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Evidence that microRNAs are associated with translating messenger RNAs in human cells

Patricia A Maroney, Yang Yu, Jesse Fisher & Timothy W Nilsen

MicroRNAs (miRNAs) regulate gene expression post-transcriptionally by binding the 3′ untranslated regions of target mRNAs. We examined the subcellular distribution of three miRNAs in exponentially growing HeLa cells and found that the vast majority are associated with mRNAs in polysomes. Several lines of evidence indicate that most of these mRNAs, including a known miRNA-regulated target (KRAS mRNA), are actively being translated.

miRNAs comprise a large family of regulatory molecules that are important in a wide array of biological processes, including developmental timing, differentiation and growth control (see refs. 1–4 for recent reviews). In animal cells, miRNAs recognize their target mRNAs by base-pairing interactions between nucleotides 2–8 of the miRNA (the seed region) and complementary nucleotides in the 3′ untranslated region (UTR) of mRNAs (for example, see refs. 5,6). Recent informatic and experimental evidence indicates that miRNA-mediated regulation is remarkably pervasive; each miRNA has hundreds of evolutionarily conserved targets and several times that number of nonconserved targets[5,6]. Both types of targets are responsive to miRNA-mediated repression[5].

Although all miRNA-mRNA interactions seem to downregulate gene expression post-transcriptionally, the absolute degree of regulation has been shown to vary substantially, from less than two-fold to greater than ten-fold, depending on the specific miRNA–mRNA target combination. Although the determinants that define the different magnitudes of regulation remain to be defined, mRNAs that are only modestly regulated must be translated. Whether the degree of translation of such mRNAs is determined by accessibility of the mRNA to miRNAs or by other factors is unknown.

Regardless of how degrees of regulation are achieved, there is yet to emerge a unifying mechanism whereby miRNAs downregulate gene expression. Early analysis indicated that regulation was at the level of translation because the abundances of regulated mRNAs did not seem to change, whereas abundances of proteins encoded by those mRNAs were greatly reduced[7,8]. Furthermore, because these regulated mRNAs were found on polysomes, the block to translation seemed to be after initiation of protein synthesis[7,8]. A recent report has demonstrated miRNA-dependent inhibition of protein synthesis after initiation, and in this case, the defect in protein synthesis has been ascribed to premature termination of translation, or 'ribosome drop-off'[9]. Nevertheless, other experiments have indicated that miRNAs can inhibit initiation of translation; in these experiments, regulated mRNAs were found to be largely unengaged with ribosomes[10,11]. The mechanistic picture is further complicated by the fact that miRNAs clearly exert other effects on mRNA metabolism, the relationship of which to translation *per se* is not yet clear. For instance, abundant evidence indicates that miRNAs can destabilize certain mRNAs (for example, see refs. 12–15). This destabilization is mediated by the CCR4–NOT deadenylase complex and the DCP1–DCP2 decapping complex[16] and is linked to the role of the P-body component GW182 in miRNA function[16–19]. However, deadenylation does not necessarily lead to mRNA degradation, and deadenylation in and of itself does not account fully for translational repression[16,20]. Although these varied observations might be explained by different fates of miRNA-targeted mRNAs in P-bodies (reviewed in ref. 21), a recent report indicates that miRNA function does not require P-body structural integrity but does require RCK/p54 (ref. 22), a DEAD-box helicase known to be essential for translational repression in yeast[23].

The very complex picture of miRNA-mediated gene regulation has been derived largely from studies of specific miRNA–mRNA target pairs. To obtain a different perspective, we examined the steady-state subcellular distribution of three abundant miRNAs in HeLa cell extracts. We found that the vast majority of these miRNAs were associated with mRNAs in polysomes. Furthermore, the sedimentation of miRNAs was sensitive to a variety of conditions that affected protein synthesis, indicating that the miRNAs were associated with actively translating mRNAs. We also found that a specific target mRNA (KRAS), which is known to be miRNA regulated, sedimented with polysomes, and several lines of evidence demonstrate that it is associated with translationally competent ribosomes. We discuss these results in light of the current understanding of miRNA function.

