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RNA polymerase III transcribes human microRNAs

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Prior work demonstrates that mammalian microRNA (miRNA or miR) expression requires RNA polymerase II (Pol II). However, the transcriptional requirements of many miRNAs remain untested. Our genomic analysis of miRNAs in the human chromosome 19 miRNA cluster (C19MC) revealed that they are interspersed among Alu repeats. Because Alu transcription occurs through RNA Pol III recruitment, and we found that Alu elements upstream of C19MC miRNAs retain sequences important for Pol III activity, we tested the promoter requirements of C19MC miRNAs. Chromatin immunoprecipitation and cell-free transcription assays showed that Pol III, but not Pol II, is associated with miRNA genomic sequence and sufficient for transcription. Moreover, the mature miRNA sequences of approximately 50 additional human miRNAs lie within Alu and other known repetitive elements. These findings extend the current view of miRNA origins and the transcriptional machinery driving their expression.

Over 450 miRNAs have been described in the human genome[1]. miRNAs are important in human development, oncogenesis and immunity, and they have drawn renewed attention to the small non-coding elements of the transcriptome. miRNAs are short (17–25 base pairs (bp)) noncoding RNAs that guide cellular machinery to specific messenger RNAs[2,3] to control expression. Initial miRNA transcripts can be several thousand base pairs in length, and they are processed to produce ~70-bp stem-loops (pre-miRNAs) before nuclear export[4]. Upon entering the cytoplasm, the RNA-induced silencing complex (RISC) cleaves and denatures pre-miRNAs to produce the functionally mature, single-stranded miRNAs[4]. Through complementary base pairing to specific protein-coding mRNA transcripts, miRNAs direct mRNA silencing by a variety of mechanisms, including message degradation[5], deadenylation[6] and translational repression[7].

The evolutionary conservation of miRNAs has been described[8], as has their use of RNA polymerase II (Pol II) promoters for control of expression[9–11]. A recent study described approximately ten mammalian miRNAs created by a repetitive element transposition and subsequent transcription across two tandem, inverted repetitive elements[12]. In this study, we tested if other miRNAs possess sequences repetitive in origin, and the transcriptional requirements of a dense cluster of human miRNAs interspersed among repetitive Alu elements on chromosome 19.

RESULTS

miRNAs localize to repeats within the human genome

We expanded prior studies by screening sequences flanking all known human miRNAs for repetitive elements (500 bp upstream and down stream) and annotating them using the Censor web server at the Genetic Information Research Institute[13]. Overall, we found that the mature sequences from ~50 human miRNAs reside within repetitive elements (**Supplementary Table 1** online). In addition, within the C19MC[14], primate-specific miRNAs and Alu repeats (Alus) account for over two-thirds of the ~100-kbp sequence (**Fig. 1a**). This miRNA-Alu arrangement is remarkable; 42 of 43 miRNAs are separated from proximal Alus by an average of 100 bp. Moreover, many of the upstream Alus retain Pol III promoter elements, and there is no intervening transcription terminator (TTTT) between the Alu-resident promoter and the miRNA. Thus, the C19MC miRNAs would be expected to constitute the 3′ ends of Alu transcripts if expressed[13,15]. Together, these observations suggest that over 20% of human miRNAs may be derived from repetitive-element sequences: the 52 aligning directly to consensus repeats and the 43 in the C19MC formed from Alu 3′ sequences.

Upstream Alus express downstream miRNAs

We used degenerate primers and thermostable polymerase to first confirm that miRNAs within the C19MC are transcribed *in vivo* (**Supplementary Table 2** online). To discern how the C19MC miRNAs are expressed, we used one of these miRNAs, miR-517a, and sequences immediately upstream, which consist of an Alu (**Fig. 1b**). Two expression constructs incorporating the 5′ Alu were generated, one consisting of the genomic sequence containing the miR-517a 5′ Alu and miR-517a hairpin (517-I), and a second in which the miRNA hairpin was replaced with a hairpin previously used to silence *Escherichia coli* β-galactosidase by RNA interference[16] (shLacZ in plasmid pAL-1; **Fig. 1c**). Transfection of plasmids and subsequent RT-PCR demonstrated expression from the miR-517a 5′ Alu (**Fig. 1d**). RNA blots of small transcripts showed hairpin production and processing from pAL-1 and 517-I (**Fig. 1e**).

