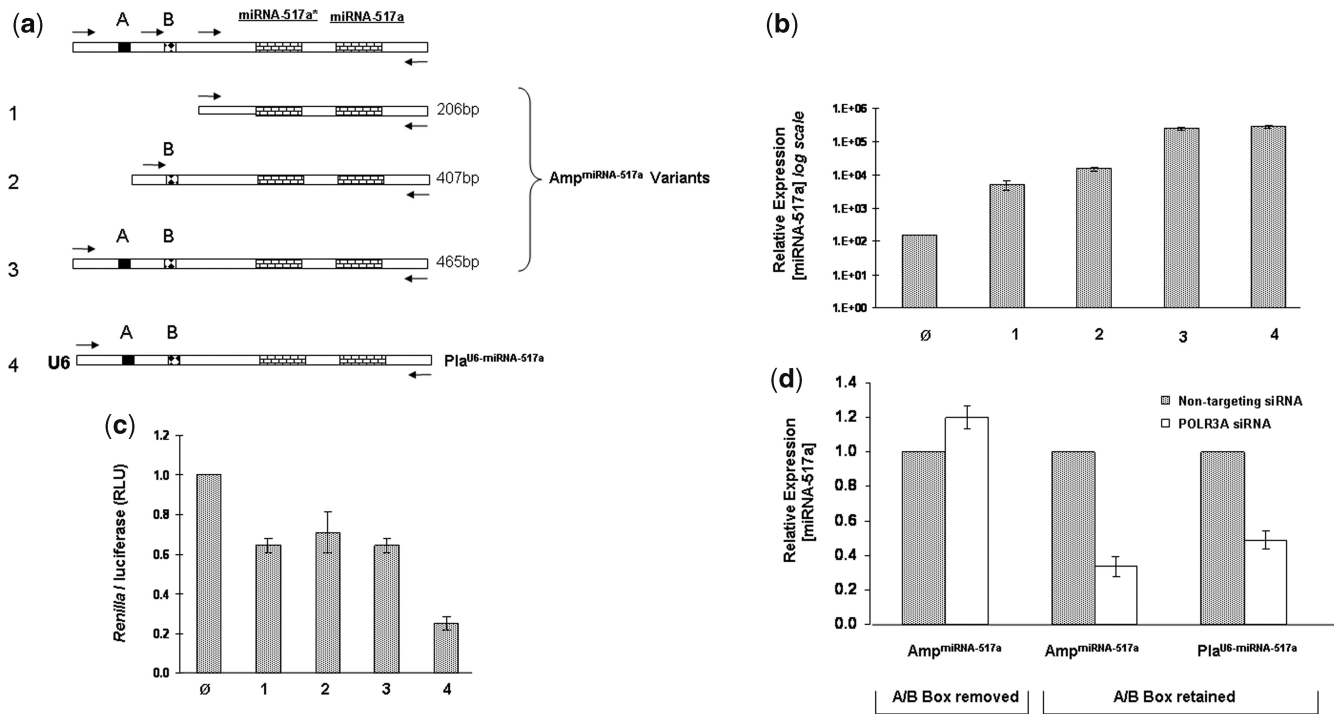


Figure 6. (a) Schema showing various sized amplicons (#1–#4) of Amp^{miRNA-517a} with and without the RNAP III specific A and B boxes. (b) Relative amount of mature miRNA-517a produced upon cellular introduction of the various constructs was quantified by real-time PCR as compared to cells transfected with an empty vector (∅). While A and B box inclusive amplicons produced the highest levels of mature miRNA, the 206-bp amplicon without A/B box retained the ability to produce mature miRNA. Experiments were performed in duplicate and constructs #1–#4 all produced significantly higher levels of mature product than ∅ ($P < 0.05$). (c) The functionality of Amp^{miRNA-517a} was assessed by sensor assays. While the RNAP III (U6)-driven expression vector retained the most potency (~80%), shorter amplicons with and without A/B boxes also reduced Renilla relative light units by ~30–40%. All constructs significantly inhibited sensor activity ($P < 0.005$). (d) Effect of silencing RNAP III on Amp^{miRNA-517a} amplicons. Cells were treated with anti-POLR3A or an irrelevant siRNA and transfected with amplicons prior to harvesting and quantifying mature miRNA-517a levels. RNAP III knockdown had no effect on Amp^{miRNA-517a} biosynthetic activity ($P = 0.17$) yet significantly decreased the synthetic capacity of both RNAP III-driven expression vector ($P = 0.0006$) and amplicons harboring A/B boxes ($P = 0.0001$).

siRNA targeting POLR3A, the same cells retained fully their ability to transcribe the shorter Amp^{miRNA-517a} that lacked the A and B boxes (Figure 6d). Thus, although wild-type miRNA-517a is under the control of RNAP III through the A and B boxes, an amplicon that does not include A and B boxes appears to operate upon transfection in a manner similar to the other amplicons we described earlier.