RESULTS

Most miRNAs are associated with polysomes

To examine the subcellular distribution of miRNAs, we arbitrarily chose three 'representative' miRNAs (miR-21, miR-16 and let-7a),

Center for RNA Molecular Biology and Department of Biochemistry, Case Western Reserve University School of Medicine, W127 10900 Euclid Avenue, Cleveland, Ohio 44106-4973, USA. Correspondence should be addressed to T.W.N. (twn@case.edu)

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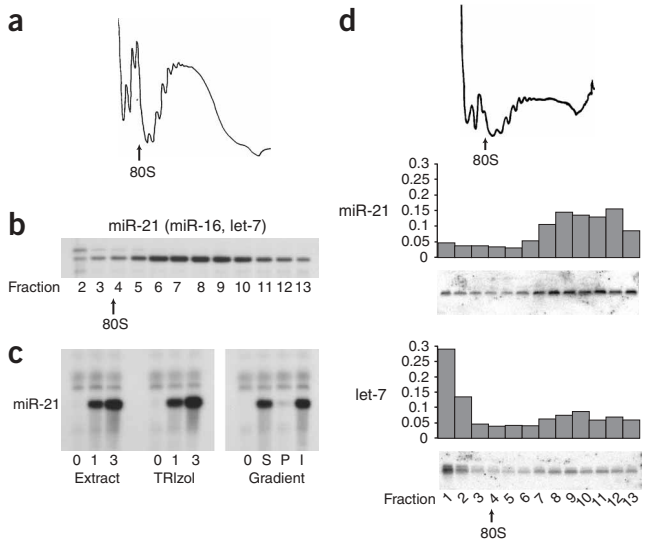

Figure 1 miRNAs sediment with polysomes in HeLa cell cytoplasmic extracts. Exponentially growing HeLa cells were harvested and cytoplasmic extracts prepared as described in Methods. An aliquot of such an extract was centrifuged through a sucrose gradient and fractionated as described in Methods. (**a**) A_{260} is shown. (**b**) Equal aliquots of each fraction were analyzed for the presence of miR-21 (shown), miR-16 or let-7 (not shown) by splinted ligation (P.A.M. and T.W.N., unpublished data); profiles of miR-16 and let-7 were identical to that of miR-21. (**c**) Equal amounts of cells were either processed as above to prepare a cytoplasmic extract (extract) or lysed with TRIzol (Invitrogen), and RNA was prepared. Equivalent aliquots of each RNA sample, corresponding to 0.01 and 0.03 of the total (1 and 3, as indicated below gel), were assayed for miR-21 as above. For the gel labeled 'gradient', an aliquot of the cytoplasmic extract was centrifuged through a sucrose gradient as in **a** and equal aliquots of the supernatant (S), pellet (P) and input (I) were assayed for miR-21 as above. (**d**) Cells were transfected with a 2′-O-me oligonucleotide complementary to let-7 as described in Methods. After 16 h, cells were harvested and extracts prepared and fractionated as above. Equal aliquots of fractions were analyzed for the presence of miR-21 or let-7 by northern blot and quantified by phosphorimaging.

each of which has previously been shown to be abundantly expressed in HeLa cells[24]. Exponentially growing cells were harvested and cytoplasmic extracts were prepared and displayed on sucrose gradients. The absorbance profiles at 260 nM reflected the pattern of ribosomes expected from rapidly growing cells; that is, there were few ribosomal subunits (40S and 60S) and few monosomes (80S); the bulk of the ribosomes sedimented in the polysomal fractions (**Fig. 1a**). Notably, the vast majority of miR-21 (as well as miR-16 and let-7) sedimented with the polysomal fractions (**Fig. 1b**). We considered the possibility that the polysome-associated miRNAs represented only a fraction of total cellular miRNAs; that is, some miRNAs might have been lost during extract preparation or pelleted during ultracentrifugation. However, this was not the case, as abundances of miRNAs in TRIzol lysates and cytoplasmic extracts were equivalent (**Fig. 1c**) and negligible amounts of miRNAs were found in the pellets of the sucrose gradients (**Fig. 1c**). To exclude the possibility that the observed sedimentation of miRNAs was due to association after lysis, cells were homogenized in buffer containing a vast excess of a 2′-O-methylated (2′-O-me) oligonucleotide complementary to let-7. Under these conditions, no change in sedimentation of any of the miRNAs was observed (data not shown). In addition, to address the possibility that sedimentation reflected nonspecific interactions of miRNAs and their associated proteins with other cytoplasmic constituents, cells were transfected with a 2′-O-me oligonucleotide complementary to let-7. In cells treated for 48 h, let-7 was not detectable (data not shown), an observation consistent with degradation induced by the complementary oligonucleotide (for example, see refs. 25,26). However, at 16 h after transfection, there was a pronounced accumulation of let-7 near the top of the gradient (**Fig. 1d**), whereas the sedimentation of miR-21 was unaltered (**Fig. 1d**). The 2′-O-me oligonucleotide effect suggests that the sedimentation of the miRNAs results from functional (that is, base-pairing–dependent) interactions.

