Translational Regulation by Short RNAs in Mammalian Cells

By

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Submitted to the Department of Biology on November 11, 2005 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology.

ABSTRACT

The large complexity of animals is thought to depend upon the regulation of gene expression and not the number of genes in a genome. Gene expression is a highly conserved process in which genes encoded by DNA are transcribed in the nucleus into messenger RNA, spliced, and exported to the cytoplasm, where they are translated into proteins. It was therefore a profound surprise when RNA itself, in the form of ~21nt short RNAs, was discovered to have major generalized roles in the regulation of mRNAs which are typically 100 times larger in size. These short RNAs, siRNAs and microRNAs, exert their influence on gene expression in mammals by RNA interference (RNAi) and translational repression.

RNAi initiated by exogenous siRNAs results in mRNA degradation whereas many endogenous microRNAs cause translational repression. Some factors which produce siRNAs from double-stranded RNA or microRNAs from hairpin precursors are shared and some are unique to each pathway. Our work shows the interaction between short RNA and target mRNA, not the origin of the short RNA, dictates the outcome of silencing. Perfectly or nearly perfectly complementary basepairing between short RNA and target mRNA causes RNAi-mediated degradation of the mRNA, whereas partial basepairing results in translational repression and a degree of mRNA degradation. This repression depends upon the number of binding sites within a target mRNA, and there is a synergistic relationship between binding site number and the degree of silencing.

Further investigation of the mechanism revealed a post-initiation block to translation by short RNAs. Translationally repressed mRNAs associate with polyribosomes, and IRES-initiated translation, which bypasses normal initiation, can be repressed by short RNA. Polyribosomes associated with repressed mRNA are sensitive to treatment with the puromycin, demonstrating that these ribosomes can complete the elongation cycle. Pulse-labeling shows that short RNAs likely act before production of complete protein. Short RNAs cause an increase of translational termination at a stop codon. After a brief inhibition of translation initiation in vivo, ribosomes dissociate more rapidly from repressed mRNAs than from active mRNAs. These results show that short RNAs repress translation after initiation by a novel mechanism which causes ribosome drop-off.

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# TABLE OF CONTENTS

Abstract .................................................................................................................. 2

Acknowledgements .................................................................................................. 4

Chapter 1. siRNAs and miRNAs ........................................................................ 6
  RNA interference
    Short interfering RNAs (siRNAs) ................................................................. 7
    RISC ........................................................................................................... 7

  microRNAs
    Discovery of miRNAs ................................................. 10
    miRNA genomics ................................................................. 10
    Biogenesis ............................................................................... 11
    Specificity of miRNA/mRNA interactions ............................................. 13
    miRNA targets ........................................................................ 14
    Range of biology ............................................................................. 15
    mRNA degradation by miRNAs and P-body localization ..................... 17
    Pathways of translation-coupled RNA decay .......................................... 20
    Eukaryotic translation ........................................................................... 21
    Translational repression by 3′ UTRs ....................................................... 23
    Mechanism of microRNA translational repression .................................. 26

References .......................................................................................................... 30

Chapter 2.
  siRNAs can function as miRNAs .......................................................... 43

Chapter 3.
  Short RNAs repress translation after initiation in mammalian cells .......... 64

Conclusions ....................................................................................................... 106

Biographical Note .............................................................................................. 113
ACKNOWLEDGEMENTS

On my committee I’d like to thank Dave Bartel and Tom Rajbhandary for advising me for 5 and 2 years respectively. I especially thank Dave for saving me at the 11th hour from a Mac to PC conversion problem during a talk in Vienna in 2003, and being a constant guiding hand in my research life. Thanks also to Victor Ambros, for many interesting discussions, and for opening my eyes to a planaria postdoc.

I can think of no better place to work than the 5th floor of the Cancer Center, and within the Sharp Lab in particular. My time here was very much brightened by the presence of brilliant and superlative peers, always willing to talk and listen, both stimulating and supportive the whole way through. Carl, Dean, Alla, Mauro, Amanda, Hristo, Will, Mike, Mike, Chonghui, KB, Derek, Lourdes, Amy, Joel, Ann, Keara, Phil, Seth, Erik, Issac, Al, Carla, Chris, Patrick, Stephan, Peter, Julian, Dave, Ruth. I’ll never forget “ice bucket soccer,” the whoopin’ stick, mornings of the daily 10x coffee “Novina Challenge” and of course, evenings at the Muddy.

John Doench traveled with me on this road, and I certainly could not have persisted without his constant encouragement and help. He’s a wonderful collaborator, and even better friend, a comrade who could bring me back when I was frustrated, and an ally throughout the journey.

Phil has been an incredible advisor, always challenging me to reach as far as I can, and helping me to become independent. Thanks for letting me follow the course I wanted to, for always inspiring me and making me remember what’s really important. Some of my favorite times here have been spent around the circular blackboard in the hallway, coffee in hand, talking about a new idea.

I’d also like to thank my musical colleagues for preserving my sanity through countless good grooves and fun gigs. Phil, Dave, Graham, John, Bob and Lee—may the Max Funk Institut go platinum next year!

I’m very thankful of my many good friends for being so incredibly supportive through the years. Christopher, you’re one of the best listeners I know, and there were times I might have given up without your encouragement. John, Phil, Seth, Andy, Erik, thanks for many great games of chance (and skill!) at Guy’s Night. Emily Levin, Slavea, Samadi, Emily Shaw, Josh, John Friskel, Lucia, Nick, Selena, Travis and Misha, you provided me a lot of stability in my home life away from lab. Matt and Melinda, thanks for being such incredible and warm friends through all these years.

Sarah, my once and future roommate, I couldn’t have asked for someone more supportive and wonderful in my life. Especially in these last days, you’ve listened more than anyone else, and really helped me through this process. Thanks. I’m so glad that I was able to overcome my fear of salsa dancing at least once a year and a half ago.

Finally, thanks so much to my wonderful family for always loving and supporting me. Even from a distance, we’ve managed to have great adventures during my time here. I’ll never forget Paris, Italy, Christmas and Summer visits to Iowa. Mom, I know no one loves me more. Dad, your thoughtfulness always inspires me. Grandma Sandholm, your vibrance is a refreshing shot in the arm, and Grandma Petersen, your warmth and grace are humbling. Andy, you’re the best kind of pal and I’m so glad you moved to Boston, and Jan-Marie, your exuberance never ceases to charm me.
For Bill Sandholm and his life of quiet dignity
Chapter One

siRNAs and microRNAs

Portions of this chapter originally appeared in Chapter 20 of The RNA World, 3rd Edition
RNA interference

**Short interfering RNAs (siRNAs)**

RNAi was discovered when researchers introduced exogenous double-stranded RNA into the nematode *C. elegans* and found that homologous genes were post-transcriptionally silenced (Fire et al. 1998). The pathway is divided into initiator and effector steps (Figure 1). RNAi is initiated by double-stranded RNA, which is recognized and cleaved at a single phosphodiester bond on each strand of the RNA by the RNaseIII enzyme Dicer to produce 21 nt double-stranded short interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999; Tuschl et al. 1999; Zamore et al. 2000; Bernstein et al. 2001; Zhang et al. 2004). In the effector step, one strand of the siRNA is incorporated into an RNA-induced Silencing Complex (RISC) that causes endonucleolytic cleavage of homologous mRNA (Hammond et al. 2000; Elbashir et al. 2001a; Nykanen et al. 2001).

SiRNAs are 19-21 nucleotide dsRNA molecules with 3’ two nucleotide hydroxyl overhangs and 5’ monophosphates (Elbashir et al. 2001a; Elbashir et al. 2001b). The guide strand of the siRNA is sensed by its reduced thermodynamic stability in the 5’ region versus the other strand (Khvorova et al. 2003; Schwarz et al. 2003). This “functional asymmetry” of a siRNA is detected by R2D2 in combination with Dicer (Liu et al. 2003; Tomari et al. 2004b). The siRNA is unwound in an ATP dependent step which requires the helicase Armitage (Tomari et al. 2004a).

**RISC**

The catalytic core of RISC is the protein Argonaute (Hammond et al. 2001; Carmell et al. 2002). Argonaute genes were among the first to be identified as required
for post-transcriptional gene silencing by dsRNA in such diverse organisms as

*Arabidopsis, Neurospora, C. elegans* and *Drosophila*. The number of Argonaute gene varies widely between organisms such that the *S. Pombe* genome has only one Argonaute protein but the *C. elegans* genome contains more than two dozen. Argonaute proteins contain two highly conserved domains, PAZ and PIWI. The PAZ domain directly contacts the 3’ overhangs of an siRNA in a crystal structure, suggesting that this domain may recognize siRNAs and deliver them into RISC (Lingel et al. 2003; Yan et al. 2003). The PIWI domain contacts the 5’ region of the siRNA guide strand when bound as an A form helix to target mRNA. This maneuvering positions catalytic residues within an RNase H-like fold of the PIWI domain near the scissile phosphate of the target mRNA, directing cleavage (Liu et al. 2004; Song et al. 2004). Contacts with the 5’ end of the siRNA are essential for catalytic function (Parker et al. 2004; Ma et al. 2005). The cleavage event produces a 5’ phosphate and 3’ hydroxyl within the target mRNA ten nucleotides from the 5’ end of the siRNA (Martinez and Tuschl 2004; Schwarz et al. 2004). In some cases, Argonaute paralogs within a genome are known to have specialized functions. For example, *C. elegans* rde-1 functions in RNA interference, while alg-1 and alg-2 function in microRNA-mediated silencing (Tabara et al. 1999; Grishok et al. 2001). Likewise, *Drosophila* ago-2 is necessary for RNAi but ago-1 is necessary for the mRNA degradation component of microRNA-mediated silencing (Rehwinkel et al. 2005). In mammals, ago-2 is required for RNAi, but the function of the other three Argonaute proteins is unknown (Liu et al. 2004).

