MicroRNAs direct rapid deadenylation of mRNA

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MicroRNAs (miRNAs) are ubiquitous regulators of eukaryotic gene expression. In addition to repressing translation, miRNAs can down-regulate the concentration of mRNAs that contain elements to which they are imperfectly complementary. Using miR-125b and let-7 as representative miRNAs, we show that in mammalian cells this reduction in message abundance is a consequence of accelerated deadenylation, which leads to rapid mRNA decay. The ability of miRNAs to expedite poly(A) removal does not result from decreased translation; nor does translational repression by miRNAs require a poly(A) tail, a 3′ histone stem-loop being an effective substitute. These findings suggest that miRNAs use two distinct posttranscriptional mechanisms to down-regulate gene expression.

let-7 | miR-125b | poly(A) | translation

A burgeoning body of evidence indicates that microRNAs (miRNAs) play an important and widespread role in regulating protein synthesis. In animal cells, these small untranslated RNAs repress gene expression by annealing to mRNAs to which they are partially complementary. Unlike perfectly complementary siRNAs, which guide mRNA cleavage at the sites to which they bind (1), imperfectly complementary miRNAs impair the ability of their mRNA targets to function as templates for protein synthesis, apparently by inhibiting translation initiation by means of a mechanism that is poorly understood (2–5). Despite this important difference, the regulatory influence of both miRNAs and siRNAs is thought to be mediated by similar protein complexes that deliver them to their mRNA targets (6–11).

Although initial reports suggested that down-regulation by partially complementary miRNAs was due entirely to decreased translation (2–4), recent studies have indicated that miRNAs can also reduce the cellular concentration of the mRNAs that they regulate, both *in vitro* and *in vivo* (12–16). For example, the interaction of miR-125b or its paralog miR-125a with two imperfectly complementary elements (miRE1 and miRE2) in the 3′ UTR of the mammalian *lin-28* message leads to significant reductions in both translation and mRNA abundance (15). This decline in mRNA concentration has been shown to occur by a posttranscriptional mechanism. In *Caenorhabditis elegans*, where *lin-28* plays an important role in larval development, a developmentally regulated miRNA homologous to miR-125b has a similar effect on *lin-28* message levels (14). These and other findings have led to suggestions that miRNAs may be able to destabilize mRNAs to which they are imperfectly complementary. However, the mechanism by which they do so is not known.

Here we report that in mammalian cells two different miRNAs, miR-125b and let-7, expedite poly(A) tail removal as an initial step in the accelerated degradation of mRNAs containing elements to which they are imperfectly complementary. This increased rate of deadenylation does not result from the diminished frequency of translation caused by miRNA binding. Conversely, although poly(A) removal appears to be a key step in miRNA-mediated mRNA decay, a poly(A) tail is not required for translational repression by miRNAs.

Results

Down-regulation of mRNA by miR-125b has been observed both in P19 mouse embryonal carcinoma cells, where the increased production of this miRNA upon differentiation into neurons contributes to a marked decline in the concentration of *lin-28* mRNA, and in 293T human embryonic kidney cells, where the synthesis of miR-125b from a transfected gene significantly reduces the cellular abundance of luciferase reporter mRNAs bearing multiple copies of either miRE1 or miRE2 in the 3′ UTR (15). To determine whether miR-125b decreases the concentration of mRNAs bearing these elements by expediting mRNA degradation, we examined the effect of *lin-28* miRE1 on the decay of a β-globin reporter mRNA (BG) expressed in 293T cells under the control of a transiently inducible c-*fos* promoter. This well established promoter-reporter system for studying mRNA decay made it possible to monitor the nearly synchronous degradation of mRNA molecules that were similar in age (17). The presence of two copies of miRE1 in the 3′ UTR (BG+2E1) markedly accelerated the decay of the reporter mRNA in cells that had been engineered to produce miR-125b at a concentration comparable with that in differentiating P19 cells (Fig. 1 *A* and *B*; see also Fig. 6, which is published as supporting information on the PNAS web site). Inserting additional copies of miRE1 resulted in even faster decay (data not shown). No such effect was observed in cells that lacked miR-125b (Fig. 1*B*; see also Fig. 6).