Besides RNAP II/III, two other polymerases are operational in mammalian cells: RNAP I and the more recently described single polypeptide nuclear RNA polymerase (spRNAP-IV) (20). RNAi-based depletion of RNAP I or spRNAP-IV had no effect upon the biosynthetic activity of Amp^{miRNA-XX} (Supplementary Figure S3).

Effect of Amp^{miRNA-XX} on endogenous miRNA expression

Numerous studies have demonstrated that ectopic expression of small RNA molecules may perturb normal cellular function through multiple mechanisms. For example, ectopic expression of shRNA appears to deregulate endogenous miRNA expression *in vitro* and perhaps more importantly, *in vivo* (21,22). To determine whether Amp^{miRNA-XX} species impacted endogenous miRNA expression, we introduced Amp^{miRNA-143} into HEK 293T cells and quantified the levels of miRNA-let7a, 125a and 125b. These three latter species are relatively abundantly expressed in HEK 293T cells. As seen in Figure 7, there was no significant difference in the endogenous expression of these three miRNA species in cells that had been transfected with Amp^{miRNA-143}

Figure 5. Continued

compared to similarly transfected cells in the absence of drug. α -Amanitin significantly reduced the biosynthetic activity of RNAP II ($P < 0.001$) and III ($P = 0.0384$)-driven constructs but had no significant effect on the activity of Amp^{miRNA-143} ($P = 1.0$). (c) RNAP II was directly localized to miRNA-143 amplicons harboring the CMV (RNAP II) promoter but not to Amp^{miRNA-143} or an amplicon harboring the U6 (RNAP III) promoter. (d) The general transcriptional inhibitor actinomycin reduced the activity of all constructs in a significant manner ($P < 0.0001$) but its effect on Amp was less pronounced. Data are shown as fold reduction in mature miRNA in drug treated/untreated cells transfected with the indicated constructs. Experiments were performed in duplicate. (e) Effect of RNAP III silencing on Amp^{miRNA-143}. HEK 293T cells were treated with siRNA against the POLR3A subunit of RNAP III or an irrelevant gene and transfected with either empty vector (∅), Pl_{U6}-miRNA-143 or Amp^{miRNA-143}. POLR3A knockdown (~50–70%) had a significant inhibitory effect ($P = 0.003$) on the transcription of RNAP III-driven expression vectors but no effect ($P = 1.0$) on Amp^{miRNA-143} as assessed by mature miRNA-143 quantification. Data are shown as fold reduction in mature miRNA compared to cells transfected with an irrelevant siRNA. Experiments were performed in duplicate.

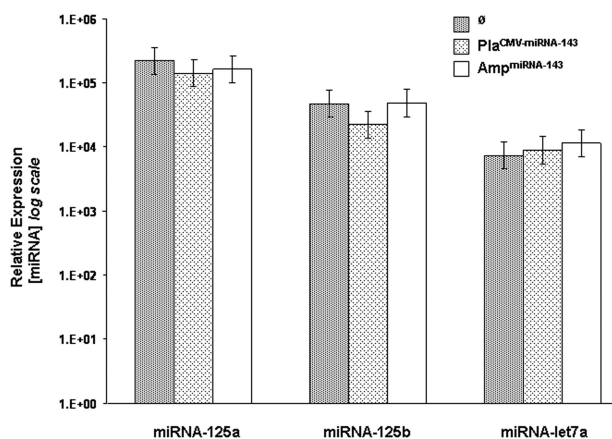


Figure 7. Effect of Amp^{miRNA-143} on endogenous miRNA levels. HEK 293T cells were transfected with empty vector (Ø), PlacMV-miRNA-143 and Amp^{miRNA-143}. Real time PCR quantification of endogenous miRNA (let 7a, 125a, 125b) in transfected cells revealed no statistically significant difference in respective mature miRNA levels between Ø, PlacMV-miRNA-143 and Amp^{miRNA-143} transfected cells.

compared to cells that had been transfected with an empty vector or a RNAP II-driven miRNA-143 expression plasmid.