Other components have been identified in RISC depending on the fractionation scheme and assay used. Although stringent fractionation of RISC activity yields a
complex approximately large enough to accommodate Argonaute and one other protein, less stringent procedures produce a RISC of about 80S (Martinez et al. 2002; Pham et al. 2004). Indeed, some components of RISC associate with L5 and L11 ribosomal proteins as well as the 5S rRNA (Ishizuka et al. 2002). The proteins VIG, FMRP, and the micrococcal nuclease Tudor SN have been identified as part of RISC, but the function of these proteins within RISC remains unknown (Caudy et al. 2002; Caudy et al. 2003). Possibly, proteins in addition to Argonaute function within the cell to properly localize RISC function or have other functions in gene silencing.

A major difference between RNAi in plants and worms versus flies and mammals is the presence of an siRNA-amplification step which requires RNA-dependent RNA polymerases (RdRPs) (Cogoni and Macino 1999; Dalmay et al. 2000; Fagard and Vaucheret 2000; Sijen et al. 2001). In these organisms, siRNAs can either perform cleavage reactions within RISC or initiate the formation of double stranded RNA using the target mRNA as a template for RdRP enzymes. These newly generated dsRNAs are substrates for Dicer and the production of more siRNAs, amplifying the RNAi effect. Despite the absolute requirement for Dicer and Ago2 homologs in the initiation and effector steps across diverse species, no homologs of RdRP are known to exist in mammals or flies. Additionally, siRNAs modified at their 3’ end to block priming by RdRPs are nonetheless functional, suggesting that an amplification step is truly not essential for RNAi in flies and mammals (Schwarz et al. 2002). Interestingly, the ability to spread silencing throughout an organism and to transmit silencing through progeny for several generations correlates with the presence of an RdRP amplification step in plants and worms. Likewise, spreading has not been observed in mammals or flies.
MicroRNAs

Discovery of miRNAs

The first microRNA was discovered when the *lin-4* gene was cloned and found to encode not a protein but a 21-nt RNA (Lee et al. 1993; Wightman et al. 1993). *Lin-4* mutants have defects in the heterochronic pathway, which is required for proper larval developmental timing (Horvitz and Sulston 1980; Chalfie et al. 1981; Ambros and Horvitz 1984). Genetically, *lin-4* was known to interact negatively with the 3’ untranslated region (UTR) of the gene encoding *lin-14*, and sequencing of *lin-14*’s 3’ UTR revealed multiple sites complementary to *lin-4* (Wightman et al. 1991; Lee et al. 1993; Wightman et al. 1993). It was suggested that the *lin-4* short RNA directly regulated gene expression through basepairing interactions with its target messenger RNA (Figure 1). Another gene within the heterochronic pathway, *let-7*, was cloned and found to encode another microRNA (Reinhart et al. 2000). This gene is conserved from worms to humans (Pasquinelli et al. 2000). One of *let-7*’s target mRNAs was discovered by forward genetics to be *lin-41*, an RBCC protein also involved in the heterochronic pathway (Slack et al. 2000).

miRNA genomics

After the discovery of siRNAs, several labs cloned RNAs approximately 21 nucleotides in length from a variety of sources to begin to define the function of endogenous short RNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). MicroRNAs are defined to be short RNAs which originate from hairpin
precursors in the genome, have 5’ phosphate and 3’ hydroxyl ends, and are Dicer products. Computational programs were also developed using validated miRNA structures to identify new candidate miRNA genes and to estimate the total number of miRNAs in genomes (Lim et al. 2003a; Lim et al. 2003b). These programs scan a genome for 60-70 nt regions that can be folded into a hairpin RNA computationally and are conserved in closely related species. Using a combination of these biochemical, genetic, and computational approaches, 116 miRNAs have been identified in C. elegans, 78 in Drosophila, 222 in humans, 224 in mouse and 112 in Arabidopsis (miRNA registry release 5.2 (Griffiths-Jones 2004)). However, some newer approaches give a higher estimate. For example, phylogenetic shadowing among primate genomes has estimated the total number of human microRNAs to be as high as 1000 (Berezikov et al. 2005).

MicroRNAs within the same organism can be clustered into families with identical sequences at positions 1-8 numbered from the 5’ end, referred to as the “seed.” The 5’ region of the microRNA is the most critical for its function (discussed below), so members of the same family probably regulate overlapping sets of genes. For example, there are 9 miRNAs in vertebrates that have an identical seed sequence with the prototype let-7 miRNA.

Biogenesis

MicroRNA genes are located within intergenic regions or within the introns of annotated genes, and are found individually or within clusters containing other microRNAs. They are derived from larger transcription units (> 0.5 kb) termed pri-microRNAs (Lee et al. 2002). Many pri-microRNAs are capped and polyadenylated,
giving further indication that most pri-microRNAs are transcribed by RNA polymerase II, although there is strong evidence that some miRNAs are processed from pri-miRNAs transcribed by RNA polymerase III (Lee et al. 2004a; Pfeffer et al. 2005).

Within the nucleus, the Microprocessor complex processes the pri-microRNA into a 60-70 nt hairpin pre-microRNA with a 2 nt 3’ overhang (Lee et al. 2002) through the activity of the RNase III enzyme Drosha (Lee et al. 2003). Drosha prefers a loop of 10 nucleotides and then cleaves 21 nucleotides along a duplex stem in a largely duplex region (Zeng et al. 2005). Another member of Microprocessor has been identified as the DeGeorge critical region 8 gene, DGCR8/Pasha, a dsRNA binding protein (Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). Processing can occur on a spliced substrate, and the pre-microRNA is ultimately exported to the cytoplasm by RanGTP and Exportin-5 (Yi et al. 2003; Kim 2004; Lund et al. 2004).

Within the cytoplasm, Dicer recognizes the 2 nt 3’ overhang produced by Drosha and cleaves the pre-microRNA on both strands near the base of the loop to create a duplex with 2 nt 3’ overhangs at both ends. One of these strands is assembled into RISC as the mature miRNA whereas the other strand, the miRNA*, is typically degraded. Strand selection follows the same principles of functional asymmetry as do exogenous siRNAs, with the selected strand having weaker basepairing at the 5’ end (Schwarz et al. 2003). As mentioned before, miRNAs in Drosophila are processed by a separate pathway from siRNAs with a dedicated Dicer, Dcr-1, which does not process siRNAs (Lee et al. 2004b). Mature miRNAs in humans are found associated with Argonaute proteins 1-4, and RNA helicases Gemin 3 and 4 (Mourelatos et al. 2002; Meister et al. 2004). In Drosophila, microRNAs also associate with Vasa-Intronic-gene (VIG), a gene
of unknown function, and Tudor SN, a micrococcal nuclease homolog (Caudy et al. 2002; Caudy et al. 2003). MicroRNAs associate with RISC and have RISC activity, but the complex responsible for translational repression (which may be RISC itself) is unknown.

A particularly intriguing miRNA-associated protein is the Fragile X Mental Retardation protein (FMRP) (Caudy et al. 2002; Ishizuka et al. 2002). FMRP, an RNA binding protein with some sequence specificity, is known to associate with polyribosomes, and the mental retardation associated with loss of this protein’s activity in humans is consistent with its role in regulating translation of particular mRNAs at neuronal synapses (Darnell et al. 2001; Antar and Bassell 2003; Veneri et al. 2004). Interestingly, the activity of FMRP in translational repression is dependent upon phosphorylation (Ceman et al. 2003). Analysis of RNAi in vitro has shown that FMRP is not required for mRNA cleavage activity, and knockdown of FMRP has only a small effect on RNAi activity in cell culture, but FMRP associates with RISC and miRNAs (Caudy et al. 2002; Ishizuka et al. 2002). It has been hypothesized that FMRP helps provide specificity for localization of RISC or RISC-like complexes to specific mRNAs for other activities, such as translational repression.

Specificity of miRNA/mRNA interactions

The 5’ region of microRNAs is more conserved than the 3’ region. Additionally, experiments of microRNA-mediated silencing in mammals, fish and flies have shown that complementarity to messenger RNA within positions 1-8 of microRNA are the most crucial for regulation (Doench and Sharp 2004; Kloosterman et al. 2004; Brennecke et al. 2004).
Extensive 3’ complementarity can enhance silencing by a microRNA if the stability of a pairing in the 5’ region is weak. Additionally, there is a statistical enrichment within mammalian 3’ UTRs for conserved 8 nucleotide sequences complementary to the seed region of microRNAs. The consensus sequence for the interaction of a microRNA and mRNA is perfect complementarity at positions 2-7 with no preferred sequence, flanked by basepaired nucleotides at position 8, and neighbored by an unpaired A at position 1 and to a lesser extent 9 (Lewis et al. 2005). The mechanistic basis for this consensus structure has not yet been determined.

**MicroRNA targets**

Although the number of identified microRNAs is large, there are still few examples of microRNA targets that have been experimentally confirmed. Strong evidence of a particular mRNA/microRNA interaction can be obtained through genetic experiments, where a phenotype due to loss of function in the microRNA can be suppressed by loss of the target gene. Genetic evidence exists showing the interaction between microRNA *lin-4* and mRNA *lin-14, lin-4 and lin-28, let-7 and lin-41, let-7 and hbl-1, lso-6 and cog-1* in worms and between *bantam* and *hid* in flies (Lee et al. 1993; Slack et al. 2000; Brennecke et al. 2003; Chang et al. 2004). Computational studies have been used to predict microRNA targets in organisms (Rhoades et al. 2002; Enright et al. 2003; Lewis et al. 2003; John et al. 2004; Jones-Rhoades and Bartel 2004; Kiriakidou et al. 2004; Lai 2004; Rajewsky and Socci 2004; Rehmsmeier et al. 2004). These approaches have yielded consistent and successful results in plants, where there are many target mRNA sequences that are highly homologous to the microRNA, and 5’ RACE has
been used to validate the expected precise 5’ mRNA cleavage product at positions 10 and 11 within the microRNA binding site (Llave et al. 2002). Unlike most animal miRNAs, murine miR-196 contains extensive complementarity to its target mRNA, HOXB8, and regulates it by mRNA cleavage (Mansfield et al. 2004; Yekta et al. 2004).