As the profile of the miRNAs could have resulted from fortuitous cosedimentation of polysomes and other rapidly sedimenting structures, extracts were pretreated with 10 mM EDTA, to dissociate ribosomes into subunits. As expected, this treatment completely disrupted the polysomes and resulted in a corresponding accumulation of 40S and 60S ribosomal subunits (**Fig. 2**, EDTA). Concomitant with polysome disaggregation, the mRNAs encoding β-actin (**Fig. 2**, EDTA) and GAPDH (data not shown) shifted from heavy fractions to the top of the gradient; miR-21 shifted in parallel to the lighter regions of the gradient (**Fig. 2**, EDTA). This result indicated that sedimentation of the miRNAs in the polysomal regions of gradients was not a result of accumulation of miRNAs in an EDTA-insensitive, high–molecular weight particle.

Association of miRNAs with polysomes is mRNA mediated

Cosedimentation of miRNAs and polysomes would be observed either if miRNAs and their associated proteins were capable of interacting directly with ribosomes or if miRNAs were present in polysomes because of their interaction with mRNAs. To distinguish between these possibilities, cytoplasmic extracts were digested under mild conditions with micrococcal nuclease. Under these conditions, ribosomes remain intact but exposed regions of mRNA are vulnerable to digestion. After micrococcal nuclease treatment, polysomes were almost completely destroyed, resulting in a large accumulation of 80S ribosomes

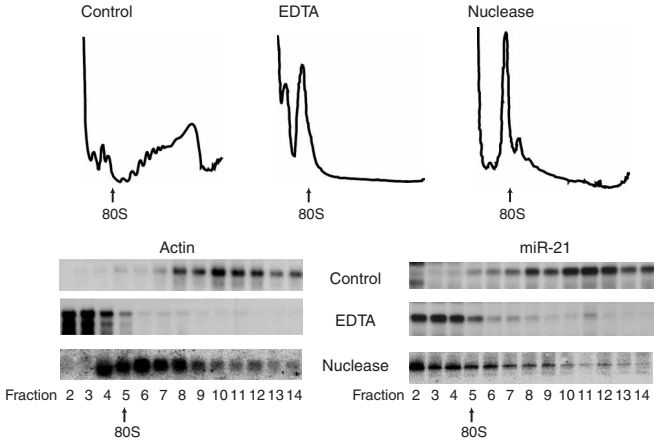

Figure 2 miRNAs are associated with mRNAs in polysomes in HeLa cells. Aliquots of cytoplasmic extracts, either untreated (Control), treated for 10 min on ice with 10 mM EDTA (EDTA) or digested with 0.75 units μl^{-1} of micrococcal nuclease (Nuclease) were centrifuged through sucrose gradients and fractionated as described in Methods. RNA was prepared from individual fractions and analyzed for the presence of miR-21 (splinted ligation for control and EDTA samples; northern blot for nuclease-treated sample) or β-actin mRNA (northern blot) as described in Methods. In nuclease-treated sample, actin signal is generated by hybridization to small ribosome-protected fragments of the mRNA.