We next made reporter constructs to test whether the hairpins expressed from the miR-517a 5′ Alu had silencing activity. These reporters (517-AS and LacZ-AS) produced *Renilla* luciferase transcripts with target sequences for miR-517a or shLacZ cloned into the 3′ UTR (**Fig. 1c**). Cotransfection of 517-I and 517-AS into cells

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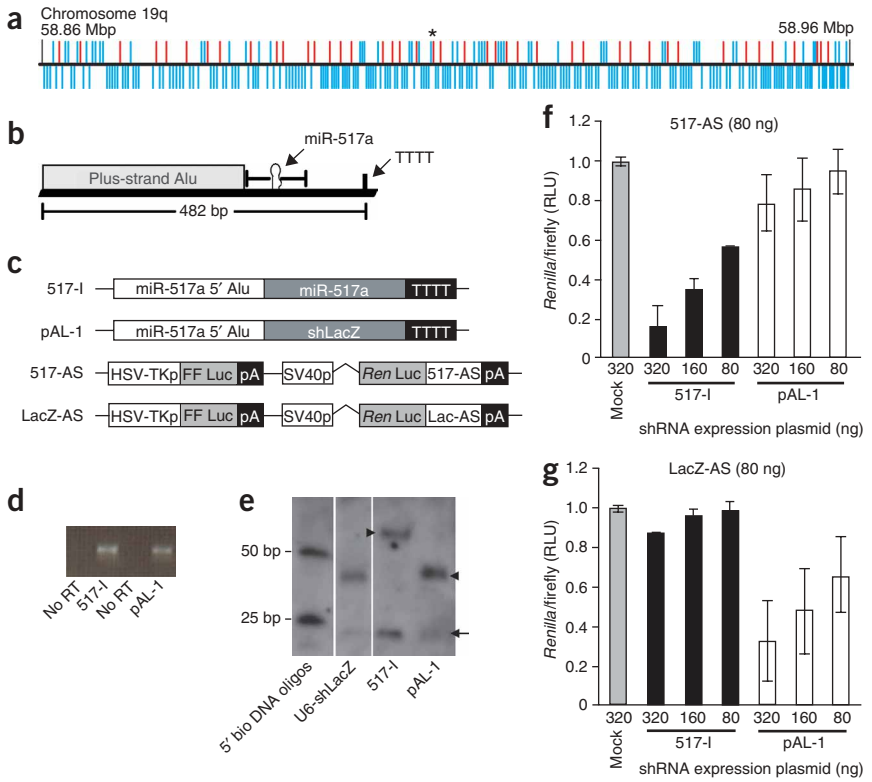

Figure 1 The C19MC cluster and assessment of 5′ Alu transcriptional activity. (**a**) Cartoon depicting the C19MC. Vertical lines indicate relative miRNA (red) and Alu (blue) positions with placement above (positive strand) or below (negative strand) the center line indicating orientation. Asterisk, miR-517a. (**b**) Scale diagram of miR-517a locus. (**c**) Diagrams of hairpin expression vectors. 517-I corresponds to a single genomic fragment containing the miR-517a hairpin and upstream Alu. In pAL-1, shLacZ replaces the miR-517a hairpin. 517-AS and LacZ-AS are respective antisense reporters. HSV-TKp, herpes simplex virus thymidine kinase promoter; SV40p, simian virus 40 promoter; FF Luc, firefly luciferase; *Ren* Luc, *Renilla* luciferase; pA, poly(A). (**d**) RT-PCR to test expression from pAL-1 and 517-I after HEK 293T transfection. (**e**) miR-517a and shLacZ small transcript northern blot (bio abbreviates biotin). Arrowhead indicates intact hairpin. Full arrow indicates mature small RNA. (**f**) Luciferase assays ($n = 4$) of A549 lysates after transfection of 517-AS and either 517-I, pAL-1 or empty vector (Mock). RLU, relative light units. (**g**) Cotransfections done as in **f**, except LacZ-AS replaced 517-AS.