Most animal microRNAs have fewer near-perfect complementary sequences within mRNAs, and instead regulate gene expression through multiple partially complementary interactions within the 3’ UTR of genes. Multiple mammalian genome alignments indicate that 30% of mammalian 3’ UTRs contain conserved potential binding sites to microRNAs. This assigns a vast regulatory function for this class of genes (Lewis et al. 2005). A similarly large number of mRNAs are targets for miRNA regulation as indicated by microarray analysis of downregulated mRNAs after the overexpression of a microRNA in a mammalian cell line (Lim et al. 2005). Some other predictions have been validated by heterologous reporter assays in mammalian cells, but most predictions await in vivo verification (Lewis et al. 2003; Lewis et al. 2005).

Range of biology

Genetically-defined microRNAs control a range of biological processes: developmental timing, apoptosis, neural asymmetry, cell division and viral replication. Overexpression studies suggest that miR-181 regulates hematopoetic differentiation in mice (Chen et al. 2004). In Drosophila, miR-14 regulates fat metabolism and apoptosis and the bantam miRNA regulates apoptosis and cell division (Brennecke et al. 2003; Xu et al. 2003). As mentioned before, in worms, developmental timing is regulated by lin-4 and let-7, and left/right asymmetry of chemoreceptor expression is regulated by lsy-6.
(Ambros and Horvitz 1984; Chang et al. 2004). Zebrafish mir-430 controls brain morphogenesis (Giraldez et al. 2005). In humans, miR-143 inhibits adipocyte differentiation (Esau et al. 2004) and miR-375 inhibits insulin production in pancreatic islet cells by interactions with Myotrophin mRNA (Poy et al. 2004). Interestingly, mammalian miR-32 can target the RNA genome of the primary foamy virus type 1 retrovirus (PVF-1) and limits the replication of this virus in cell culture, indicating that miRNAs may target exogenous RNAs as well as endogenous mRNAs (Lecellier et al. 2005). Conversely, the liver-specific miR-122 interacts with the 5' region of the Hepatitis C Virus genome to allow replication of that genome (Jopling et al. 2005).

Many microRNAs show tissue-specific expression patterns, and are likely to be involved in cell-type specific regulation.

MicroRNAs have also been assigned functions in previously known forms of mRNA regulation. For example, silencing by 3' UTR AU-rich elements (AREs) within the 3' UTR of tumor necrosis factor-α (TNF-α) mRNA is dependent upon Ago1, Ago2 and Dicer-1 in Drosophila. This silencing also requires miR-16, which is complementary to TNF-α ARE sequences (Jing et al. 2005). These results suggest that microRNAs may direct gene regulation through other AREs as well. MicroRNAs may also be involved in regulation of mRNA localization. For example, the dsRNA binding protein Staufen is required for proper localization and translation of mRNAs in Drosophila embryos and mammalian neurons (Li et al. 1997; Kiebler et al. 1999). Interestingly, Staufen-dependent localization of oskar mRNA in Drosophila embryos also requires splicing of oskar mRNA (Hachet and Ephrussi 2004). Although Staufen has not been assigned a role in microRNA-mediated silencing, it interacts with FMRP and has recently been
shown to direct an mRNA surveillance response dependent on the nonsense-mediated decay protein Upf1, but independent of Upf2 and Upf3 (Ohashi et al. 2002; Kim et al. 2005). Possibly, microRNAs are involved in many processes involving proper localization or quality-control of mRNAs.

MicroRNAs comprise upwards of 4% of mammalian genes yet are predicted to regulate 30% of protein-coding mRNAs. This prediction has been supported for two microRNAs by array analysis of the decreases in mRNA levels following transfection of siRNAs corresponding to two tissue specific microRNAs (Lim et al. 2005). Each microRNA downregulated the mRNA levels of about 100 genes. The microRNAs studied in this series were cell type specific for expression in muscle (miR-1) and brain (miR-124), and they preferentially targeted genes that are not highly expressed in the cell types where the microRNA is expressed. These observations might suggest that microRNAs generally function as “micro-managers” to shape cell type specific gene expression (Bartel 2004). However, further examination of predicted microRNA targets will be necessary to show whether only a subset of potential targets are substantially regulated at the protein level.

**mRNA degradation by miRNAs and P-body localization**

Recent experiments investigating the relationship between the mRNA decay pathway and RNA silencing support the notion that microRNAs cause a variable degree of mRNA degradation of their targets. The major mRNA degradation pathway in yeast involves deadenylation by Ccr4 and Pat1 exonucleases, recruitment of Sm proteins Lsm1-7 and decapping enzymes Dcp1 and Dcp2, and finally, 5’ exonucleolytic degradation initiated by Xrn1 (Muhlrad et al. 1994; Tharun and Parker 2001; Tucker et
al. 2001; Coller and Parker 2004). 3’ exonucleolytic degradation also occurs on the deadenylated mRNA and is catalyzed by a complex of at least 10 exonucleases termed the exosome (Anderson and Parker 1998; Allmang et al. 1999). Most of these components localize to cytoplasmic foci termed P-bodies or GW-bodies in eukaryotic cells (Ingelfinger et al. 2002; Eystathioy et al. 2003; Sheth and Parker 2003; Cougot et al. 2004; Kshirsagar and Parker 2004). Moreover, experiments have been performed to suggest that P-bodies are the site of degradation activities and not a storage compartment for inactive populations of the degradation machinery. First, mRNA degradation intermediates stabilized by loss of function mutations in Xrn1 or Dcp1 localize to P-bodies (Sheth and Parker 2003; Cougot et al. 2004). Additionally, inhibition of translation elongation with cycloheximide leads to mRNA stabilization and the disappearance of P bodies (Sheth and Parker 2003; Cougot et al. 2004).

RNAi proteins and small RNAs have also been observed to localize near or within P-bodies. Mammalian Argonaute 1 and Argonaute 2 have been shown to localize within P bodies (Liu et al. 2005; Pillai et al. 2005; Sen and Blau 2005). Additionally, transfected reporter mRNAs undergoing RNAi silencing with a perfectly complementary siRNA or translational silencing with partially complementary siRNA or miRNA show localization to P-bodies that also stain positive for Lsm1 (Liu et al. 2005). Interestingly, miRNAs expressed by nuclear injection of precursors show localization near but not within P-bodies (Pillai et al. 2005). It is not clear whether localization to P-bodies is a cause or a consequence of silencing by short RNA. RNAi requires endonucleolytic cleavage by Ago2, and also requires involves the activities of Xrn1 to degrade the 3’ cleavage fragment and the Rrp4, Csl4, and Ski2 components of the exosome to degrade
the 5’ fragment, suggesting that exonucleolytic degradation occurs subsequent to endonucleolytic cleavage (Orban and Izaurralde 2005). However, it is still not clear whether the majority of Argonaute 2 activity is present within the P-bodies or elsewhere in the cell before subsequent P-body localization and repression. To this end, the extent of P-body localization of a silenced reporter has not been quantitated and it has not been shown whether core P-body components are required for silencing by RNAi.

Similarly, microRNAs and their target mRNAs have been observed to concentrate at P-bodies, which is consistent with accounts of mRNA degradation occurring during microRNA-mediated silencing (Liu et al. 2005; Pillai et al. 2005). The extent of this mRNA degradative effect appears variable. For example, in studies of repression of lin-14 by lin-4 miRNA, ribonuclease protection assays (RPAs) showed that repression coincided with a 2-fold mRNA decrease and a 15-fold protein decrease (Olsen and Ambros 1999). However, another study used Northern analysis to quantitate an mRNA loss of 5-fold for repressed lin-14 mRNA and noted the presence of mRNA cleavage products during the repression (Bagga et al. 2005). It is not yet clear whether translational repression by microRNAs preceeds P-body localization, whether translational repression is a result of P-body localization, or whether partial P-body localization occurs independently of translational repression. However, RNAi depletion of GW-184, a component of P-bodies, reduces the mRNA degradative effect of miRNAs by 6-fold in Drosophila S2 cells, and likewise, depletion of Dcp-1 and Dcp-2 reduces this effect by 4-fold, supporting the notion that mRNA degradation by miRNAs in fact occurs within P-bodies (Rehwinkel et al. 2005). A possible explanation for all of the data is that microRNAs cause translational repression and subsequent P-body localization and
mRNA degradation. Alternatively, mRNA degradation within P-bodies and translational repression may be independent pathways controlled by miRNAs. Importantly, the extent of P-body localization for an mRNA undergoing repression by small RNA has not yet been accurately assessed. Further studies will be necessary to define the precise relationship between translational repression, mRNA degradation and P-body localization for microRNA-mediated silencing.

**Pathways of translation-coupled RNA decay**

Several distinct pathways have emerged in which mRNAs undergo quality control dependent upon translation. Nonsense mediated decay (NMD) is a process in which mRNAs possessing a premature termination codon located upstream of the last intron are rapidly degraded (Neu-Yilik et al. 2004). Destabilization of an mRNA by NMD requires its open reading frame to be translated and the pathway is present in yeast, worms, flies and humans. In another translation-dependent proofreading pathway documented only in *S. cerevisiae*, mRNAs with no stop codon are downregulated by mechanism termed non-stop mRNA decay (NSD) (Frischmeyer et al. 2002; van Hoof et al. 2002). This process requires ski7, an essential component of the exosome. Ski7p contains a GTPase domain homologous to that found within EF1A and eRF3 (see discussion below), which are known to interact with the A site of the ribosome during elongation and termination. Interestingly, the GTPase domain of Ski7p is dispensable for exosome function but required for NSD, raising the possibility that ribosomes which translate to the end of a mRNA without encountering a stop codon end up with an empty A site which is recognized by Ski7.
Two emerging translation-related RNA decay pathways are No-Go Decay (NGD) and Nonfunctional rRNA Decay (NRD), both of which have been observed in S. cerevisiae. Strong stem loops positioned in the coding region of mRNAs are known to inhibit translation elongation, and it recently has been observed that such mRNAs are rapidly degraded independently of Dcp2p decapping enzyme or Ski7p component of the exosome. Placement of an additional stem loop in the 5’ UTR to inhibit translation initiation restores the stability of the mRNA, suggesting that translation is required for the mRNA degradation caused by the internal stem loop. Interestingly, the proteins Dom34p and Hbs1p, sequence and structural homologs of eRF1 and eRF3 respectively, are required for this process, suggesting that these factors release poorly elongating ribosomes and trigger decay of the mRNA (Meenakshi Kshirsagar and Dr. Roy Parker, personal communication). NRD is a process of quality control for functional ribosomal RNA. It has been observed that small and large subunits of ribosomal RNA containing point mutations expected to render them non-functional in translation are degraded post-transcriptionally. The process apparently does not inhibit processing of ribosomal RNA subunits from their nuclear precursors, and does not impair the rRNA’s assembly with ribosomal proteins to form the 40S and 60S subunits. Therefore, the ability of rRNAs to perform translation itself is proofread by the cell (Rederick LaRiviere and Dr. Melissa Moore, personal communication).