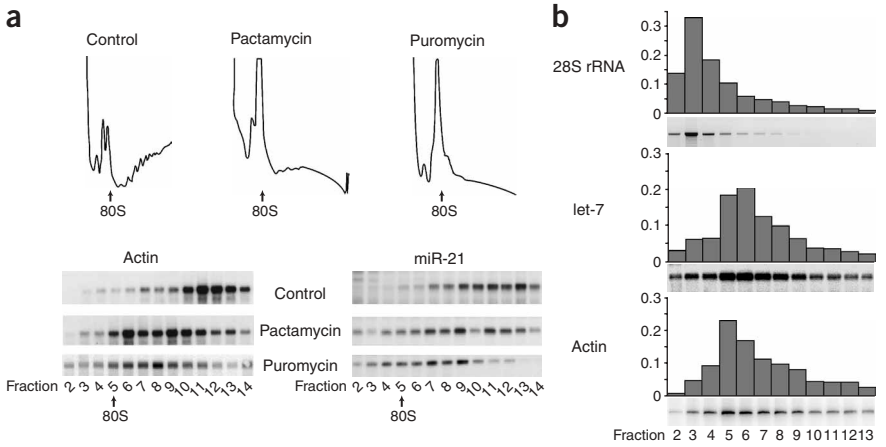

Figure 3 miRNAs are associated with mRNAs engaged in active translation. (**a**) Cytoplasmic extracts from control cells, cells treated with 0.3 μM pactamycin for 30 min or cells treated with 100 μg ml^{-1} puromycin for 10 min, as indicated. Extracts were prepared, fractionated and analyzed as in **Figure 1**. β-actin mRNA was detected by northern blot, miR-21 by splinted ligation. (**b**) In a separate experiment, cells were treated with puromycin as in **a** and fractionated, and positions of 28S ribosomal RNA, let-7 miRNA and β-actin mRNA were determined and quantified. Height of each bar represents fraction of total signal present.

(**Fig. 2**, Nuclease). Ribosome-protected fragments of actin mRNA also accumulated and, as expected, these fragments cosedimented with the bulk of ribosomes (**Fig. 2**, Nuclease). miR-21 was remarkably resistant to digestion and, notably, its sedimentation profile was distinct from that of ribosomes or ribosome-protected mRNA fragments; the bulk of miR-21 sedimented near the top of the gradient (**Fig. 2**, Nuclease). These results, together with those of the 2′-O-me oligonucleotide experiment (**Fig. 1d**), strongly support the conclusion that miRNAs are present in polyribosomes because of their specific associations with nuclease-sensitive regions of mRNAs, presumably the 3′ UTRs.

miRNAs are associated with translating mRNAs

To assess the translational status of mRNAs associated with miRNAs, we treated cells with pactamycin, a small molecule that at low concentrations predominantly blocks initiation of protein synthesis (for example, see ref. 27). In the presence of pactamycin, polysomes were reduced and monosomes increased greatly (**Fig. 3a**, Pactamycin). Coincident with the reduction in heavy polysomes, control mRNAs shifted to lighter polysomes; the sedimentation of miR-21 mirrored that of mRNAs. Moreover, as was seen after micrococcal nuclease treatment, the sedimentation of the miRNA did not coincide with that of bulk ribosomes, providing additional confirmation that miRNAs were associated with mRNAs, not ribosomes *per se*. Notably, the response to pactamycin indicated the miRNAs were bound to mRNAs engaged with elongating ribosomes; if the miRNAs had been associated with translationally arrested mRNAs, no shift would have been observed.

To extend and confirm this interpretation, we treated cells with puromycin, a drug that inhibits protein synthesis by a different mechanism. Puromycin is a small molecule that mimics acyl–transfer RNA and serves as a polypeptide chain terminator. In cells treated with puromycin, we observed extensive disaggregation of polysomes and accumulation of 80S monosomes. As with pactamycin, puromycin treatment caused a shift of mRNAs to lighter polyribosomes, and miRNAs showed a parallel shift (**Fig. 3a**, Puromycin). As observed with the inhibitor of initiation, the sedimentation of miRNAs did not mirror that of ribosomes but paralleled that of mRNAs (**Fig. 3b**). Because puromycin affects only elongating ribosomes, these results reinforce the conclusion that bulk miRNAs are associated with mRNAs undergoing translation.

miRNA distribution after stress and recovery

Both pactamycin and puromycin are irreversible inhibitors of protein synthesis. To assay miRNA distribution under more dynamic conditions, we exposed cells to hypertonic media, a treatment that causes rapid cessation of protein synthesis (for example, see ref. 28). Inhibition of protein synthesis is correlated with dephosphorylation of the initiation factors eIF4G and eIF4E as well as the regulatory protein 4E-binding protein-1 (4E-BP1), accompanied by an increase in the eIF4E–4E-BP1 complex. The net result is a decrease in availability of eIF4F[28] and consequent inhibition of initiation of protein synthesis. Importantly, inhibition of initiation of translation by hypertonic shock is readily reversible by return to isotonic media. When cells were exposed to 150 mM external NaCl, a strong inhibition of protein synthesis was observed (greater than 99%, as monitored by [^{35}S]methionine incorporation), accompanied by a corresponding decrease in polysomes (**Fig. 4**, NaCl). As expected from previous results, there was a pronounced shift of mRNAs and miRNAs from heavy polysomal fractions to lighter ones. Upon return to isotonic media, protein synthesis recovered to levels indistinguishable from those in untreated cells, and control mRNAs returned to heavy polysomes (**Fig. 4**, Recovery). Notably, the sedimentation of miRNAs again paralleled that of the mRNAs. These results indicate that