resulted in a dose-dependent reduction in *Renilla* activity, with ~80% knockdown at a 4:1 shRNA/reporter transfection ratio and ~40% reduction at a 1:1 ratio, unlike cotransfection of the 517-I and LacZ-AS controls (**Fig. 1f**). Similarly, cotransfection of pAL-1 and LacZ-AS resulted in a dose-dependent reduction in *Renilla* activity, with ~70% knockdown at a 4:1 transfection ratio and ~35% reduction at a 1:1 ratio (**Fig. 1g**). Cotransfection of control constructs pAL-1 and 517-AS into cells resulted in no marked silencing. Together, our results suggest that the miR-517a 5′ Alu can drive expression of downstream noncoding short hairpin RNAs (shRNAs) that contain RNA-interference activity.

As presented above, C19MC miR-517a is expressed endogenously (**Supplementary Table 2**). To confirm that endogenous miR-517a is active, 517-AS or control vectors were transfected into cells with measurable miR-517a expression (HEK 293T cells), and reporter activity was assessed. *Renilla* activity in 517-AS–transfected cells (~1,500 copies of reporter DNA per cell) was reduced to ~30% of control-transfected cells (data not shown).

To test whether transcription from 5′ Alu promoters occurs at C19MC miRNA loci other than miR-517a, we generated pAL-4 from the miR-517c upstream Alu, which, similarly to miR-517a, is separated from the miR-517c hairpin by only 40 bp. In addition, we deleted the A and B boxes (responsible for Pol III recruitment)[15,17] from our miR-517a and miR-517c 5′ Alu expression constructs (creating pAL-7 and pAL-5, respectively; **Fig. 2a**). The pALs or 517-I were individually cotransfected with 517-AS or LacZ-AS. As expected, pAL-1 silenced the *Renilla* activity of LacZ-AS by ~80% but had little to no effect on 517-AS. 517-I silenced the *Renilla* activity of 517-AS by ~80% but had limited effect on LacZ-AS. pAL-4 (containing the 517c upstream Alu promoter) silenced shLacZ-AS *Renilla* activity by >60%. In contrast, constructs with A- and B-box deletions (pAL-5 and pAL-7) did not silence (**Fig. 2b**). Northern blots confirmed that only pAL-1 and pAL-4 expressed transcripts

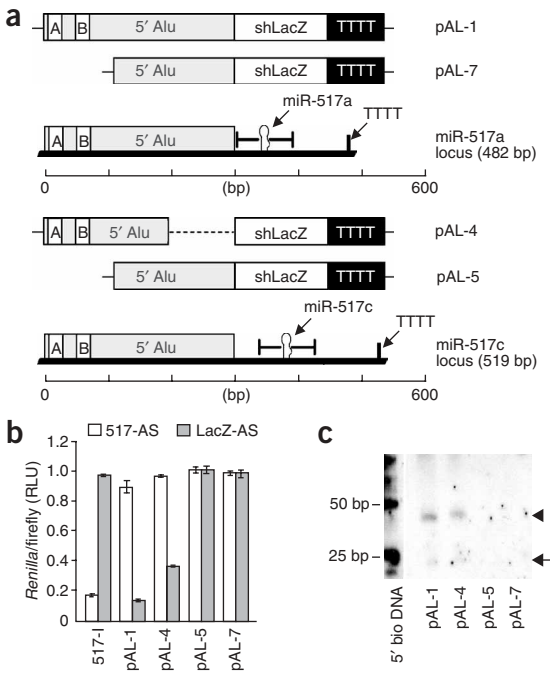

Figure 2 Analysis of miR-517a and miR-517c 5′ promoter elements. (**a**) Scale diagram of the miR-517a and miR-517c loci. Sequences used to construct pAL-1, pAL-4, pAL-5 and pAL-7 are illustrated. A and B indicate Alu promoter elements (A and B boxes; see **Fig. 3b**). (**b**) Luciferase assays as in **Figure 1f,g**, with 320 ng of expression vector. (**c**) Northern blot as in **Figure 1e** using LacZ-AS probe.