Further investigation showed that the increased rate of mRNA decay did not result from endonucleolytic cleavage within the *lin-28* element. Luciferase reporter mRNAs that bore either miRE1 or a synthetic element (element *P*) perfectly complementary to miR-125b were extracted from 293T cells containing miR-125b and analyzed by ligation to a synthetic oligoribonucleotide and RT-PCR with primers related to the oligoribonucleotide or complementary to a 3′ UTR segment downstream of the regulatory element. This procedure made it possible to detect any 3′-terminal degradation intermediates that might result from mRNA cleavage within the target element, as the 5′ end of such intermediates could be joined to the oligoribonucleotide by T4 RNA ligase (18). Although miR-125b directed cleavage within the RNA element to which it was perfectly complementary, no such cleavage could be detected in or near the imperfectly complementary miRE1 element, which nonetheless mediated significant reductions in luciferase mRNA and protein levels (Fig. 1*C* and other data not shown; see also Fig. 7, which is published as supporting information on the PNAS web site). We conclude that miR-125b accelerates the decay of mRNA containing imperfectly complementary elements by a mechanism that is distinct from the site-directed endonucleolytic process characteristic of RNA silencing mediated by perfectly complementary siRNAs (1).

Closer examination of the decay of BG+2E1 mRNA revealed that, in the presence of miR-125b, it underwent noticeable shortening within 3 h after its transient synthesis (Fig. 1*B*).

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Abbreviation: miRNA, microRNA.

See Commentary on page 3951.

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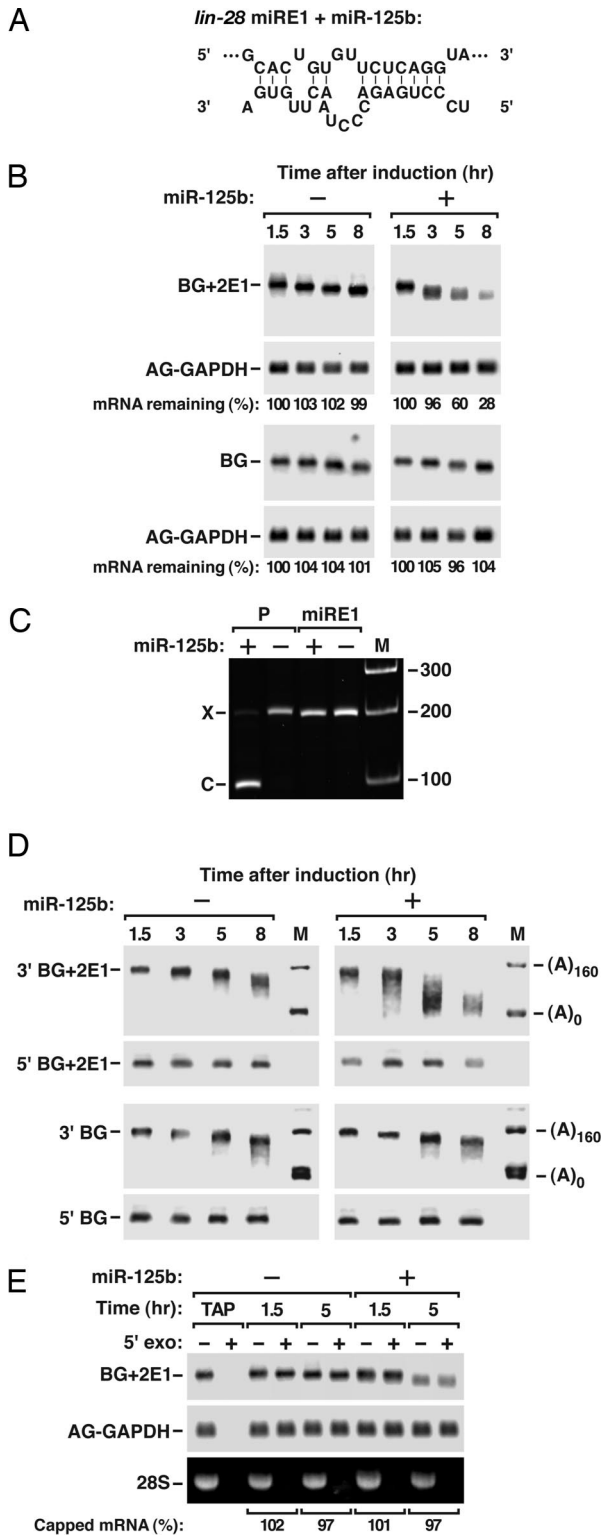