Engineered miRNA-like molecules do not participate in atypical transcription

Our experiments suggested that the miRNA species examined above possessed either sequence and/or structural determinants that enabled the recruitment of an unconventional transcriptional machinery. To gain mechanistic insight into the perplexing nature of atypical transcription, we first compared the nucleotide sequence of the various miRNA associated with functional amplicons and could not identify a conserved sequence motif. All amplicons, however, shared two major attributes upon transcription: their hairpin-like RNA structure and subsequent stereotypic processing by RNAase III enzymes into shorter fragments of mature and (*) miRNA and possibly other 21–22-nt fragments, such as the recently characterized miRNA-offset RNA (23,24). We reasoned that as long as the hairpin structure and Dicer/Drosha processing sites were maintained, the primary sequence of any given Amp^{miRNA-XX} species could be altered yet remain functional. Previously, we and others have demonstrated that the miRNA backbone can be modified to include sequences of a given siRNA and thereby serve as efficient vectors of delivery upon incorporation into RNAP III-driven expression cassettes (25). Thus, we introduced sequences encoding a previously validated siRNA targeting HIV-1 *tat* into the backbone of miRNA-143 (Figure 8a). Unexpectedly, when miRNA-143/*tat* was PCR-amplified and introduced as a promoter-less Amp, we could not detect *tat* siRNA expression. However, introduction of an RNAP III-driven expression cassette harboring miRNA-143/*tat* into HEK 293T cells led to *tat* shRNA detection, as assessed by northern blot (Figure 8b). Thus, the sequence replacement did not prevent the molecule's entry into and processing by the cellular RNAi machinery

upon placement of the construct under the direction of a strong promoter. Similar negative results were encountered when *tat* siRNA sequences were embedded in miRNA-30a (data not shown). The only seeming distinction between functional Amp^{miRNA-XX} versus non-functional Amp^{miRNA-*tat*} was the replacement of mature and (*) miRNA sequences with those encoding the *tat* siRNA guide and passenger strand, respectively. It was entirely unclear to us why engineered miRNA/*tat* molecules failed to undergo atypical transcription when delivered as Amp. We next modified the miRNA-143 backbone such that mature and (*) sequences were replaced with those encoding miRNA-145. Introduction of this hybrid molecule led to the production of mature, functional miRNA-145 in a statistically significant manner (Figure 8c and d). Thus, alteration of the miRNA backbone in and of itself does not appear to impact the biosynthetic potential of Amp as much as the choice of cargo.

DISCUSSION

In summary, we report our findings of atypical self-transcription upon cellular introduction for several DNA segments, collectively referred to here as Amp^{miRNA-XX}, which contain the precursors for known human miRNA that are devoid of any known exogenous promoters. The majority of miRNA (8/12) that we studied were intergenic and thus expected to be transcribed with their host genes (15). A recent promoter analysis localized transcription start sites of miRNA-26a, 30a, 107 and 122 at several thousand nucleotides from the beginning of their respective pre-sequences. Our experiments, which included these and other miRNA, however, indicated that a cryptic transcriptional element was present in the sequence of the pri/pre-sequences of the intronic and intergenic miRNA genes examined. Our analysis also revealed altered sensitivity of Amp^{miRNA-XX} to pharmacologic and/or genetic knockdown of RNAP I/II/III, thus raising the possibility of an atypical transcriptional program involving pri-miRNA gene fragments. The absence of any apparent shared sequence features among the different amplicons that we employed suggests the formation of a likely novel scaffold that is able to recruit the necessary factors required for transcription. The precise components of this scaffold await identification but replacement of mature and (*) sequences of a *bonafide* miRNA with those encoding siRNA leads to a non-functional molecule. These results raise the intriguing possibility that mature and (*) sequences are perhaps themselves involved in enabling miRNA fragment transcription but this conjecture clearly needs vigorous investigation.

From the experiments described earlier, we conclude that at least some of the miRNA precursors that are currently known in the literature have the ability to self-transcribe their own sequence fragments, and can do so in a number of different human cell types. Transcription efficiency is lower by comparison to that of plasmid systems or PCR amplicons employing RNAP