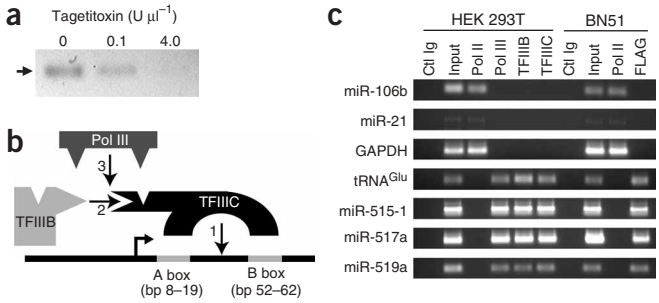

Figure 3 C19MC 5′ Alus are transcribed by Pol III. (**a**) *In vitro* transcription of pAL-1 is reduced in extracts containing the Pol III–specific inhibitor tagetitoxin. Bands correspond to full-length pAL-1 transcript. Northern blots of the products using probes specific for shLacZ confirmed transcript identity (data not shown). (**b**) Cartoon of Alu A and B boxes and transcription factor binding (transcription factor subunits depicted in **Supplementary Fig. 1**). (**c**) ChIPs performed in HEK 293T cells and BN51 cells (expressing a Flag-tagged Pol III subunit). Amplicons were verified by sequencing. Pol III ($tRNA^{Glu}$) and Pol II (GAPDH, miR-21, miR-106b) controls are indicated. Ctl Ig, control immunoglobulin.

(**Fig. 2c**), further supporting the conclusion that an intact upstream Alu Pol III promoter is sufficient for expression.

miRNAs in the C19MC are transcribed by Pol III

Pol III transcription of Alus is well established[15,17]. Therefore, we directly assayed the miR-517a upstream Alu for Pol III transcription by cell-free transcription and chromatin immunoprecipitation (ChIP). *In vitro* transcription of pAL-1 was performed in cell extracts in the presence or absence of the Pol III–specific inhibitor tagetitoxin[18,19]. Treating extracts with tagetitoxin attenuated transcript production from the miR-517a construct, pAL-1 (**Fig. 3a**).

ChIPs were performed using antibodies specific for Pol II, Pol III, TFIIIB and TFIIIC (**Fig. 3b** and **Supplementary Fig. 1** online). ChIP analysis of sequences upstream of miR-21 (previously shown to be transcribed by Pol II[9,10]) and miR-106b (presumably processed from an intron occurring ~600 bp downstream of a minichromosomal maintenance protein (MCM7) transcription start site[1]) showed enrichment for Pol II, but not Pol III. Conversely, the $tRNA^{Glu}$ promoter was enriched for Pol III, TFIIIB and TFIIIC, but not Pol II. Notably, ChIP analysis of sequences ~300 bp upstream of miRNAs representing distinct C19MC subfamilies[14]–miR-515-1, miR-517a and miR-519a-1–were each enriched for Pol III, TFIIIB and TFIIIC, but not Pol II. This demonstrates specific occupation of these loci with Pol III transcriptional machinery. To confirm our results, we used antibodies specific for Pol II and Flag for ChIP analysis of DNA harvested from BN51 cells. The BN51 cell line is a modified HeLa line constitutively expressing a Flag-tagged Pol III subunit[20]. We found sequences immediately upstream of miR-515-1, miR-517a and miR-519a-1 enriched for Flag-Pol III but not Pol II (**Fig. 3c**). Together, our results show that miRNAs within the C19MC are transcribed by Pol III.