Fig. 1. Effect of miR-125b on the degradation of mRNA bearing *lin-28* miRE1. (*A*) Duplex expected for *lin-28* miRE1 (top) base-paired with miR-125b (bottom). (*B*) Decay of BG+2E1 and BG mRNA in the presence or absence of miR-125b. Total cytoplasmic RNA was extracted from transfected 293T cells at time intervals after serum stimulation to induce transient transcription of each reporter gene from its c-*fos* promoter. Equal amounts of each RNA sample were analyzed by electrophoresis and blotting. The relative quantity of reporter mRNA remaining at each time was calculated after normalization to AG-GAPDH mRNA (a cotransfected internal standard). (*C*) Inability of miR-125b to direct endonucleolytic cleavage within miRE1. Luciferase reporter mRNAs bearing miRE1 or a synthetic element (element *P*) perfectly complementary to miR-125b were extracted from 293T cells that did or did not produce miR-125b, ligated to an RNA oligonucleotide, and amplified by RT-PCR, using primers corresponding to sites within the ligated oligonucleotide or 0.08 kb downstream of the inserted element. The RT-PCR products were analyzed by gel electrophoresis beside DNA size markers (M). DNA sequencing confirmed that band C represented a degradation intermediate resulting from miR-125b-directed mRNA cleavage in the middle of the perfectly complementary element *P*. Band X, which resulted from miR-125b-independent mRNA cleavage upstream of the insertion site of miRE1 or element *P*, served as an internal standard; its low intensity in the leftmost lane is due to the diminished concentration of this reporter mRNA in the presence of miR-125b and competition with band C for PCR amplification. Calibration is in base pairs. (*D*) Deadenylation mediated by miR-125b. Equal amounts of the total cytoplasmic RNA samples examined in *B* were subjected to site-specific cleavage by RNase H in the presence of an oligodeoxynucleotide complementary to codons 74–81 within the coding region of BG+2E1 and BG mRNA. The 5′ and 3′ RNA fragments thereby produced were analyzed by electrophoresis and blotting, using markers (M) that corresponded in size to reporter mRNA 3′ fragments bearing no poly(A) or a 160-nt poly(A) tail. (*E*) Retention of the 5′ cap on mRNA undergoing deadenylation. The 1.5 and 5 h BG+2E1 mRNA samples examined in *B* were treated or not treated with a 5′-phosphate-dependent exonuclease and analyzed by electrophoresis and blotting. Prior treatment of the 1.5 h sample with tobacco acid pyrophosphatase (TAP) released the 5′ cap and rendered the mRNA susceptible to 5′ exonuclease digestion. 28S rRNA, which lacks a 5′ cap, served as an additional positive control for exonuclease activity. AG-GAPDH mRNA was used as a normalization standard. The percentage of BG+2E1 mRNA that was capped was calculated from the ratio of band intensities in the presence versus the absence of exonuclease treatment.

Site-specific cleavage *in vitro* by RNase H before gel electrophoresis showed that this decrease in length was due to trimming of the 3′ poly(A) tail (Fig. 1*D*), most likely by an exonuclease. By measuring poly(A) length as a function of time after inducing transient transcription in cells containing miR-125b, we found that the presence of miRE1 caused extensive deadenylation and that decay of the transcribed portion of BG+2E1 mRNA did not begin until the shortest poly(A) tails had reached a length of 20–30 nt or less. No such effect of miR-125b was observed for the poly(A) tail of an otherwise identical reporter mRNA (BG) lacking miRE1; nor did miRE1 expedite deadenylation in the absence of miR-125b. That BG+2E1 mRNA undergoing miR-125b-mediated deadenylation still retained its 5′ cap was evident from its resistance to 5′-exonucleolytic digestion *in vitro*, which was lost upon treatment with tobacco acid pyrophosphatase to remove the cap (Fig. 1*E*). These findings indicate that miR-125b can destabilize mRNA by interacting with imperfectly complementary 3′ UTR elements and hastening deadenylation as an initial step in accelerated decay.

To identify other transcripts down-regulated by miR-125b, we examined its effect on mRNA levels in P19 cells, which produce this miRNA when induced to differentiate into neurons (15, 19). Cytoplasmic RNA extracted from undifferentiated P19 cells that had or had not been transfected with chemically synthesized miR-125b (Fig. 6) was used to probe a mouse genome microarray. With a high degree of confidence ($P < 0.003$), 22 mRNAs were found to decrease in abundance by at least a factor of 1.4 within 24 h after exposure to miR-125b. As expected, these included *lin-28* mRNA, whose concentration fell by a factor of 1.7, a change comparable with the 2-fold increase in Lin-28 protein levels previously observed when the function of miR-125b was inhibited in differentiating P19 cells (15). Most of the affected mRNAs contained one or more 3′ UTR elements with the potential to interact productively with miR-125b, whereas such elements were comparatively rare in P19 mRNAs judged not to be affected (Fig. 2*A*; see also Table 1, which is published as supporting information on the PNAS web site).