Eukaryotic translation

Normal eukaryotic translation initiation occurs by a multi-step process (Sonenberg and Dever 2003). First, eIF4E binds directly to the 5’ cap of the mRNA and
recruits eIF4G and eIF4A to form the eIF4F complex. Next, the 40S small ribosomal subunit, in association with methionine tRNA, eIF2 and eIF3, binds the mRNA through interactions between eIF3 with eIF4E and eIF4G. The interaction between eIF4E and eIF4G is stabilized by poly-A binding protein PABP, which makes interactions with eIF4G and accounts for the synergy observed between cap and polyA tail in translation. After binding messenger RNA, the complex scans across the 5’ UTR of the mRNA in an eIF4A-dependent manner until encountering the first AUG. In a final step, the 60S subunit of the ribosome is recruited after GTP hydrolysis by eIF2 and subsequent dissociation of eIF2 and 3, and the elongation steps of translation commence. In translational elongation, two factors promote association of amino acyl-tRNA with the ribosome, EF1A and EF1B, whereas one factor, EF2, catalyzes translocation (Browne and Proud 2002). EF1A (functionally equivalent to bacterial EF-Tu) binds GTP, and is responsible for bringing amino acyl-tRNA to the ribosomal A site. Once the amino acyl-tRNA is properly positioned in the A site, GTP hydrolysis occurs and EF1A is released from the ribosome. The EF-Ts homolog EF1B promotes GDP/GTP exchange on EF1A, recycling it for further use. Removal of EF1A promotes the peptidyl transferase reaction catalyzed by the large subunit of the ribosome. eEF2 then catalyzes translocation of the peptidyl-tRNA from the A site to the P site of the ribosome, and deacylated tRNA from the P site to the E site, and another round of elongation begins. Translation termination requires two proteins, eRF1 and eRF3 (Kisselev et al. 2003). eRF1 recognizes the stop codon while binding within the A site of the ribosome and stimulates hydrolysis of the peptidyl-tRNA bond. eRF3 stimulates eRF1 activity through an unknown mechanism dependent upon hydrolysis of GTP by its GTPase domain.
Interestingly, eRF3 has been implicated in diverse processes. In yeast, the N-terminal domain of eRF3 has prion properties and causes the [PSI+] infected state (Doel et al. 1994). Because eRF3 is sequestered in the [PSI+] state, normal termination becomes inefficient, promoting readthrough of normal stop codons and enhanced variation due to the addition of C-terminal extensions to many proteins (Wilson et al. 2005). ERF3 is also required for the mitotic G1/S transition in *S. cerevisiae* (Kikuchi et al. 1988). In humans, there are two paralogs of eRF3, eRF3a/GSPT1 and eRF3b/GSPT2. eRF3a is expressed ubiquitously whereas eRF3b is expressed in proliferating cells and neurons, although they can functionally complement each other’s translation termination activity (Chauvin et al. 2005). N-terminal polyglycine expansions in eRF3a/GSPT1 are associated with gastric cancer and eRF3 is frequently overexpressed in intestinal type carcinomas (Brito et al. 2005; Malta-Vacas et al. 2005). eRF3a is inhibited by RNaseL, the RNase activated by long double-stranded RNA in the PKR response (Le Roy et al. 2005). The N-terminal domain of eRF3 is not required for translation termination and interacts with poly(A) binding protein (PABP), and this interaction is required for deadenylation-mediated mRNA decay (Hosoda et al. 2003). Interestingly, tethering of PABP or eRF3 but not eRF1 can stabilize a mRNA undergoing nonsense-mediated decay in yeast. (Amrani et al. 2004).

**Translational Repression by 3' UTRs**

3' UTRs contain a plethora of sites to control translation. Broadly, translational control by 3' UTRs can be divided into four types of mechanisms: regulation of polyA tail length, inhibition of ribosomal small subunit recruitment by impairment of
preinitiation complex formation, inhibition of large ribosomal subunit recruitment, and inhibition after initiation. Deadenylation of mRNA generally decreases its translational capacity. The best characterized 3’ UTR regulatory sequence governing polyadenylation is the CPE (cytoplasmic polyadenylation element) recognized by CPEB protein. Several mRNAs involved in cell cycle regulation in *Xenopus* (mitotic cyclins, cdk2, wee1 and Aurora A) contain CPE elements and are translationally activated during oocyte maturation (Mendez and Richter 2001). Within the oocyte, progesterone activates the kinase Aurora A/Eg2 to phosphorylate CPEB on serine 174, which then recruits cytoplasmic polyadenylation specificity factor (CPSF) and thereby poly(A) polymerase (PAP) (Hake and Richter 1994; Mendez et al. 2000a; Mendez et al. 2000b). Subsequent polyadenylation results in translational activation. However, germ cells may represent a unique compartment for this type of regulation because deadenylation within most somatic cells causes rapid mRNA decay, as discussed above.

Another class of translational repression mechanisms involves inhibition of ribosomal small subunit association with mRNA by the disruption of eIF4E interactions with eIF4G. Several proteins binding to the 3’ UTR of mRNAs are known to prevent eIF4E interactions with eIF4G and thereby inhibit initiation. For example, *Drosophila caudal* mRNA is repressed by Bicoid protein binding to Bicoid-binding region elements (BBR) within the 3’ UTR of *caudal* mRNA. Bicoid contains an eIF4E binding motif that is necessary for association with eIF4E and translational repression. More typically in this type of regulation of translation initiation, a 3’ UTR binding protein recruits an intermediate protein that makes direct contacts with eIF4E. For example, in addition to directing transcript-specific polyadenylation, CPEB also represses translation of cyclin
B1 mRNA by recruiting the Maskin protein, which in turn binds eIF4E and thereby excludes eIF4G binding (Stebbins-Boaz et al. 1999; Cao and Richter 2002). Likewise, translational repression elements within the 3’ UTR of Drosophila oskar mRNA bind the protein Bruno, which in turn recruits Cup, a competitor for eIF4E binding to eIF4G (Wilhelm et al. 2003; Nakamura et al. 2004). Similarly, nanos mRNA is repressed by 3’ UTR sequences which bind the protein Smaug, which in turn recruit Cup to inhibit eIF4G binding to eIF4E (Ostareck-Lederer et al. 1994; Ostareck-Lederer and Ostareck 2004).

3’ UTRs are also capable of inhibiting translational initiation at the step of large subunit recruitment. 15-lipoxygenase protein degrades the mitochondrial membranes during the final stage of erythrocyte development and its mRNA is translationally regulated by ten 19-nt differentiation control elements (DICE) within its 3’ UTR (Ostareck-Lederer et al. 1994; Ostareck-Lederer and Ostareck 2004). These elements bind hnRNP K and hnRNP E1 which block 60S subunit joining through an unknown mechanism (Ostareck et al. 2001; Ostareck-Lederer et al. 2002).

Finally, post-initiation steps of translation can be inhibited by 3’ UTRs. Although both oskar and nanos mRNAs contain regulatory elements that recruit primary and secondary proteins which inhibit eIF4E association with eIF4G, both mRNAs are localized to polysomes during translational repression (Clark et al. 2000; Braat et al. 2004). Treatment of either of these repressed mRNAs with puromycin releases the mRNAs from polyribosomes into the monosome and free RNP fractions of sucrose gradients (Clark et al. 2000; Braat et al. 2004). Because puromycin mimics peptidyl tRNA and is added to the growing polypeptide chain to cause ribosome release, ribosomes associated with these mRNAs are likely to be unimpaired in their ability to
complete the elongation cycle (Blobel and Sabatini 1971). Accordingly, ribosomes have
been observed to dissociate from the repressed mRNA in cell extracts recapitulating
nanos repression upon inhibition of translation initiation with the drug pactamycin (Clark
et al. 2000). No studies of these systems have distinguished between models of ribosome
drop-off or destabilization of the nascent polypeptide chain. However, the nascent
polypeptide associated complex (NAC) is required for translational repression of oskar
mRNA, but this protein could be acting to facilitate either type of repression (Braat et al.
2004). Interestingly, the helicase Armitage, which is required for RNA interference in
Drosophila, is also required for oskar repression translational repression, indicating that
microRNAs might contribute to oskar regulation (Cook et al. 2004; Tomari et al. 2004a).