DISCUSSION

We show that the human C19MC 5′ Alus can function as miRNA promoters and that miRNAs arising from repetitive sequences are more abundant than previously appreciated. Recent work has suggested that miRNAs encoded by mouse γ herpesvirus 68 use tRNA promoters, and hence Pol III, as they are expressed and they lie immediately downstream from tRNA sequences[21]. Our data now demonstrate directly that Pol III transcription of miRNAs does occur.

In addition to C19MC miRNAs, we noted 18 additional human miRNAs that are strong candidates for Pol III–mediated transcription (**Supplementary Table 3** online). Notably, these miRNAs reside near upstream tRNA sequences, Alu sequences or mammalian-wide interspersed repeat (MWIR) sequences. Most candidate promoters retained both intact A and B boxes, but all retained intact B-box sequences. B boxes are sufficient for transcriptional activity, which A boxes enhance[22]. Because Pol III–mediated transcription from tRNA, MWIR or Alu promoter elements can be constitutive, induced upon cellular stress or activated by transcription factors[23,24], the miRNA residing downstream may be controlled similarly.

Another intriguing feature of the 43 miRNA loci comprising the C19MC (**Fig. 1a**) is the occurrence of a negative-strand Alu downstream of each hairpin (**Fig. 4**). Notably, Pol III terminators (TTTT) occur in 74% of the loops of the C19MC miRNA hairpins if expressed from the negative-strand Alu (**Supplementary Table 4** online), suggesting that transcription of the full miRNA would probably be confined to one strand. However, it is tempting to speculate that two transcripts may be produced from C19MC loci: one from the positive strand, generating a miRNA, and a second from the negative strand, consisting of an Alu terminating with sequences antisense to one miRNA stem. One possible role for the antisense sequence may be to inhibit retrotransposition of the Alu pre-miRNA–containing transcript, presuming it is transpositionally active[22]. Indeed, this was recently reported for LINE-1 elements[25]. In contrast to that report, however, we show that functional miRNAs are produced from a single strand (517-I). Additionally, Alus found upstream of the miRNAs within C19MC belong to either the AluJ or AluS subfamilies, and, as evolutionarily older Alu sequences, they are probably not retrotranspositionally active. Moreover, the overlap between the two transcripts is minimal. An alternative role for the 3′ antisense transcript would be to provide target, or seed, sequences for miRNA regulation. For example, if during evolution the negative-strand Alu transposed and inserted into a Pol II–transcribed gene, the miRNA expressed from the positive strand could translationally control that mRNA. Target sequences would be retained if the miRNA-target relationship imparted increased fitness.

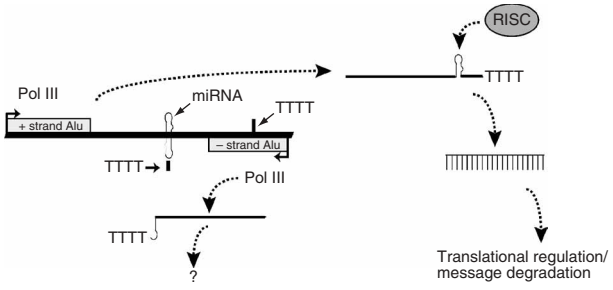

Figure 4 Cartoon depicting the arrangement of C19MC miRNAs with Alus. The majority of C19MC miRNAs are sandwiched between positive-strand and negative-strand Alus (relative to miRNA transcription). The separation of C19MC miRNA hairpins from downstream Alus is generally less than 100 bp. Transcripts arising from these 3′ Alus would typically consist of only one stem of the pre-miRNA, as approximately 74% contain a Pol III terminator (TTTT) between the two stems of the pre-miRNA (**Supplementary Table 4**) when read from the negative-strand Alu.

In summary, we show that the C19MC miRNAs miR-515-1, miR-517a, miR-517c and miR-519a-1 are expressed using Pol III; bioinformatic analyses suggest that additional C19MC miRNAs, and human miRNAs with upstream Alu-, tRNA- or MWIR-based promoter elements, may be similarly transcribed. These findings, together with other reports[12], suggest that the connection between miRNAs and transposable elements is more pronounced than previously appreciated. More broadly, they support an important role for repetitive elements in human miRNA origin and expression.