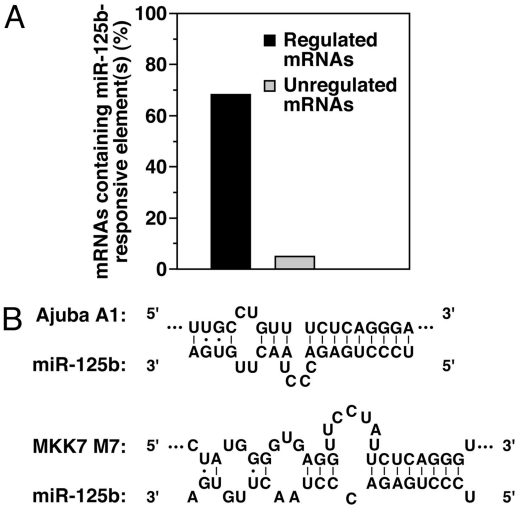

Fig. 2. miR-125b-responsive elements in down-regulated mRNAs of P19 cells. (*A*) Correlation between reduced mRNA concentration and the presence of probable miR-125b-responsive elements. Mouse Genome 430A 2.0 arrays (Affymetrix) were used to compare the relative concentrations of mRNAs in undifferentiated P19 cells 24 h after mock transfection or transfection with a chemically synthesized miR-125b duplex (see Fig. 6). Two groups of mRNAs were identified whose concentration could be said with a high degree of certainty to have decreased significantly (by at least a factor of 1.40, with ≥95% confidence that the change was by at least a factor of 1.30) (see Table 1) or to have remained unchanged (≥95% confidence of a concentration change no greater than ±5%) in the presence of miR-125b. These mRNAs (22 in the first group, and 669 in the second) were then examined for 3′ UTR elements with the potential to interact productively with miR-125b [complementarity to nucleotides 2–8 or to nucleotides 1 and 3–9 of miR-125b (15, 31)], and the percentage of mRNAs with one or more such elements was calculated for each group. (*B*) Duplexes expected to result from base pairing of the Ajuba A1 element or the MKK7 M7 element with miR-125b.

Besides *lin-28*, the P19 messages down-regulated by miR-125b included Ajuba mRNA, whose 3′ UTR contains a likely miR-125b-responsive element in both mice and humans (A1; Fig. 2*B*). Ajuba is a LIM-domain protein that plays an important role in controlling the entry of vertebrate cells into mitosis and meiosis (20, 21). In P19 cells induced to differentiate, the concentration of this protein initially rises and then declines (22), suggesting a delayed-onset repression mechanism to which miR-125b may contribute as it accumulates over time. Like *lin-28* miRE1, the Ajuba A1 element was found to direct rapid deadenylation and subsequent decay of a reporter mRNA in transfected 293T cells that produced miR-125b (Fig. 8, which is published as supporting information on the PNAS web site). mRNA destabilization involving fast poly(A) removal was likewise observed for an element derived from MKK7 (MAPK kinase 7) mRNA, another message down-regulated by miR-125b in P19 cells (Fig. 2*B*; see also Fig. 9, which is published as supporting information on the PNAS web site). These findings indicate that miR-125b can expedite the deadenylation and decay of many different mRNAs bearing a variety of elements to which it is imperfectly complementary.

A second miRNA, let-7, also helps to down-regulate *lin-28* gene expression in differentiating P19 cells by interacting with a distinct 3′ UTR element (L7; Fig. 3*A*) (15). In combination, L7 and let-7 proved to be as effective as miRE1 and miR-125b at repressing protein synthesis (15), as judged from the 89% decrease in expression of an L7-containing luciferase reporter (Luc+6L7) in the presence of let-7a. Furthermore, down-regulation of protein synthesis was accompanied by a smaller yet substantial decline in mRNA concentration (Fig. 3*B*). This reduction in mRNA was a consequence of accelerated deadenylation and decay, as evidenced