Mechanism of miRNA translational repression

MicroRNA regulation also seems to occur at two different separate steps in
translation. The lin-4 microRNA regulates lin-14 and lin-28 after translation initiation in
C. elegans because repressed lin-14 or lin-28 mRNAs associate with polyribosomes
(Olsen and Ambros 1999; Seggerson et al. 2002). However, a reporter mRNA
synthesized in vitro and transfected into Hela cells is repressed by let-7 in a cap-
dependent manner, and an IRES-containing construct cannot be repressed, arguing for
repression at a stage early in initiation (Pillai et al. 2005). The reporter mRNA in this case
did not associate with polyribosomes, another possible indication of a block to initiation.
However, we provide evidence that a reporter of translational silencing by short RNAs in
mammalian cells is repressed after translation initiation through a process that involves
ribosome drop-off (see Ch. 3). Because both oskar and nanos mRNAs are capable of
being regulated by their 3’ UTRs at the level of initiation and also after initiation (see above), it may be that different mechanisms of repression occur in different developmental contexts or that regulation of two stages of translation simultaneously allows for tight control of gene expression. Indeed, evidence is accumulating that multiple steps of translation can be repressed on the same mRNA. For example, the 3’ UTR of *Drosophila male specific lethal-2 (msl-2)* mRNA inhibits recruitment of small subunit preinitiation complexes to the mRNA and the 5’ UTR inhibits scanning by the subunits which have escaped this block (Beckmann et al. 2005). Additionally, Fragile X protein FMRP (see above) has been shown to be capable of regulating translational initiation by inhibiting 80S complex formation *in vitro*, but *in vivo* is associated with polyribosomes and represses translational elongation on some of its targets (Ceman et al. 2003; Khandjian et al. 2004). On the other hand, the critical experiments performed to date in support of the initiation model for microRNA repression—experiments in which uncapped IRES-containing mRNAs cannot be translationally repressed—have not been rigorously shown to be a translational phenomenon and may not capture the full extent of regulation which occurs for an mRNA which is transcribed within the nucleus, processed and exported.

The genetic requirements for translational repression are also not fully known. Although mammalian Argonaute 2 is required for RISC cleavage of mRNA, it is dispensable for translational repression (Liu et al. 2004). However, either Argonaute 2, 3 or 4 can cause a repression of translation when tethered to the 3’ UTR of a reporter mRNA in the absence of short RNA (Pillai et al. 2004). This observation suggests that perhaps all mammalian Argonaute genes can function redundantly to repress translation
and these activities are typically targeted to specific mRNAs through interaction with microRNAs.
Figure 1. mRNA cleavage and translational repression by short RNAs. On the left, exogenous or endogenous dsRNA is processed by Dicer to yield an siRNA with a 19 nt duplex, 2 nt 3' overhangs, and 5' phosphates. Dicer and R2D2 load one strand into the RNA-Induced Silencing Complex (RISC) based on the asymmetry of thermodynamic stability at each end of the duplex, while the other strand is rapidly degraded after duplex unwinding. On the right, pri-miRNAs are transcribed from microRNA genes in the nucleus and the RNase III enzyme Drosha acts with DGCR8 to processes these into 60-70 nt hairpin pre-miRNAs with a 2 nt 3' overhang which are then exported into the cytoplasm by Exportin-5 and RanGDP. The pre-miRNA is processed to a transient siRNA-like duplex by Dicer, and one strand is chosen for assembly into the miRNP while the other is rapidly degraded. The miRNP, which contains an Argonaute protein, binds multiple sequences in target gene 3' UTRs with partial complementarity and silences translation at a step after initiation without significantly degrading the mRNA. MiRNAs can perform target mRNA cleavage, and siRNAs can mediate translational repression.
References


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Chapter Two

siRNAs Can Function as miRNAs

This chapter is presented in the context of its contemporary science, and originally appeared in *Genes and Development* 17: 438-42 (2003).

The experiments described here were performed in collaboration with John G. Doench.
Abstract

With the discovery of RNA interference (RNAi) and related phenomena, new regulatory roles attributed to RNA continue to emerge. Here we show, in mammalian tissue culture, that a short interfering RNA (siRNA) can repress expression of a target mRNA with partially complementary binding sites in its 3’ UTR, much like the demonstrated function of endogenously encoded microRNAs (miRNAs). The mechanism for this repression is synergistic, distinct from the catalytic mechanism of mRNA cleavage by siRNAs. The use of siRNAs to study translational repression holds promise for dissecting the sequence and structural determinants and general mechanism of gene repression by miRNAs.
Introduction

The RNA interference (RNAi) pathway was first recognized in *Caenorhabditis elegans* as a response to exogenously introduced long double stranded RNA (dsRNA) (Fire et al. 1998). An RNase III enzyme, Dicer, cleaves the dsRNA into duplexes of 21-23 nt termed short interfering RNAs (siRNAs), which then guide a multicomponent complex known as RISC (RNA Induced Silencing Complex) to mRNAs sharing perfect complementarity and target their cleavage (Hamilton and Baulcombe 1999; Tuschl et al. 1999; Zamore et al. 2000; Hammond et al. 2000; Bernstein et al. 2001; Elbashir et al. 2001a). The RNAi pathway has been implicated in silencing transposons in the *C. elegans* germline (Tabara et al. 1999; Ketting et al. 1999), silencing Stellate repeats in the *Drosophila* germline (Aravin et al. 2001), and serving as an immune response against invading viruses in plants (reviewed in Baulcombe 2001). Very little, however, is known about the intrinsic biological role of RNAi in mammalian systems; indeed, no endogenous siRNAs have been identified in mammals. Nevertheless, transfection of mammalian cells with exogenous siRNAs has rapidly been adopted as a technology for targeted gene silencing (Elbashir et al. 2001a).

A related short RNA species, microRNAs (miRNAs), has been identified in organisms ranging from plants to nematodes to mammals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart et al. 2002). These endogenous RNA species are first transcribed as a long RNA and then processed to a pre-miRNA of ~70 nt (Lee et al. 2002). This pre-miRNA forms an imperfect hairpin structure which is processed by Dicer to produce the mature, single strand ~22 nt miRNA (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). Despite the large library of miRNAs
now known in animals, only two have a known function; *lin-4* and *let-7* regulate endogenous genes involved in developmental timing in *C. elegans* by partially basepairing to the 3’ UTR of target mRNAs such as *lin-14* and *lin-41*, respectively (Lee et al. 1993; Wightman et al. 1993; Ha et al. 1996; Reinhart et al. 2000; Slack et al. 2000). This interaction does not affect the stability of the target mRNA but rather represses gene expression through an unknown mechanism known as translational repression (Olsen and Ambros 1999). The polysome profile of the target mRNA does not change upon gene silencing, suggesting that this repression occurs after initiation of translation, and potentially occurs post-translationally (Olsen and Ambros 1999). This form of regulation is likely to be conserved in mammalian cells since overexpression of miR-30 can repress a reporter gene with partially complementary miR-30 binding sites in its 3’ UTR without affecting mRNA stability (Zeng et al. 2002).

The RNAi pathway of siRNA-directed mRNA cleavage and the miRNA-mediated translational repression pathway are genetically and biochemically distinct. In addition to different outcomes, the two pathways have differential requirements for Paz-Piwi domain (PPD) proteins in *C. elegans*. Translational repression by *lin-4* and *let-7* depends on *alg-1* and *alg-2* for miRNA processing and/or stability yet these genes are not required for RNAi (Grishok et al. 2001), while *rde-1* is needed in RNAi but is not necessary for translational repression (Tabara et al. 1999). In HeLa cells, Gemin 3 and 4 proteins immunoprecipitate with RISC activity (Hutvagner and Zamore 2002) and miRNAs (Mourelatos et al. 2002), but have not been detected as components of purified RISC activity from S100 extracts (Martinez et al. 2002).
In addition to requiring Dicer processing to generate the short RNA, RNAi and translational repression share common components. The PPD protein eIF2C2 both immunoprecipitates with miRNAs from HeLa cells (Mourelatos et al. 2002) and co-purifies with RISC activity (Martinez et al. 2002). Additionally, endogenous let-7 in HeLa extracts is capable of directing cleavage of a perfectly complementary target mRNA, suggesting that RNAi and translational repression share common entry points if not overlapping machinery (Hutvagner and Zamore 2002). Because of these similarities, we reasoned that siRNAs may be capable of repressing gene expression via the miRNA-mediated pathway.
Results and Discussion

To test the ability of siRNAs to function like miRNAs in repressing gene expression, we designed a binding site that would basepair to the antisense strand of a siRNA known to be active in vivo for cleavage of the cell-surface receptor CXCR4 mRNA (Fig. 1A). Notably, this binding site contains a central bulge, thereby precluding RISC-directed mRNA cleavage (Elbashir et al. 2001a; Holen et al. 2002). We introduced four of these binding sites as consecutive repeats separated by four nucleotides into the 3’ UTR of the Renilla reniformis luciferase reporter gene (Rr-luc); we also made a similar 3’ UTR construct with a single binding site with perfect complementarity, to serve as a positive control for RNAi activity. Transfection of HeLa cells and subsequent luciferase assays revealed that the CXCR4 siRNA induced at least ten fold silencing of both of these constructs (Fig. 1B). RT-PCR showed that the two constructs were suppressed by two different mechanisms, as the perfectly complementary antisense siRNA:mRNA interaction resulted in a significant decrease in the steady state mRNA level, while the bulged interaction did not significantly reduce the mRNA level (Fig. 1C). Trace radiolabeling of an independent RT-PCR experiment was also used to better quantitate RNA levels, normalizing first within a sample to the control Photinus pyralis luciferase (Pp-luc) and then across samples to the (-) siRNA transfection. Such quantitation revealed that the perfectly complementary construct, targeted for RNAi, showed a greater than ten fold decrease in RNA level, while the bulged construct showed only 1.2 fold reduction in RNA level (data not shown). Interestingly, the sense strand of the same CXCR4 siRNA was capable of repressing a mRNA with four bulged binding sites (Fig. 1D). However, in this case the level of repression was only four fold as compared to the
ten fold repression observed above (data not shown). As an additional control, the four bulged CXCR4 binding sites (Fig. 1A) were introduced into the Pp-luc vector. Luciferase assays showed six fold repression (data not shown). Northern analysis of cytoplasmic RNA confirmed that the bulged binding sites do not cause a decrease in mRNA levels, relative to the β-actin control (Fig. 1E). Thus, we conclude that a siRNA can function like a miRNA, repressing gene expression without a concordant decrease in mRNA stability.

Cloning efforts in many labs have revealed a large library of miRNAs, yet C. elegans lin-4 and let-7 remain the only miRNAs with known mRNA targets for translational repression in animals, and no such interactions are known in mammals. Computational prediction of targets is difficult because the rules for miRNA:mRNA pairing which function in translational repression have not been determined. Systematic manipulation of genes encoding miRNAs to explore these rules is complicated because the mutant genes must be processed by Dicer and the rules for this cleavage are not known. However, the ability of a siRNA to function by a miRNA-type pathway allows direct investigation of sequence and structure requirements for translational repression in the absence of Dicer processing.