METHODS

Oligonucleotides. Sequences are detailed in **Supplementary Table 5** online.

Small transcript northern blots. HEK 293Ts were cultured in 12-well plates in DMEM (10% v/v FBS, 1% (v/v) penicillin-streptomycin (PS). At 90% confluence, cells were transfected with Lipofectamine 2000 (Invitrogen), as suggested by the manufacturer. Total RNA was isolated at 48 h using Trizol (Invitrogen). A 15% acrylamide/bis-acrylamide (29:1) gel containing 8 M urea (48% (w/v)) and 1× TBE was prerun for 30 min at 100 V in a vertical mini-PROTEAN tank (Bio-Rad). Gels were flushed and loaded with 10 μg of total RNA in 1× Gel Loading Buffer 2 (Ambion), then run at 200 V until the bromophenol blue dye front reached the gel bottom. As a size reference, 1 μl of pooled, commercially synthesized biotin 5′ end–labeled DNA oligonucleotides (18, 25, 50 and 75 bp each at 25 mM) was also loaded in Gel Loading Buffer 2. Positive controls consisted of a DNA shLacZ hairpin oligonucleotide and miR-517a antisense DNA oligonucleotide loaded at four times the concentration of the biotin-labeled oligonucleotides. After electrophoresis, RNA was electrotransferred (Transblot SD, Bio-Rad) to Hybond N+ Nylon membranes (GE Healthcare) for 2 h at 400 mA in 0.25× TBE. After removal from the transfer stack, membranes were gently washed in 1× TBE for 15 min on an orbital shaker, then UV cross-linked (Stratalinker, Stratagene). Prehybridization was performed in UltraHyb Oligo hybridization buffer (Ambion) at 42 °C for 30 min, after which 1 μg of each appropriate biotin 5′ end–labeled oligonucleotide was added directly to the hybridization buffer as probe. Blots were hybridized overnight with gentle rotation. The hybridization buffer was removed the following day, and membranes were washed in Northern Max Low Stringency Wash Solution (Ambion) three times for 5 min at room temperature. Membranes were finally developed using the Brightstar system (Ambion), following the manufacturer protocol, and exposed to film (BioMax MS and Kodak) at room temperature.

Luciferase assays. A549 cells were cultured in MEM (10% (v/v) FBS and 1% (v/v) PS) in 12-well plates. At 90% confluence, we transfected cells by following the Lipofectamine 2000 (Invitrogen) protocol. At 35 h, existing media was replaced with 1 ml fresh media. At 36 h, cells were scraped from well bottoms and transferred to 1.5-ml Eppendorf tubes. Eppendorfs were centrifuged at 400*g* for 3 min, and then supernatant was aspirated and cells resuspended in 300 μl PBS. Cells were lysed by freeze-thaws and debris removed by centrifuging at 750*g* for 3 min. Supernatant (50 μl) was transferred to a 96-well MicroLite plate (MTX Lab Systems), and then firefly and *Renilla* luciferase activities were measured using the Dual-glo Luciferase Reporter System (Promega) and a 96-well plate luminometer (Dynex). Relative light units were calculated as the quotient of *Renilla*/firefly relative light units and normalized to a mock assay.

Eukaryotic *in vitro* transcriptions. pAL-1 *in vitro* transcriptions were performed in HeLa S100 extracts with α-amanitin added to all reactions to inhibit Pol II transcription. Each reaction consisted of parts A and B, which were later combined. Part A contained 2.0 μl of additional water and no NTPs. Part B contained 7.2 μl of additional water and no pAL-1. (Standard reaction in order: 4.0 μl 5× Pol III transcription buffer, 0.2 μl DTT (0.1 M), 0.2 μl α-amanitin (1 μg μl^{-1}; Alexis Biochemicals no. 350-270-M001), 0.2 μl RNase inhibitor (20 units μl^{-1}), 2.0 μl NTPs (5 mM), 7.2 μl pAL-1 (0.14 μg μl^{-1}), 4.0 μl S100 extract (6 μg μl^{-1}; Protein One no. P0004-01) and 2 μl tagetitoxin (20 units μl^{-1}; Epicentre no. T9705H; replaced by water in controls.) Part A was incubated at 30 °C for 30 min, during which *in vitro* transcription Part B remained on ice. After 30 min, Part B was added to Part A and the mixture incubated at 30 °C for 30 min. After reaction completion, RNA was isolated using 1 ml Trizol (Invitrogen) and resuspended in 1× Tris-EDTA buffer and products resolved by formaldehyde-agarose electrophoresis (500 ng per sample) and visualized with ethidium bromide.