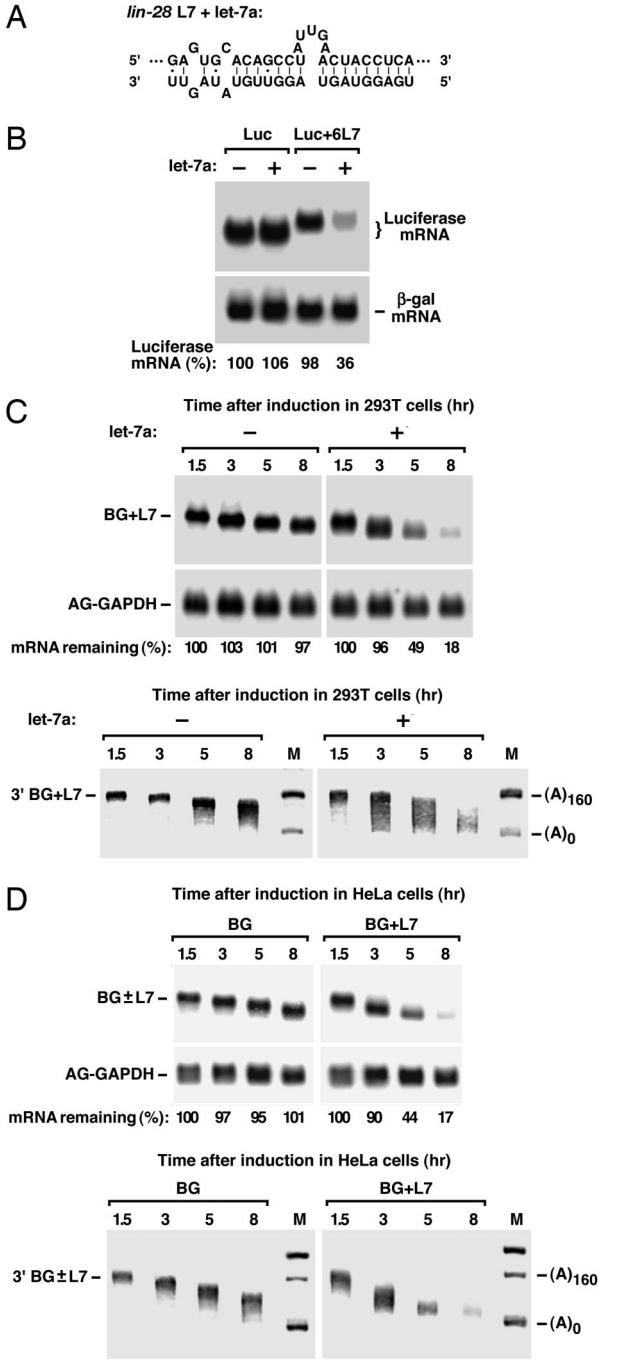

Fig. 3. Effect of let-7 on the deadenylation and decay of mRNA bearing the *lin-28* L7 element. (*A*) Duplex expected for the *lin-28* L7 element (top) base-paired with let-7a (bottom). (*B*) Influence of let-7a on the concentration of Luc+6L7 mRNA. Luciferase mRNA levels were analyzed by electrophoresis and blotting of total cytoplasmic RNA from 293T cells that had been transiently cotransfected with a luciferase reporter gene containing 0 or 6 copies of the *lin-28* L7 element in its 3′ UTR, a gene encoding (+) or not encoding (−) human let-7a, and a β-galactosidase gene (internal standard). (*C*) Influence of let-7a on the degradation of BG+L7 mRNA in 293T cells. Analyses of mRNA deadenylation and decay similar to those in Fig. 1 were performed with RNA samples from 293T cells that had been transiently cotransfected with a BG+L7 reporter gene, a gene encoding (+) or not encoding (−) human let-7a, and a gene encoding AG-GAPDH mRNA (internal standard). (*D*) Deadenylation and decay of BG+L7 and BG mRNA in HeLa cells. Analyses of mRNA degradation similar to those in Fig. 1 were performed by using RNA samples from HeLa cells that had been transiently cotransfected with a reporter gene containing (BG+L7) or lacking (BG) one copy of L7 and a gene encoding AG-GAPDH mRNA (internal standard).

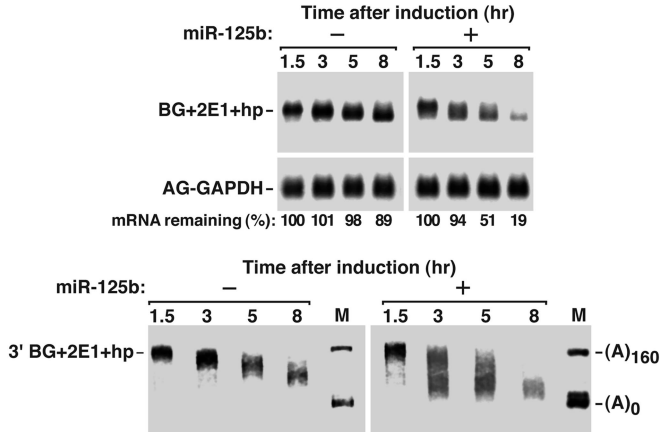

Fig. 4. Accelerated deadenylation and decay in the absence of translation. Analyses of mRNA degradation similar to those in Fig. 1 were performed with RNA samples from 293T cells that had been transiently cotransfected with a modified BG+2E1 gene bearing a 40-bp inverted repeat in the 5′ UTR (BG+2E1+hp), a gene encoding (+) or not encoding (−) miR-125b, and a gene encoding AG-GAPDH mRNA (internal standard).

by the ability of one copy of L7 to cause rapid poly(A) shortening and subsequent degradation of BG+L7 mRNA in 293T cells transfected with a gene encoding let-7a (Fig. 3*C*; see also Fig. 6). That these effects were not cell-type-specific and could be caused by an endogenous miRNA was demonstrated by showing a similar influence of L7 on mRNA deadenylation and decay in HeLa cells (Fig. 3*D*), which produce let-7 naturally (23).