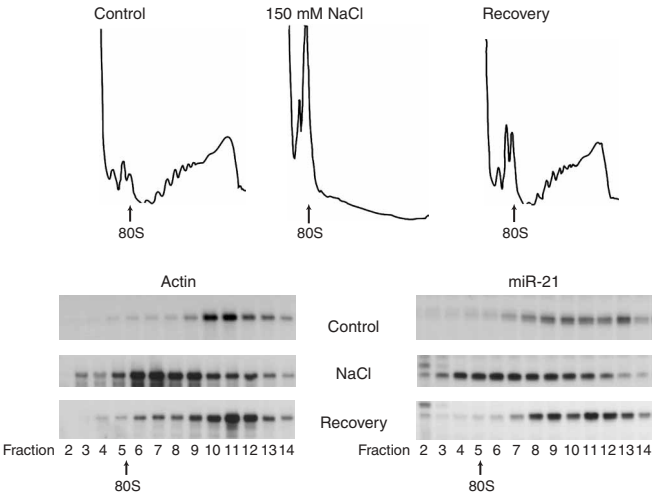

Figure 4 Parallel polysomal distribution of miRNAs and mRNAs after exposure to and recovery from hypertonic stress. Cytoplasmic extracts were prepared from control cells, cells exposed to 150 mM NaCl for 60 min, or cells exposed to 150 mM NaCl for 60 min and allowed to recover for 45 min in isotonic media, as indicated. Extracts were prepared, fractionated and analyzed for the presence of β-actin mRNA (northern blots) or miR-21 (splinted ligation) as described in Methods.