To begin to define these rules, different siRNA sequences were tested for their ability to repress reporters in the luciferase assay. Because both the more effective strand of the CXCR4 siRNA (Fig. 1A) and the only previously studied example of miRNA repression in mammalian cells (Zeng et al. 2002) had a 3’-AGG-5’ bulge in the siRNA strand when paired to the target mRNA, we tested the importance of this sequence. Two constructs were designed which would basepair to the sense or antisense strand of a
siRNA previously used to effectively target GFP mRNA for cleavage. The siRNA:mRNA interaction with the AGG bulge was two fold more effective than that with the ACC bulge (Fig. 2, comparison of A & B). By using a different siRNA, the AGG bulge of the siRNA:mRNA interaction in figure 2A was replaced with an ACC bulge, and the ACC bulge of the siRNA:mRNA interaction in figure 2B was replaced with an AGG bulge. (We note that in Fig. 2A the two 3’ bases of the siRNA were changed from UU to CC.) Surprisingly, none of these changes had an effect on the degree of repression. Therefore, by this assay the sequence of the bulge is not the major determinant of translational repression activity.

Since in Drosophila embryo extracts the antisense strand of the siRNA sets the ruler for cleavage of target mRNA, at the ninth nucleotide from its paired 5’ end (Elbashir et al. 2001b), the position of the bulge may be a critical determinant of translational repression activity. However, both the most effective and least effective bulges tested (Fig. 1A and 2B, respectively) position the bulge eight basepairs from the 5’ end of the siRNA. Furthermore, another active construct positioned the bulge nine basepairs from the 5’ end (Fig. 2A). We speculate that a combination of these sequence and structural parameters govern the ability of a siRNA/miRNA to induce translational repression, but that an expanded study will be necessary to define them.

The number of miRNA binding sites in a target mRNA is a likely determinant of the effectiveness of translational repression. Indeed, the lin-14 3’ UTR contains seven potential lin-4 miRNA binding sites, and the lin-41 3’ UTR contains one lin-4 miRNA and two let-7 miRNA binding sites (reviewed in Banerjee and Slack 2002). To investigate this possibility, a series of Rr-luc reporters with an increasing number of
binding sites—0, 2, 4, and 6—were transfected into HeLa cells with increasing concentrations of CXCR4 siRNA. The level of repression increased with increasing number of binding sites and with increasing concentrations of siRNA (Fig. 3A). To compare the effectiveness of translational repression to mRNA cleavage by siRNAs, a series of Pp-luc reporters with an increasing number of binding sites—0, 1, 2, and 3—perfectly complementary to the CXCR4 siRNA were transfected with increasing concentrations of siRNA. Like the translational repression effect observed above, the level of gene silencing by RNAi increases with increasing number of perfectly complementary binding sites and with increasing concentration of siRNA (Fig 3B). As might be expected from a mechanism that results in cleavage of the mRNA, RNAi silences gene expression to a greater extent than translational repression.

The mechanism of mRNA cleavage in RNAi implies that each siRNA:binding site interaction will function independently of another interaction; once a mRNA is cleaved it is expected to be rapidly degraded, and thus a second cleavage event would have little if any effect on gene expression. To assess this, we divided the repression observed for each construct in figure 3B by the number of binding sites on that mRNA, at each concentration of siRNA. These values were then normalized to the repression observed for a single binding site to assess the relative contribution of each site (Fig. 3D). As expected, the relative effectiveness of each site remained the same as the number of binding sites increased. Addition of more binding sites likely only increases the probability of the single necessary cleavage event, and thus multiple binding sites function independently of one another. This same analysis was applied to the translational repression constructs in figure 3A, normalizing to the construct with two
binding sites (Fig. 3C). Strikingly, the degree of repression achieved by increasing the number of sites is not simply additive, as each site in the construct with four binding sites conferred twice as much repression as each site in the construct with two binding sites. The effectiveness of each binding site in the construct with six sites was similar to that of the construct with four sites. These results suggest that the effects of binding multiple miRNA complexes to the 3’ UTR are likely to be synergistic. Ribonucleoprotein complexes could either mutually stabilize one another or cooperatively interact to more effectively inhibit translation or both. As with other cooperative interactions in gene regulation, this would allow a cell to fine-tune the expression of a mRNA by regulating the degree of binding of different miRNAs to the 3’ UTR of the mRNA.

The discovery that siRNAs can function in translational repression as miRNAs, and that the sequence requirements for this interaction are less stringent than those for RNAi, may help to explain non-specific effects sometimes observed in experiments utilizing siRNAs for targeted gene silencing. Using an arbitrary 21 nt sequence, BLAST searches against the mRNAs predicted from the human genome identify multiple inexact matches with 16-18 nt complementarity. Combined with the potential of GU wobble basepairs, and depending on the overall sequence rules for translational repression, there may be translational repression of a number of off-target genes by the introduction of a siRNA intended to knock-down the expression of only the target gene. However, the mechanistic finding that several binding sites are needed to produce a significant effect on protein expression may make non-specific siRNA effects the exception rather than the rule, and to date siRNAs have certainly been used with ostensible specificity.
Materials and Methods

DNA constructs and siRNAs

3’ UTR binding sites for the siRNAs were constructed by a multimerization of DNA oligonucleotides (IDT), gel purification, PCR amplification, and restriction digestion. The products were inserted into the Xbal site immediately downstream of the stop codon in either the pRL-TK vector coding for the *Renilla reniformis* luciferase (Rr-luc) or the pGL3 control vector coding for the *Photinus pyralis* luciferase (Pp-luc) (Promega). siRNAs were purchased as single strands, deprotected, and annealed according to the manufacturer (Dharmacon). All sequences for siRNAs and 3’ UTR constructs used in this study are available on the Sharp Lab website at http://web.mit.edu/sharplab/RNAi/sequences.html

Cell culture and transfections

Adherent HeLa cells were grown in 10% IFS in DMEM, supplemented with glutamine in the presence of antibiotics. For all transfections, except those noted below, cells were transfected with Lipofectin and the PLUS reagent (Invitrogen). On the day before transfection, exponentially growing cells were trypsinized and plated into 24-well plates at a density of 3x10^4 cells/well in antibiotic-free media. The next day the cells were transfected with 0.2 μg DNA and 25 nM siRNA in a final volume of 250 μL. For Fig. 1E and Fig. 3, cells were transfected with Lipofectamine 2000, as during the course of this study we found that this reagent delivers effective doses of siRNAs at lower concentrations. On the day before transfection, cells were trypsinized and plated into 24-well plates at a density of 8x10^3 cells/well in antibiotic-free media. The next day cells
were transfected with 0.8 μg DNA and 5 nM siRNA, unless noted, in a final volume of 500 μL.

*Luciferase assays*

Dual-Luciferase assays (Promega) were performed 24 hours post-transfection according to the manufacturer’s protocol and detected with an Optocomp I Luminometer (MGM Instruments). *Rr*-luc target vectors were co-transfected with control pGL3, and *Pp*-luc target vectors were co-transfected with a pRL-CMV control (Promega). Transfections were harvested 24 hours post-transfection, and the two luciferase activities consecutively assayed.

*RT-PCR*

Total RNA was harvested from transfected HeLa cells using the RNAeasy kit (Qiagen). Total RNA was DNase treated twice with DNase-Free (Ambion) and reverse transcribed using Omniscript reverse transcriptase (Qiagen) with a DNA primer complementary to a region near the SV40 polyadenylation sequence found in both the *Pp*-luc and *Rr*-luc reporter vectors (5’-GCATTCTAGTTGTGGTTTGTCC). Trace radiolabeled PCR products were detected via autoradiography, and quantitated with ImageQuant software v. 1.2 (Molecular Dynamics).

*Northern Analysis*

Cytoplasmic RNA was harvested by hypotonic lysis without detergent and subsequent needle homogenization of HeLa cells 24h after transfected using Lipofectamine 2000. Nuclei were pelleted at 1500 x g for 15 min and the supernatant treated with proteinase K, extracted in phenol:chloroform and again in chloroform, precipitated with isopropanol and washed with 70% ethanol. Samples were then treated with DNase-Free (Ambion).
Northern analysis was performed using the NorthernMax kit (Ambion). 10 μg of RNA from the (+) siRNA or (-) siRNA samples were separated by electrophoresis on a 1% formaldehyde agarose gel and transferred onto Hybond N+ nitrocellulose by downward transfer (Amersham Pharmacia). The 1.5 kb ORF of the *Pp*-luc cDNA was generated by restriction digest of the pGL3 control vector with XbaI and HindIII (New England Biolabs), and used with DECA-Prime II (Ambion) in the presence of $^{32}$P-dATP to generate a random-primed DNA used to probe the membrane. The membrane was stripped and reprobed with β-actin probe, generated from DECAtemplate-β-actin-mouse (Ambion).