To demonstrate that the rapid deadenylation and decay mediated by miRNAs is not a consequence of the reduced frequency with which ribosomes transit the coding region, translation of BG+2E1 mRNA was abolished by inserting a large (40-nt) stem-loop structure within its 5′ UTR to create BG+2E1+hp. This stem-loop had previously been shown to prevent assembly of 80S ribosomes when present at the same location within another mRNA containing an identical 5′ UTR and coding region (24), and its inhibitory effect on translation of BG+2E1+hp mRNA was confirmed by in-frame fusion to a luciferase coding region, which showed it to reduce protein synthesis by >99.5%. Blocking translation initiation in this manner caused only a slight increase in the slow rate of poly(A) shortening previously observed for BG+2E1 in the absence of miR-125b, and this change was dwarfed by the much larger increase in the rate of deadenylation and decay caused by miR-125b (Fig. 4). Thus, impaired translation *per se* is not sufficient to accelerate deadenylation markedly, nor is translation required for miR-125b to increase the rate of poly(A) removal.

Conversely, a poly(A) tail is not required for miR-125b to repress translation. This was shown by comparing the effect of miR-125b on luciferase reporter mRNAs bearing miRE1 and either a 3′ poly(A) tail (Luc+6E1) or a 3′-terminal stem-loop derived from a histone mRNA (Luc+6E1.HSL). As expected, miR-125b diminished the abundance of only the polyadenylated reporter mRNA (Fig. 5 *A* and *B*). Interestingly, assays of luciferase protein synthesis showed that, whereas the overall degree of repression was greater for the polyadenylated reporter, the inhibitory effect of miR-125b on translation efficiency (protein synthesis per mRNA molecule) was the same for both mRNAs (Fig. 5*C*). This finding suggested that the regulatory effects of translational repression and accelerated decay were additive, such that decreases in translation efficiency and mRNA concentration contributed equally to down-regulation of the polyadenylated reporter, whereas repression of the reporter