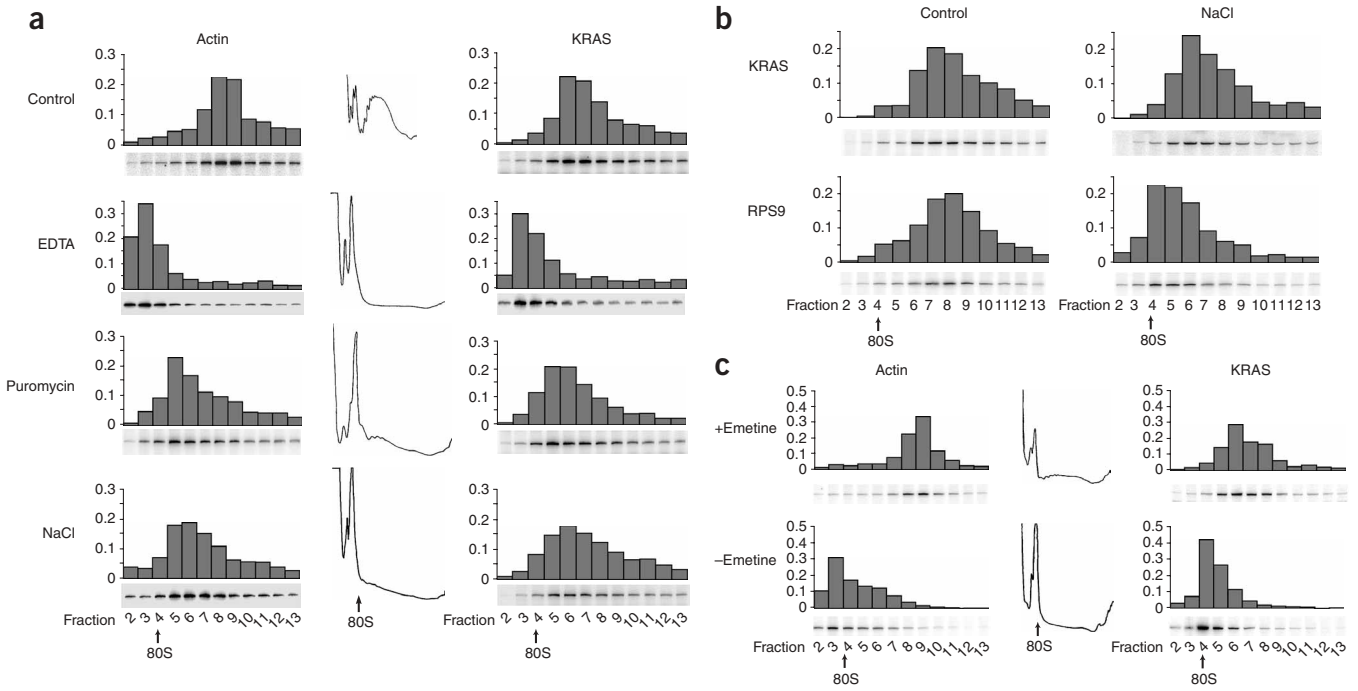

Figure 5 KRAS mRNA sediments with polysomes and is associated with translationally competent ribosomes. (**a**) HeLa cells were either untreated or treated with hypertonic media (NaCl) or puromycin as in **Figures 3** and **4** before being harvested. Extracts were prepared and fractionated as described in Methods. EDTA indicates that extracts prepared from untreated cells were incubated with 10 mM EDTA before fractionation, as in **Figure 2**. Positions of β-actin and KRAS mRNAs were determined by semiquantitative reverse-transcription PCR as described in Methods; bars represent fraction of total signal for each mRNA in a specific gradient fraction. (**b**) Extracts from control cells or cells treated with hypertonic media were fractionated and positions of KRAS and RPS9 mRNAs determined and quantified as in **a**. (**c**) Extract was prepared from cells as described in Methods, except that emetine was omitted before cell disruption. Aliquots of this extract were then incubated under protein-synthesis conditions in the presence or absence of emetine as described in Methods. After incubation, reaction mixtures were fractionated and positions of KRAS and β-actin mRNAs determined and quantified as in **a**.

mRNAs bound to miRNAs can resume active translation even after exposure to conditions that disrupt protein synthesis.

A specific miRNA-targeted mRNA is present in polysomes

Because the experiments above indicated that bulk miRNAs are associated with actively translating mRNAs, we wished to determine whether a specific characterized miRNA target behaves similarly. For this analysis, we chose the KRAS mRNA, as KRAS has been shown to be regulated by the let-7 miRNA in HeLa cells[22,29]. Notably, most of the KRAS mRNA was found to sediment in the polysomal region of sucrose gradients (**Fig. 5a**, Control), and, as would be expected if it were associated with ribosomes, the mRNA moved to the top of the gradient upon treatment with EDTA (**Fig. 5a**, EDTA).

To determine whether the KRAS mRNA is engaged with actively translating ribosomes, cells were treated with either puromycin or hypertonic media (see above). Under both conditions, the KRAS mRNA shifted to lighter fractions of the gradient (**Fig. 5a**), indicating that the mRNAs were being translated. We noted, however, that the shift was much smaller than that observed with β-actin mRNA (**Fig. 5a**). β-actin mRNA has a coding sequence of 1,128 nucleotides (encoding 376 amino acids) and is occupied by 10–14 ribosomes under control conditions; after hypertonic shock, the number of ribosomes is reduced to 3–5. KRAS mRNA has a coding sequence approximately half the length of actin's (567 nucleotides, encoding 189 amino acids) and, as judged by sedimentation, is occupied by 6–8 ribosomes under control conditions; after hypertonic shock, the number is reduced to 3–5. These data could be interpreted to suggest that protein synthesis is slowed on the KRAS mRNA. Alternatively, 3–5 ribosomes could represent an end point of dissociation achievable under hypertonic conditions; although protein synthesis is completely arrested under these conditions, we never observed complete dissociation of mRNAs from ribosomes, for reasons that are not understood (**Fig. 5a**, compare EDTA with puromycin and NaCl).

To distinguish between these possible interpretations, we compared the behavior of KRAS with that of an mRNA that contained a coding region of equivalent size, encoding ribosomal protein S9 (RPS9; coding region of 585 nucleotides, encoding 195 amino acids). The RPS9 mRNA is occupied by 6–8 ribosomes under control conditions, the same number as KRAS mRNA (**Fig. 5b**). After exposure to hypertonic conditions, the RPS9 mRNA shifts to a lighter position on the gradient (1–3 ribosomes; **Fig. 5b**) than KRAS mRNA. This result suggests that the sedimentation of KRAS mRNA after hypertonic shock does not reflect an end point but rather the rate of translation of the KRAS mRNA is somehow slowed relative to that of control mRNAs.