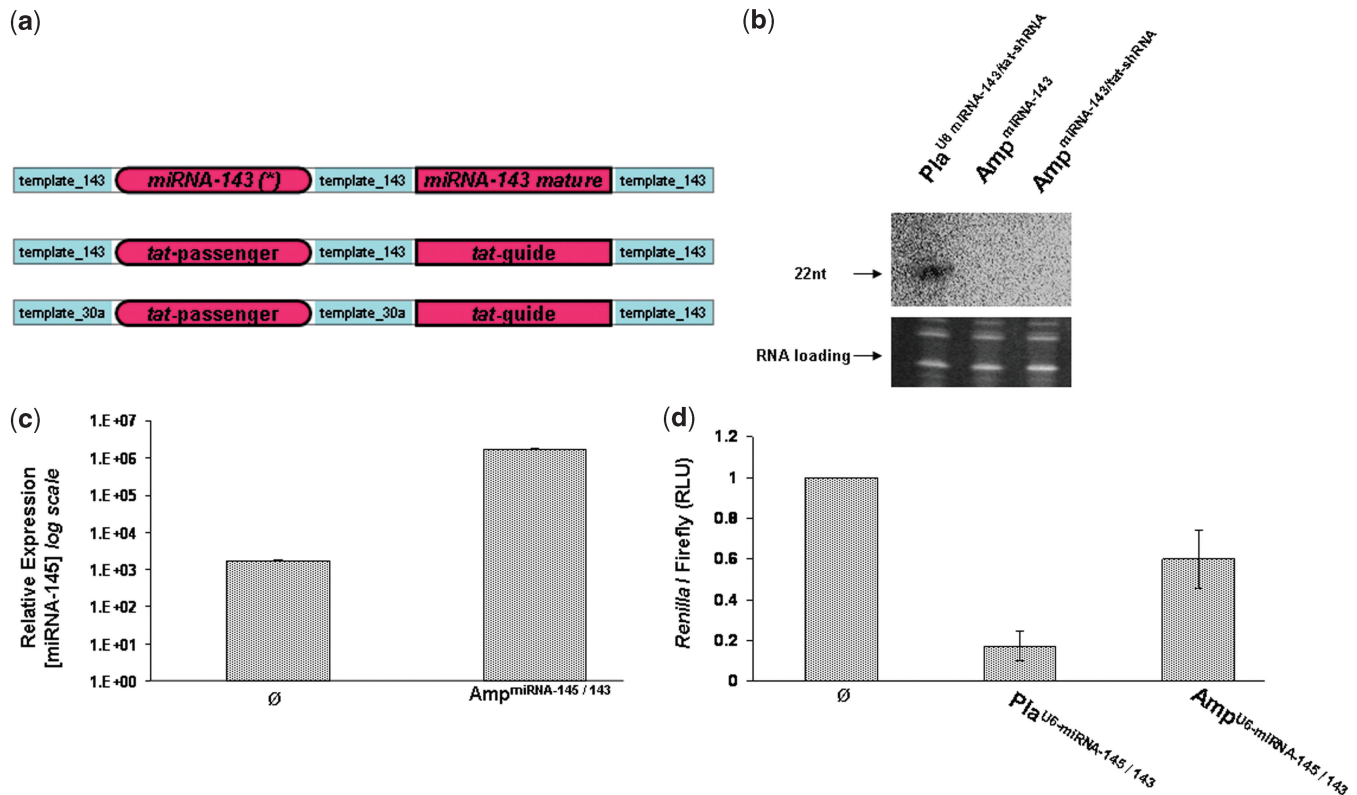


Figure 8. Activity of miRNA-like amplicons. (a) We created two Amp^{miRNA-XX} variants in which the mature and (*) sequences of miRNA 143 and 30a were replaced by those encoding the guide and passenger strand of siRNA targeting the HIV-1 transactivator (*tat*) protein. (b) Northern blot was used to validate the activity of Amp^{miRNA-143/tat}. When miRNA-143/*tat* was placed under the direction of RNAP III (U6), we could detect *tat* siRNA. However, when the construct was introduced as a promoter-less Amp^{miRNA-143/tat}, *tat* siRNA could not be detected. Similar results were obtained for Amp^{miRNA-30a/tat}. (c, d) The failure of Amp^{miRNA-xx/tat} prompted us to manipulate the backbone of miRNA-143 by replacing its mature and (*) sequences with those encoding miRNA-145. Real-time PCR was used to quantify mature miRNA-145 levels in cells that had been transfected with either empty vector (∅) or Amp^{miRNA-143/145}. Amp species were associated with a statistically significant 1000-fold increase ($P < 0.001$) in mature miRNA-145 levels compared to cells that had been treated with ∅. (d). miRNA-145 sensor assays were used to quantify the activity of Amp^{miRNA-143/145}. Compared to a U6-driven construct, Amp^{miRNA-143/145} reduced sensor activity in a statistically significant manner ($P < 0.05$).

II/III promoters; consequently, the phenomenon can be observed readily only against a backdrop of minimal expression for the studied miRNA.

To date, means of expressing miRNA in cells include their delivery in plasmid or viral vectors as well as direct delivery as synthetic molecules. Our findings suggest that it might be possible to engineer expression of at least a handful of miRNAs simply by introducing the corresponding miRNA precursor fragments in the form of synthetic or PCR-generated DNA automatons devoid of traditional promoters into targeted cells *in vitro* or entire organisms *in vivo*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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