Acknowledgements

The authors thank A. Grishok for helpful discussion, and C. Novina, D. M. Dykxhoorn, H. Houbaviy, D. Tantin, and R. Bodner for comments on the manuscript. J.G.D. is a Howard Hughes Medical Institute Predoctoral Fellow. C.P.P. is a National Science Foundation Predoctoral Fellow.
Figure 1
Figure 2
Figure 3
**Figure Legends**

**Figure 1.** siRNAs translationally represses a target mRNA. (A) Schematic of the proposed interaction between a binding site engineered into the 3’ UTR of the target mRNA and the antisense strand of the CXCR4 siRNA. The thymidines at the 3’ end of the siRNA are deoxynucleotides. (B) Dual Luciferase assay of transfected HeLa cells. Three *Renilla reniformis* luciferase (*Rr*-luc) constructs were used in this assay. One was unmodified (“no sites”), one contained a binding site perfectly complementary to the siRNA strand shown in (A) (“1 perfect”), and one contained four of the binding sites shown in (A) in tandem repeat (“4 bulged”). *A Photinus pyralis* luciferase (*Pp*-luc) served as an internal transfection control. The cells were transfected with no siRNA (black bars), a non-specific (targeting GFP) siRNA (white bars), or the CXCR4 siRNA (gray bars). The ratios of *Rr*-luc to *Pp*-luc expression were normalized to the no siRNA transfections, +/- S.E. from three independent experiments. (C) RT-PCR of harvested RNA. Total RNA was harvested from cells transfected with the constructs described in (B), transfected with or without the CXCR4 siRNA. Control experiments demonstrate that DNA was successfully removed from the RNA preparation and that the PCR was in the linear range of amplification (data not shown). (D) Schematic of the proposed interaction between the sense strand of the CXCR4 siRNA and a designed binding site. (E) RNA analysis of *Pp*-luc with four bulged CXCR4 binding sites (shown in A), targeted for translational repression, transfected either with the CXCR4 siRNA (+) or no siRNA (-). RNA was detected by Northern analysis, probing for either *Pp*-luc or β-actin.
Figure 2. Analysis of sequence and structure rules for siRNA:mRNA interaction. HeLa cells were transfected with constructs containing four binding sites in tandem repeat with imperfect complementarity to either the antisense (A) or sense (B) strand of a GFP siRNA. The effect on luciferase expression is shown by the white bars, +/- S.E. from two independent experiments, normalized to cells transfected with no siRNA (black bars). A different siRNA was then used to produce different bulges, shown in gray with arrows. These new interactions were assayed and are depicted with gray bars.

Figure 3. Comparison of RNAi and translational repression. (A) Titration of Rr-luc constructs containing 0 (O), 2 (■), 4 (X), or 6 (○) of the bulged binding sites, for pairing with the antisense strand of the CXCR4 siRNA, as depicted in Fig. 1A. The level of repression achieved is plotted, normalized to cells transfected with no siRNA. (B) Titration of Pp-luc constructs containing 0 (O), 1 (■), 2 (X), or 3 (○) binding sites perfectly complementary to the antisense strand of the CXCR4 siRNA (see Fig. 1A). (C) Analysis of the relative repression each site contributes for the data presented in (A), normalized to the construct with two binding sites, +/- S.E. (D) Analysis of the relative repression each site contributes for the data presented in (B), normalized to the construct with one binding site, +/- S.E.
References


Chapter Three

Short RNAs repress translation after initiation in mammalian cells

This chapter is presented in the context of its contemporary science, and has been submitted for publication.
Abstract

MicroRNAs are predicted to regulate the translation of 30% of mammalian protein-encoding genes by interactions with their 3’ untranslated regions (UTRs). We use partially complementary siRNAs to investigate the mechanism by which short RNAs mediate translational repression in human cells. Repressed mRNAs are associated with puromycin-sensitive polyribosomes. Circumvention of cap-dependent initiation by the HCV IRES, as well as scanning and Met-tRNA\textsubscript{met} binding by the CrPV IRES indicates that mRNAs can be repressed by short RNAs after translational initiation. Metabolic labeling shows that silencing occurs before completion of the nascent polypeptide chain. Silencing by short RNAs causes a decrease in translational readthrough at a stop codon and ribosomes associated with repressed mRNAs dissociate rapidly under a block of translation initiation. These results suggest short RNA translational repression causes ribosome drop-off.
Introduction

MicroRNAs are a class of short ~21 nt RNAs which regulate diverse cellular and molecular processes in plants and animals (Petersen et al., 2005). They constitute upwards of 4% of human genes and are predicted to regulate as many as 30% of protein-encoding genes (Berezikov et al., 2005; Lewis et al., 2005; Xie et al., 2005). MicroRNAs are involved in normal development in worms, flies, mammals and have been implicated in cancer and infectious disease, so it is of great interest to understand the mechanisms underlying their action (Bartel, 2004).

Short RNAs are capable of silencing gene expression by at least two different mechanisms in the cytoplasm. Perfectly complementary interactions between short RNA (siRNA or miRNA) and target mRNA result in mRNA degradation. In contrast, mismatched interactions between either siRNA or miRNA and the target mRNA can result in translational repression (Doench et al., 2003; Zeng et al., 2003). The degradation mechanism requires the endonucleolytic activity of Argonaute 2, whereas Argonaute 2, 3 or 4 proteins are sufficient to cause translational arrest if tethered to mRNA (Liu et al., 2004; Pillai et al., 2004). Although microRNAs direct cleavage of endogenous and artificial substrates containing highly complementary binding sequences, the majority of interactions between microRNA and mRNAs in animals are only partially complementary and thus translational inhibition predominates (Hutvagner and Zamore, 2002; Lewis et al., 2003; Yekta et al., 2004). Indeed, the earliest identified miRNAs, *lin-*4 and *let-*7, act to repress translation of their target mRNAs *lin-14, lin-28, lin-41* (Brennecke et al., 2003; Olsen and Ambros, 1999; Seggerson et al., 2002). More recently, it has been reported that *bantam* miRNA represses translation of *hid* mRNA and
the miR-2 family represses *hid*, *grim*, *rpr*, and *skl* in drosophila (Brennecke et al., 2003; Jorgensen and Kurland, 1990). In addition, miR-17 and miR-20 act together to repress translation of E2F1 in Hela cells (O'Donnell et al., 2005).

The mechanism of translational repression by short RNA has been studied previously. *Lin-14* mRNA is associated with polyribosomes, and its polysomal distribution is not altered during repression, suggesting that *lin-4* miRNA acts after translation initiation to repress gene expression (Olsen and Ambros, 1999; Seggerson et al., 2002). Additionally, microRNAs have been observed to co-sediment with polyribosomes in worms and mammalian cells (Kim et al., 2004; Nelson et al., 2004; Olsen and Ambros, 1999). Translational repression by short RNA is accompanied by localization of the mRNA and short RNA at or near P-granules, known sites of mRNA degradation (Liu et al., 2005; Pillai et al., 2005; Sen and Blau, 2005). Indeed, this localization is consistent with the observation that microRNAs *lin-4* and *let-7* cause a degree of mRNA degradation of their targets (Bagga et al., 2005).

To investigate the mechanism of translational regulation by short RNAs, we have used a well-characterized system in which siRNA is targeted through imperfectly complementary interactions to multimerized sequences within the 3’ UTR of luciferase mRNAs (Doench et al., 2003). This system has been shown to recapitulate many of the phenomena associated with microRNA translational silencing. First, siRNA causes a decrease in luciferase protein without a significant decrease in mRNA levels, and additional binding sites and higher concentrations of siRNA increase repression. Second, complementarity to messenger RNA within the 5’ region of the siRNA is critical for regulation, a phenomenon verified in other systems and organisms (Brennecke et al.,
2005; Doench and Sharp, 2004; Kloosterman et al., 2004). This particular system using an siRNA designed against the CXCR4 mRNA follows the consensus sequence for predicted mammalian microRNA target sites: perfect complementarity at positions 2-7 with no preferred sequence, flanked by basepaired nucleotides at position 8, and neighbored by an unpaired A at position 1 and to a lesser extent position 9 (Figure 1B) (Lewis et al., 2005). Additionally, the catalytic subunit of RISC, Argonaute 2, is required for CXCR4 silencing of a luciferase reporter with one perfectly complementary binding site but dispensable for silencing a luciferase reporter with six bulged binding sites (Liu et al., 2004). Finally, this reporter localizes to P-granules upon silencing by complementary siRNA (Liu et al., 2005).

Using this system, we dissect the mechanism of short RNA translational silencing in mammalian cells. These results support a model in which short RNAs cause ribosome drop-off.

Results

Repressed mRNAs associate with polyribosomes

We have previously described a system in mammalian cells that recapitulates many of the features of microRNA silencing. In this system, multiple binding sites are inserted in the 3’ UTR of a luciferase reporter to which the CXCR4 siRNA can bind creating a central bulge (Figure 1A and B). Transfection of the CXCR4 siRNA along with the reporter plasmid causes inhibition of protein production as measured by luciferase assay without a corresponding loss of luciferase mRNA, mimicking the known outcome of lin-4 miRNA’s interaction with lin-14 mRNA in C. elegans (Olsen and
Ambros, 1999). With this system, we observe a 30-fold decrease in protein expression in 293T cells at 20 hours post-cotransfection with 5 nM CXCR4 siRNA and a Renilla vector driven by the TK promoter containing 6 target sites. Quantitation by RPA of mRNA loss due to CXCR4 siRNA shows no more than a 2-fold decrease in overall messenger RNA levels, and thus the repression occurs primarily at a translational or post-translational step in gene expression (Figure 1C).

We next examined the polyribosome profile of the active versus repressed mRNAs in cytoplasmic extracts made from 293T cells transfected with the above Renilla reporter and Firefly reporter used as an internal control (lacking binding sites for short RNA). Cells were extracted with lysis buffer containing 1% NP-40 and 1% deoxycholate (DOC) and separated by ultracentrifugation on 15-50% linear sucrose gradients (UV absorbance profiles are shown in figure 1D). Under these conditions, both active and repressed Renilla mRNAs cosediment with polyribosomes, and the distribution of the mRNA over these fractions was identical within experimental error (Figure 1D). As a control, sedimentation of an internal Firefly control mRNA which was not translationally inhibited also did not change significantly (Figure 1D). Because these mRNAs are associated with polyribosomes, it is likely that some step after translation initiation is altered during inhibition by short RNA. As a control for the specificity of observed cosedimentation of repressed mRNA with polyribosomes, puromycin release experiments were performed (see below, Figure 2).