We then attempted to recapitulate this apparent regulation *in vitro*. For these analyses, cytoplasmic extracts were prepared in the absence of inhibitors of protein synthesis. Although some ribosome run-off during extract preparation was observed (**Fig. 5c**), both KRAS and β-actin mRNAs were present predominantly in polysomal fractions, at the expected positions. Extracts then were or were not treated with emetine (which prevents elongation) and were incubated under protein-synthesis conditions. Both mRNAs remained associated with polysomes when protein synthesis was arrested, but both moved markedly to lighter fractions under conditions permissive for protein synthesis (**Fig. 5c**). Although these experiments are not informative

regarding potential mechanisms of KRAS regulation, they provide unambiguous evidence that KRAS mRNAs are associated with translationally competent ribosomes.

DISCUSSION

We have shown that, at steady state, most miRNAs in exponentially growing HeLa cells cosediment with polyribosomes. Several lines of evidence indicate that this cosedimentation results from the association of miRNAs with mRNAs, not with ribosomes *per se*. Sensitivity of sedimentation to conditions that alter translation in predictable ways further indicates that most of the miRNAs we have examined are bound to mRNAs that are being translated. Because a complementary 2′-O-me oligonucleotide blocks association of a miRNA with polysomes, the association is most probably a consequence of base-pairing between the miRNAs and complementary target sites on mRNAs. We have also shown that a specific mRNA (KRAS), known to be translationally downregulated in a miRNA-dependent manner[22,29] is present in polysomal fractions. *In vitro* run-off experiments indicate that this mRNA is associated with translationally competent ribosomes.

The observed localization of miRNAs to polysomes is consistent with several previous studies. The mRNA targets of the founding members of the miRNA family in *Caenorhabditis elegans* were shown to be in polysomes (for example, see refs. 7,8), and subsequent studies showed that the bulk of miRNAs in *Drosophila melanogaster* cells and in *C. elegans* sediment with ribosomes[30–32]. Additional analyses have documented polysome-associated miRNAs and short interfering RNAs (for example, see refs. 33–35), and, in one case, evidence for miRNA–mRNA target interaction in polysomes has been presented[33].

Even though most miRNA-mRNA interactions do not seem to affect protein synthesis rates greatly, they most probably have a regulatory function. However, low levels of translation inhibition would not be apparent in the experiments we have performed and effects on mRNA turnover would be invisible to polysome analyses. This interpretation is consistent with a large body of informatic and experimental data, which indicates that a substantial fraction of all mRNAs are miRNA targets, yet are translated (for example, see refs. 5,12,36). Indeed, the regulatory effects of many miRNA-target interactions seem to be subtle (for example, see refs. 5,12,36,37). Although it is possible that different degrees of regulation could be achieved by modulating the accessibility of miRNAs to their targets, the localization of most miRNAs to polysomes suggests that this is unlikely to be the case. An alternative possibility is that the interplay of miRNAs and their associated factors with the complement of proteins bound to specific 3′ UTRs determines both the mechanism and extent of regulation. Consistent with this, one recent study has demonstrated that miRNA-mediated regulation can be modulated by protein binding to the 3′ UTR[38] and another has documented mRNA-specific fates (enhanced turnover, translational inhibition or a combination of both) that occur as a consequence of miRNA-mediated regulation[16]. The notion that specific interactions of messenger ribonucleoprotein particles with the translational machinery determine these fates is also consistent with the observation that miRNA-mediated repression requires the participation of Rck-P54 (ref. 22), the yeast homolog of which acts only on mRNAs that are translated[23].

Whereas the bulk miRNA data clearly indicate that miRNA–mRNA target interactions are compatible with active translation, the results with KRAS mRNA are more complex. In particular, both the relative lack of sensitivity of sedimentation of this mRNA to conditions that inhibit initiation of protein synthesis and the modest sensitivity to puromycin treatment suggest that elongation rates on the KRAS mRNA might be slowed relative to those of other control mRNAs. However, this interpretation is complicated by the *in vitro* results, which did not reveal any apparent difference between translation of KRAS and translation of actin or RPS9 (**Fig. 5c** and data not shown). The results are reminiscent of those obtained in a previous analysis of miRNA function in *C. elegans*, where *in vitro* run-off of a miRNA-regulated mRNA from polysomes was observed[8].