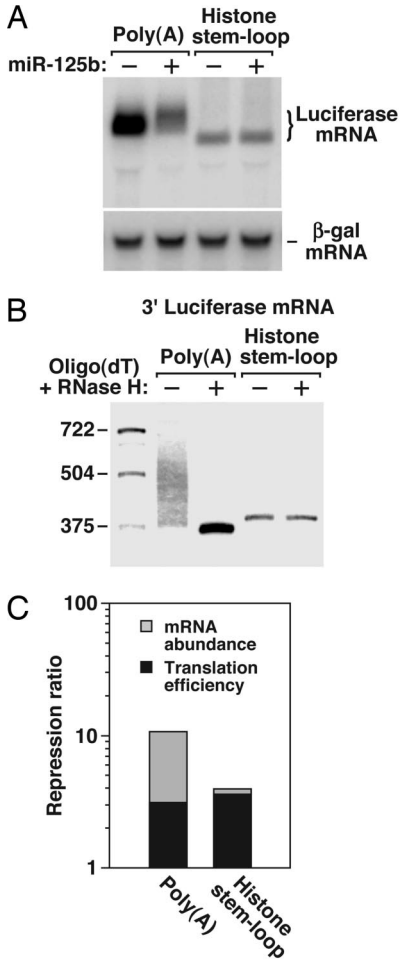

Fig. 5. Translational repression in the absence of a poly(A) tail. (*A*) Differential effect of a 3′ histone stem-loop or a poly(A) tail on the reduction in mRNA abundance caused by miR-125b. Luciferase mRNA levels were analyzed by electrophoresis and blotting of total cytoplasmic RNA from 293T cells that had been transiently cotransfected with a gene that encoded a luciferase reporter mRNA bearing six copies of miRE1 in the 3′ UTR and ending with either a 3′ poly(A) tail (Luc+6E1) or a 3′-terminal histone H1.3 stem-loop (Luc+6E1.HSL), a gene that encoded (+) or did not encode (−) miR-125b, and a β-galactosidase gene (internal standard). (*B*) Confirmation of the dissimilar nature of the 3′ ends. The Luc+6E1 and Luc+6E1.HSL RNA samples from cells lacking miR-125b were also analyzed by electrophoresis and blotting after treatment, in the presence or absence of oligo(dT), with RNase H and an oligodeoxynucleotide complementary to a segment 361–386 nt upstream of the poly(A) addition site of Luc+6E1 or 376–395 nt upstream of the 3′ end of Luc+6E1.HSL (right). Calibration is in nucleotides. (*C*) Contributions of translation inhibition and diminished mRNA abundance to repression by miR-125b. After normalizing the concentrations of Luc+6E1 and Luc+6E1.HSL mRNA to β-galactosidase mRNA, the ratio of each in the absence or presence of miR-125b was calculated and superposed on a bar graph showing the overall degree of repression of the same reporters, as judged from relative luciferase protein levels (normalized to β-galactosidase). The effect of miR-125b on translation efficiency (black bars) corresponds to the ratio of its overall effect on protein synthesis versus its effect on mRNA concentration (gray bars). In the case of the polyadenylated reporter (Luc+6E1), which was repressed 10.7-fold, expression of miR-125b reduced translation efficiency by a factor of 3.1 ± 0.3 and mRNA abundance by a factor of 3.5 ± 0.2. In the case of the reporter ending in a histone stem-loop (Luc+6E1.HSL), which was repressed 3.9-fold, miR-125b reduced translation efficiency by a factor of 3.6 ± 0.3 and mRNA abundance by a factor of 1.1 ± 0.1.

bearing a histone stem-loop was achieved entirely at the level of translation. We conclude that, whereas the mRNA decay mechanism triggered by the interaction of miR-125b with miRE1

involves accelerated deadenylation, the mechanism of translational repression mediated by the same interaction does not require a poly(A) tail.

Discussion

The discovery that both miR-125b and let-7 can expedite poly(A) removal as an initial step in the rapid degradation of mRNAs bearing imperfectly complementary elements suggests that accelerated deadenylation and decay are likely to be frequent consequences of the interaction of mammalian miRNAs with their targets. By analogy to known pathways for mRNA degradation (25), poly(A) tail loss would be expected to facilitate decay of the transcribed portion of targeted mRNAs by exposing either their 5′ ends to cap removal and subsequent degradation by the 5′ exonuclease Xrn1 or their 3′ ends to exonucleolytic degradation by the exosome. The former pathway would help to explain the detection of 3′-terminal decay intermediates resulting from let-7-mediated mRNA degradation in *C. elegans* (14).

mRNA decay triggered by rapid deadenylation appears to be an important aspect of the mechanism by which miR-125b and let-7 down-regulate gene expression. Thus, replacing an mRNA poly(A) tail with a 3′ histone stem-loop not only eliminates the effect of miR-125b on message abundance but also diminishes its influence on protein synthesis by an amount equal to its effect on the concentration of an otherwise identical mRNA bearing a poly(A) tail. This finding suggests that, by reducing both the translation efficiency and the concentration of targeted mRNAs that are polyadenylated, miRNAs can have a greater overall impact on gene expression than if down-regulation were limited to translational repression. Moreover, unlike translation inhibition, the regulatory consequences of mRNA degradation are irreversible. By extrapolation, accelerated deadenylation may also contribute to the off-target effects of siRNAs, which can likewise reduce the abundance of mRNAs to which they are imperfectly complementary (26).

Although it is possible that miRNA-mediated deadenylation and translational repression may both result from a single precipitating event, our findings imply that these two regulatory mechanisms can operate independently. Translation is not required for miRNAs to hasten poly(A) shortening, nor is a poly(A) tail needed for them to down-regulate translation efficiency. The latter observation is of particular significance in view of recent evidence that miRNAs inhibit translation initiation (5), as poly(A)-binding protein (PABP) facilitates translation initiation by interacting with a protein component of the 5′ cap binding complex (27, 28). Thus, miRNA-mediated poly(A) tail loss might have been expected to impair translation efficiency were the deadenylated messages not rapidly degraded. The ability of miR-125b to inhibit translation of a reporter that ends in a histone stem-loop instead of a poly(A) tail suggests that translational repression by miRNAs occurs by a mechanism that is unrelated to PABP loss or at a step in translation initiation where PABP and the protein that binds histone stem-loops function analogously (28, 29).

Materials and Methods

Plasmid Construction, Cell Culture, and Transfection. Methods for plasmid construction, cell culture, and DNA transfection are published as *Supporting Materials and Methods* on the PNAS web site.

