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-μl reaction mixture containing forward and reverse primers at 2 μM, 0.5 U of pfu DNA polymerase (Stratagene) and dNTP at 200 μM. Constructs created in this manner are denoted as AmpmiRNA-XX. Human genomic DNA from HEK 293T cells (0.2 μg) was used as the sole template for creation of AmpmiRNA-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 BamH1 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′ Pst1 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...
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 pSCE2 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 Amxaxa system (Amxax 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-32P)-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 (TGTGCTGCGAAGCGAGCAC) 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 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 psichek 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 (Ø) 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'-accaggggaacctgtgtagag-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'-cacaagtgttgtagtgagtc-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'-acttaccacttccaggctgatg-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 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 (AmpmiRNA-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 AmpmiRNA-143 was indeed contributing to the increased levels of mature miRNA-143, we sought to distinguish the product derived from AmpmiRNA-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 AmpmiRNA-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 AmpmiRNA-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 AmpmiRNA-143 that preserves its ability to self-transcribe

The amplicon used in the above-described experiments was 316nt 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 AmpmiRNA-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
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, OligomiRNA-143-B led to mature miRNA-143 levels comparable to those associated with the PCR generated AmpmiRNA-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 AmpmiRNA-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 AmpmiRNA-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, U6...
HeLa) that had been treated with Amp\textsuperscript{miRNA-143} readily confirmed its biosynthetic capacity in these cell types as well (Figure 3a). In each case, Amp\textsuperscript{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 (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\textsuperscript{miRNA-143-B} were commercially synthesized, annealed and introduced into HEK 293T cells. Oligo\textsuperscript{miRNA-143-B} retained similar biosynthetic capacity as compared to Amp\textsuperscript{miRNA-143-B}. Furthermore, the activity of Amp\textsuperscript{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.

Figure 2. Transcriptional activity of shorter Amp\textsuperscript{miRNA-143} fragments. (a) Schema showing relative lengths of six (A–E) shorter versions of Amp\textsuperscript{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\textsuperscript{miRNA-143-B} were commercially synthesized, annealed and introduced into HEK 293T cells. Oligo\textsuperscript{miRNA-143-B} retained similar biosynthetic capacity as compared to Amp\textsuperscript{miRNA-143-B}. Furthermore, the activity of Amp\textsuperscript{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.

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\textsuperscript{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\textsuperscript{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\textsuperscript{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 $\text{Amp}^{\text{miRNA-143}}$ 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 (Ø) with associated 95% CI. All experiments were performed in duplicate, independent experiments. In each cell type, Amp$^{\text{miRNA-143}}$ 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 (Ø) and 95% CI. Each Amp species led to a statistically significant increase in the levels of the respective miRNA compared to Ø ($P < 0.0001$). (c) The biosynthetic potential of a given amplicon was quantifiable in cells with relatively low endogenous levels of the miRNA studied. For example,
transfected with other amplicons including those encoding miRNA-363 and 181a-2 (Supplementary Figure S2).

**Functionality of Amp**<sup>miRNA-XX</sup>

While quantitative assays revealed the biosynthetic activity of Amp<sup>miRNA-XX</sup>, 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 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 ± SD compared to control conditions. (a) Functional activity of Amp<sup>miRNA-143</sup>, Amp<sup>CMV-miRNA-143</sup> and Pla<sup>CMV-miRNA-143</sup>. Co-transfection of the miRNA-143 sensor and Amp<sup>miRNA-143</sup> 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<sup>miRNA-143-A/B/C/D/E/F</sup>. 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<sup>miRNA-XX</sup>. 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<sup>miRNA-143</sup> 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.

**Figure 3.** Continued

Amp<sup>miRNA-125a</sup> significantly (P < 0.0001) increased levels of mature product in Huh-7 cells (miRNA-125a<sup>low</sup>) but not in HEK 293T cells (miRNA-125a<sup>high</sup>). Conversely, Amp<sup>miRNA-122</sup> produced significantly (P = 0.0008) increased levels of mature product only in HEK 293T cells (miRNA-122<sup>low</sup>) but not in Huh-7 cells (miRNA-122<sup>high</sup>). (d) A hybrid amplicon consisting of miRNA-143 and miRNA-125a (Amp<sup>miRNA-125a + 143</sup>) 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<sup>miRNA-XX</sup> 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-26a, 110, 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 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 6a and b). 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\textsuperscript{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\textsuperscript{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\textsuperscript{miRNA-143} as well as RNAP II/III-driven expression constructs. Cells were treated with α-amanitin (50 ug/ml) and actinomycin (2 ug/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.
siRNA targeting POLR3A, the same cells retained fully their ability to transcribe the shorter Amp mimRNA-517a that lacked the A and B boxes (Figure 6d). Thus, although wild-type mimRNA-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 mimRNA-XX (Supplementary Figure S3).

**Effect of Amp mimRNA-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 mimRNA-XX species impacted endogenous miRNA expression, we introduced Amp mimRNA-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 mimRNA-143 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 mimRNA-143 (P = 1.0). (e) RNAP II was directly localized to mimRNA-143 amplicons harboring the CMV (RNAP II) promoter but not to Amp mimRNA-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 mimRNA-143 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 mimRNA-XX. HEK 293T cells were treated with siRNA against the POLR3A subunit of RNAP III or an irrelevant gene and transfected with either empty vector (Ø), Pla-U6-miRNA-143 or Amp mimRNA-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 mimRNA-143 as assessed by mature mimRNA-143 quantification. Data are shown as fold reduction in mature miRNA compared to cells transfected with an irrelevant siRNA. Experiments were performed in duplicate.
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.

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 21–22nt 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 AmpmiRNA-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 AmpmiRNA-XX versus non-functional AmpmiRNA/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 AmpmiRNA-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 AmpmiRNA-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
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 automats 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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