Although the mechanism by which KRAS expression is regulated by miRNAs is not yet clear, it seems to be distinct from previously documented inhibitory pathways. In particular, regulation cannot be due to sequestration in P-bodies, because these structures lack ribosomes (reviewed in ref. 21). It is also unlikely to be due to inhibition of initiation of protein synthesis[10,11], as essentially all of the mRNA is associated with polysomes. Finally, the evidence suggests that enhanced ribosome drop-off[9] does not occur, because the mRNA remains on polysomes under conditions where initiation is blocked. It seems unlikely that such diverse mechanisms could result from differences in technical approaches. Rather, it seems that miRNA-mediated translational repression can be manifested in distinct ways depending upon specific interactions of messenger ribonucleoprotein particles with the translational machinery. Accordingly, it will be of considerable interest to determine the complement of proteins associated with 3′ UTRs that seem to be regulated by dissimilar mechanisms.

METHODS

Extracts and fractionation. HeLa cells were maintained in DMEM supplemented with 10% (v/v) FCS and harvested when approximately 80% confluent. Ten minutes before harvesting, cells were treated with 0.1 mg ml^{-1} emetine to irreversibly block protein synthesis during extract preparation. To prepare cytoplasmic extracts, cells were swelled in three volumes of hypotonic buffer without detergent[39] and disrupted by dounce homogenization. After addition of 0.5% (v/v) Nonidet P40, nuclei were removed by centrifugation at 1,200*g* for 5 min. For the experiment shown in **Figure 1d**, cells were transfected using Oligofectamine (Invitrogen) with 150 nM of a 2′-O-me oligonucleotide (5′-GCAGACCUUCCGAUAACUAUACAACCUACUACCUCA-3′) complementary to let-7, which contained a 14-base 5′ extension of irrelevant sequence to prevent its comigration with the miRNA. Aliquots of the resultant supernatant were centrifuged through 15%–45% (w/w) sucrose gradients at 40,000 r.p.m., ~275,000*g*, for 75 or 90 min in a Beckman SW41 rotor. After centrifugation, gradients were fractionated from the top by displacement through a flow cell, and A_{260} was monitored with a recording spectrophotometer.

RNA analysis. After fractionation, individual fractions were deproteinized with phenol chloroform and RNA was recovered by ethanol precipitation. For mRNA analysis, equal aliquots were separated on 1% agarose gels containing formaldehyde, transferred to GeneScreen Plus membranes and hybridized with a random primed probe prepared from β-actin complementary DNA. miRNAs were assayed by either northern blot or splinted ligation (P.A.M. and T.W.N., unpublished data), as indicated in the figure legends. Because of the low abundance of the KRAS mRNA, the RNA analyses in **Figure 5** for β-actin, KRAS and RPS9 mRNAs were done by semiquantitative reverse-transcription PCR. cDNA was synthesized using random primers and Moloney murine leukemiavirus reverse transcriptase (USB). PCR reactions (17 cycles for β-actin, 25 cycles for KRAS, 18 cycles for RPS9; linear conditions) were carried out in the presence of [α-^{32}P]dCTP, analyzed on nondenaturing 6% acrylamide gels and quantified using a PhosphorImager. Primers were as follows: KRAS forward, 5′-GACTGAATATAAACTTGTGG-3′; KRAS reverse, 5′-CTGTTTT GTGTCTACTGTTC-3′; actin forward, 5′-AGAAAATCTGGCACCACACC-3′; actin reverse, 5′-CTCCTTAATGTCACGCACG-3′; RPS9 forward, 5′-GACGCTT GATGAGAAGGACC-3′; RPS9 reverse, GATGTTCACCACCTGCTTGC-3′. An *in vitro*–transcribed fragment of the KRAS mRNA designed to produce a PCR product 96 bases shorter than the endogenous mRNA was added to each gradient fraction as a control for RNA recovery, cDNA synthesis and PCR.

Polysome run-off analysis. For the *in vitro* run-off experiments in **Figure 5c**, extract was prepared as above except that emetine was omitted before harvesting and after homogenization the extract was centrifuged at 30,000*g* for 5 min. For run-off analyses, 200-μl reactions contained 100 μl extract and 2 mM ATP, 0.3 mM GTP, 0.05 mM complete amino acid mix, 20 mM creatine phosphate, 60 units ml^{-1} creatine phosphokinase, 0.1 mM spermidine, 3 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT, 0.5 units μl^{-1} RNasin (Promega), and 10 mM HEPES (pH 7.5), either with or without 0.1 mg ml^{-1} emetine. Reaction mixtures were incubated at 30 °C for 30 min before fractionation on sucrose gradients as described above.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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