Monitoring the Decay of β-Globin Reporter mRNAs. DNA mixtures for transient transfection contained a β-globin reporter plasmid (pBG, pBG+2E1, pBG+A1, pBG+M7, or pBG+L7; 0.8 μg), a plasmid encoding or not encoding a miRNA (pMIR125b or pMIR125bΔ; pLET7a or pLET7aΔ; 1.0–3.0 μg), and a plasmid encoding an α-globin-GAPDH mRNA chimera (AG-GAPDH) under the control of a constitutive SV40 promoter (pSVα1-GAPDH; 0.2 μg), which served as an internal standard. After serum-starving the transfected cells for 24 h (0.5% serum), transcription from the c-*fos* promoter of the reporter gene was transiently induced by increasing the serum concentration to 20%, and total cytoplasmic RNA was isolated at time intervals and analyzed by electrophoresis (1.5% agarose) and blotting, as described in ref. 15.

To monitor the degradation of 5′- and 3′-terminal segments of reporter mRNAs, equal amounts of cytoplasmic RNA were treated with RNase H in the presence of an oligodeoxynucleotide (oligo BBB243: GGTTGTCCAGGTGACTCAGACCCTC) complementary to codons 74–81 within the β-globin coding region, and the digested RNA samples were analyzed by electrophoresis and blotting, using a 5% polyacrylamide/8 M urea gel. Size markers for fully deadenylated mRNA and for mRNA bearing a 160-nt poly(A) tail were generated by treating reporter mRNAs in cytoplasmic extracts with RNase H, oligo(dT), and either oligo BBB243 or an oligodeoxynucleotide (oligo BBB81: CCTCACCACCAACTTCTTCCACATT) complementary to codons 20–27 within the β-globin coding region. Alternatively, RNA markers of the same size and sequence were generated by *in vitro* transcription with T7 RNA polymerase.

Similar procedures were used to examine let-7-mediated deadenylation and decay in HeLa cells, except that the cells were transfected with a β-globin reporter plasmid (pBG or pBG+L7; 0.8 μg), pSVα1-GAPDH (0.2 μg), and pUC19 (1.0 μg).

To test for the presence of a 5′ cap, cytoplasmic RNA samples (10 μg) were treated with Terminator 5′-phosphate-dependent exonuclease (1 unit; EPICENTRE Biotechnologies) for 2 h at 30°C, according to the manufacturer's protocol. As a positive control, samples were pretreated with tobacco acid pyrophosphatase (5 units; EPICENTRE Biotechnologies) for 2 h at 37°C to release the 5′ cap.

Luciferase mRNA and Protein Assays. Relative steady-state levels of luciferase and β-galactosidase mRNA or protein were assayed in extracts of transiently transfected 293T cells by RNA electrophoresis and blotting or by measuring enzyme activity, as described in ref. 15. To confirm the differential nature of the 3′ ends of Luc+6E1 and Luc+6E1.HSL mRNA, cytoplasmic RNA from 293T cells transfected with pCL-6E1 or pCL-6E1.HSL was treated, in the presence or absence of oligo(dT), with RNase H and an oligodeoxynucleotide complementary either to a segment 361–386 nt upstream of the poly(A) addition site of Luc+6E1 (oligo HIVRH: CCGTTCACTAATCGAATGGATCTGTC) or to a segment 376–395 nt upstream of the 3′ end of Luc+6E1.HSL (oligo LUC32: TTCCGCCCTTCTTGGCCTTT). The resulting RNA samples were analyzed by electrophoresis on a 5% polyacrylamide/8 M urea gel beside a set of radiolabeled RNA size markers, followed by blotting and probing.

A description of the use of oligoribonucleotide ligation and RT-PCR to determine whether miR-125b can direct cleavage of a luciferase reporter mRNA within miRE1 can be found in *Supporting Materials and Methods*.

Microarray Analysis of mRNA in P19 Cells. Triplicate cultures of undifferentiated P19 cells growing in α-MEM (GIBCO) supplemented with 10% FBS were mock-transfected or transfected with a chemically synthesized miR-125b duplex (5′-UCCCUGAGACCCUAACUUGUGA-3′ and 5′-ACAAGUUAGGGUCUCAGGGAUU-3′, 40 nM; Dharmacon) in the presence of Lipofectamine 2000 (Invitrogen; see manufacturer's protocol). After 12 h, the transfection medium was replaced with fresh

medium, and the cells were grown for an additional 12 h before total cytoplasmic RNA was extracted (15). The resulting RNA samples were processed for gene array analysis and used to probe Mouse Genome 430A 2.0 arrays (Affymetrix). The microarrays were scanned with an Affymetrix GeneChip Scanner 3000, and the raw data were processed with Affymetrix GCOS software. Calculations of relative mRNA concentration, including normalization and model-based analysis, were performed by using DCHIP software (30).

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