Chromatin immunoprecipitations. HEK 293T cells and BN51 cells were cultured in DMEM (10% (v/v) FBS, 1% (v/v) PS) in 60-mm plates. At 90% confluence, cells were heat-shocked for 30 min at 40 °C, then incubated at 37 °C for 2 h. Next, cells were dislodged by scraping, transferred to 15-ml Falcon tubes and incubated for 10 min in 10 ml DMEM with 1% (v/v) formaldehyde. ChIPs were performed using the EZ-ChIP kit (Upstate USA, no. 17371) and standard manufacturer protocol. Antibodies used for immunoprecipitation were as follows: normal mouse IgG negative control (Upstate USA, no. 12371B), anti–RNA polymerase II (Upstate USA, no. 05623B), anti-Flag (Sigma-Aldrich, no. F7425), anti-RPC 53 (Pol III-specific), anti-RPB 90 (TFIIIB-specific) and anti-TFIII c63 (TFIIIC-specific) (see ref. 20 and Acknowledgments). Recovered DNA was quantified using the Picogreen dsDNA Quantitation kit (Molecular Probes), then diluted to 0.1 ng μl^{-1}. Standard PCR reactions were performed for 25, 30 or 35 cycles (as described in next section). Starting material was standardized across all reactions (0.1 ng eluted DNA), excluding inputs, which were titrated from 0.5 ng to 10 ng per reaction. Following amplification, products were resolved by 1.5% agarose gel electrophoresis. The identities of all amplicons were verified by subsequent cloning and sequencing.

Vector construction. Unless otherwise indicated, PCR amplifications were performed in 40-μl reactions at standard concentrations (1.5 mM $MgCl_2$, 0.2 mM dNTP, 1× Biolase PCR buffer, 0.5 units Taq polymerase (Biolase USA; 0.5 μM for each primer)) and using standard cycling parameters (initial cycle of 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C; final cycle of 3 min at 72 °C), then cloned into Topo PCR 2.1 (Invitrogen). All reverse transcriptions were performed at 65 °C using MonsterScript Reverse Transcriptase (Epicentre, no. MSTA5110), then amplified with gene-specific or degenerate primers. Putative promoters were first cloned and verified, then reamplified using the forward primer from the first reaction and a vector-specific reverse primer tailed with the shLacZ hairpin and Pol III terminator. Resultant amplicons were cloned into Topo PCR 2.1 and sequenced. Antisense reporters, LacZ-AS and 517-AS, were constructed by oligonucleotide primer extension (25 cycles with 10 s extensions) with primers containing 5′ XhoI and 3′ SpeI restriction sites immediately flanking sequences perfectly complementary to shLacZ and miR-517a. After digestion, amplicons were ligated into the *Renilla* luciferase 3′ UTR of psiCheck2 vector (Promega) that had been linearized with XhoI and SpeI. The presence of an independently transcribed firefly luciferase in these reporters allowed normalization for transfection efficiency.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

G.M.B. conceived the study, collected the experimental data and contributed to the identification of repetitive element–microRNA associations, design and planning of the wet-lab experimentation, analysis of the experimental and computational findings and writing of the manuscript. W.L. contributed to the identification of repetitive element–microRNA associations and computational analysis. B.L.D. contributed to design and planning of the wet-lab experimentation, analysis of the experimental and computational findings and writing of the manuscript. All authors edited and approved the final version of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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