Ribosomes associated with repressed mRNA exhibit peptidyl transferase activity
We next tested whether the repressed mRNAs are undergoing active translation by measuring their responsiveness to a brief incubation (3 minutes) with puromycin. Puromycin is a chain-terminator that requires ribosomal peptidyl-transferase activity to be added to the end of the nascent polypeptide chains (Blobel and Sabatini, 1971). Any off-target effects of puromycin are likely to be minimized during this short (3 minute) exposure. Polyribosome fractionation and RPA analysis were performed on cells transfected with targeting or control siRNA and treated or not treated with puromycin for 3 minutes. The profiles of both active and repressed mRNAs similarly shift to fewer polysomes after a 3 minute incubation with puromycin (Figure 2). Since this drug causes premature termination of protein synthesis, these results indicate that polysomes on repressed mRNAs are actively involved in peptidyl transferase activity. In addition, the sensitivity of active and repressed mRNAs to puromycin importantly demonstrates that their rapid sedimentation in the gradient reflects a genuine association with polyribosomes and not with other large non-ribosomal complexes.

**IRES initiated translation can be repressed by short RNA**

If the mechanism of inhibition was post initiation then functionally bypassing processes important for normal translational initiation should not affect the inhibition. To test this prediction, we constructed bicistronic luciferase vectors separated by the Hepatitis C virus (HCV) or Cricket Paralysis virus Intergenic Region (CrPV-IGR) IRES sequences. HCV and CrPV-IGR IRES-driven translation requires a subset of initiation factors that are necessary for cap-dependent translation. HCV IRES directly binds the 40S ribosomal subunit (thereby circumventing the cap requirement for initiation) and
requires eIF3, eIF2 and methionine tRNA for positioning the 40S at the AUG codon (Kieft et al., 2001; Pestova et al., 1998). CrPV-IGR IRES-driven translation bypasses all steps in translational initiation and lacks a requirement for cap-binding, scanning, and all initiation factors (Wilson et al., 2000).

HCV or CrPV-IGR reporter plasmids were transfected with targeting or control siRNAs into 293T cells and luciferase assays were performed the following day. In these experiments, a novel inhibitor of translation initiation, called hippuristanol, was used. This compound selectively inhibits eIF4A RNA binding activity, inhibiting cap-dependent protein synthesis, but not affecting HCV- or CrPV-mediated translation initiation (Bordeleau et al., submitted). Cells were transfected with bicistronic IRES constructs and siRNA for 4 hours, exposed to 1 uM hippuristanol or DMSO for 10 hours, and luciferase assays were performed. Hippuristanol caused a significant 20-fold reduction of cap-dependent translation of upstream Firefly luciferase but only a 2-fold decrease of downstream Renilla activity driven by either IRES sequence (Figure 3A). This modest 2-fold decrease could be due to an indirect effect of the inhibition of translational processes since it was observed with both IRESes. To determine whether IRES-dependent translation could be repressed by short RNA, bicistronic constructs were transfected in the presence of targeting CXCR4 or control siRNA and treated with hippuristanol or DMSO for 10 hours and luciferase assays were performed. In the presence or the absence of the initiation inhibitor, upstream Firefly and downstream Renilla expression were repressed similarly by short RNA (Figure 3B).
**Repression occurs before synthesis of full length polypeptide**

To assess the fate of polypeptides synthesized under normal and siRNA silencing conditions, we performed metabolic labeling experiments on cells transfected with a *Firefly* reporter bearing 6 imperfect CXCR4 binding sites and an N-terminal Flag epitope. Following a 3 minute pulse of radiolabeled methionine and immunoprecipitation with anti-FLAG conjugated beads, label accumulated in a 65 kDa band, the full-length of tagged *Firefly* polypeptide and also in a faster-migrating smear typical of nascent polypeptide chains. In pulse-chase experiments, the faster-migrating smear was observed to be short-lived and thus likely represents labeling of nascent polypeptide chains (data not shown). As expected, no labeled polypeptides were observed in the untransfected cells (Figure 4). In contrast to the control, translationally repressed *Firefly* mRNA produced neither labeled full-length peptide nor labeled nascent polypeptide chains during the 3 minute incubation. Because the pulse labeling was done in such a short period of time, the results of this experiment argue strongly against a model of repression in which short RNAs cause post-translational degradation of the completed polypeptide chain. Therefore, this experiment suggested that repression occurs before the completion of the nascent polypeptide chain.

**Silencing by short RNAs increases termination**

We addressed whether silencing by this system is capable of influencing translation termination. Bicistronic luciferase vectors were constructed to measure the incidence of translational readthrough at a codon, which is inversely proportional to the
frequency of translational termination. The *Renilla* luciferase with 6 bulged CXCR4 binding sites in its 3’UTR was cloned downstream of Firefly luciferase ORF, and each of the three stop codons were inserted in-frame between the two ORFs. 293T cells were transfected with the plasmids and with targeting CXCR4 or control siRNA, and luciferase assays were performed 20 hours post-transfection. Translational readthrough was calculated by dividing downstream *Renilla* expression by the total *Renilla* plus Firefly expression as described previously (Grentzmann et al., 1998). In the absence of inhibition by siRNA, the incidence of translational readthrough was about 4.5% for each of the three stop codons, similar to what was reported previously for this kind of assay (Orlova et al., 2003). The addition of siRNA caused a 2-fold decrease in the efficiency of translation readthrough for any stop codon (Figure 5A). If this decrease in readthrough was due to an increase in termination while the ribosome was paused at the stop codon, the expression of a suppressor tRNA should reduce the degree of termination by reducing the pause. Consistent with this hypothesis, expression of amber suppressor tRNA produced an 8-fold increase in readthrough and more importantly eliminated the 2-fold decrease in readthrough caused by the siRNA (Figure 5B). These results indicate that short RNA translational repression interacts negatively with translational elongation at a location distant from the binding sites (1 kb) and suggest that short RNAs may accentuate premature termination or early exit of ribosomes within the open reading frame.

**Ribosome drop-off on repressed mRNAs**

To test the hypothesis of premature termination, we performed *in vivo* run-off experiments with the eIF4A inhibitor hippuristanol. Cells co-transfected with the *Renilla*
reporter mRNA downstream of the CMV promoter and either the targeting CXCR4 or control siRNA were treated either with DMSO or 1 uM hippuristanol for 4 or 5 minutes at 37 degrees. Under these conditions, protein repression of this Renilla construct by the CXCR4 siRNA was 12 fold and mRNA loss was less than 2 fold, as measured by Northern analysis of cytoplasmic mRNA (data not shown). Although as a drug hippuristanol may theoretically have off-target effects, the short-term nature of these experiments should minimize them. Further, hippuristanol displayed the expected properties of a translation initiation inhibitor in these experiments as treatment for 5 minutes resulted in a profound decrease in total cellular polyribosomes and an accumulation of 80S monosomes as compared to the DMSO control (Figure 6, absorbance profiles). Treatment for longer times results in complete dissociation of polyribosomes (data not shown). Northern analysis of fractionated Renilla mRNA showed that inhibition of translation initiation by hippuristanol resulted in a more rapid loss of repressed mRNA (Figure 6, targeting siRNA) from the polyribosome regions of the gradient than that observed for active mRNAs (Figure 6, control siRNA). These results indicate that the ribosomes on repressed mRNAs are released from their association with the mRNA more rapidly than are ribosomes on active mRNAs. The simplest interpretation of these results is that ribosomes exit prematurely from repressed mRNAs.
Repressed mRNA cofractionates with membrane-associated cytoplasmic compartment

To test whether the mRNA changes its subcellular localization, cell fractionation by differential detergent solubility was performed. This method has been used previously to define subcellular compartments containing individual mRNAs (Lerner et al. 2003). Free cytoplasm was first solubilized in the mild detergent digitonin, and the remaining membrane-associated cytoplasm was solubilized in 1% NP-40 and 1% deoxycholate (Figure 8A). The remaining insoluble material contained nuclei. Western blotting of the free cytoplasmic GAPDH and ER resident TRAPalpha proteins shows that the free and membrane compartments were efficiently separated (Figure 8B).

To determine the subcellular distribution of active versus repressed mRNAs we performed Ribonuclease Protection Assays (RPA) on each subcellular fraction taken from 293T cells which were transfected with Renilla microRNA silencing reporter and Firefly control plasmids and either targeting CXCR4 or control siRNA (Fig 8C). In this assay, cytoplasmic Renilla mRNA has a slightly greater abundance (65%) in the membrane compartment compared to Firefly mRNA, which is more equally distributed. These results are consistent with previous experiments which show that even mRNAs which do not encode secreted or membrane proteins can have significant abundance in membrane fractions (Lerner et al. 2003). Strikingly, siRNA causes a 6-fold decrease in mRNA levels in the free cytoplasm but a 1.5-fold reduction in the membrane and nuclear compartments. This result can be interpreted in two ways. Either siRNA causes mRNA degradation specifically of the free cytoplasmic reporter mRNA or it causes a shift in the
subcellular distribution of repressed mRNA such that it is more difficult to extract and co-fractionates with the ER.

**Discussion**

This study shows that short RNAs repress translation via a novel mechanism in eukaryotic cells. Our results strongly indicate that repression occurs after the initiation of translation because IRES-dependent translation can be repressed and because repressed mRNAs associate with polyribosomes that actively undergo the peptidyl transferase reaction. However, pulse-labeling experiments indicate that repression occurs before the completion of full-length polypeptide chain. Silencing by partially complementary short RNAs increases termination at a distant stop codon and also causes ribosomes associated with repressed mRNAs to dissociate more rapidly following inhibition of translational initiation than those associated with active mRNAs. Together, these observations strongly suggest that short RNAs repress translation by causing ribosomes to exit prematurely from their associated mRNAs (Figure 7).

Ribosome drop-off by microRNAs could result indirectly from an inhibition of elongation rates at every position, favoring stochastic release processes, a phenomenon described for bacterial RNA polymerase (von Hippel and Yager, 1991). In this case, a very small decrease of elongation processivity at every step (0.01%) would be sufficient to cause a significant reduction of the synthesis of full-length polypeptides. Our observed decrease in efficiency of read-through at a stop codon due to translational inhibition by short RNAs is consistent with this possibility. Alternatively, microRNA complexes could directly cause premature termination by recruitment of known termination factors.
such as eRF3 or induction of processes which result in termination, such as a decrease in the activities of charged tRNA. In either case, the drop-off model of miRNA silencing could explain why translationally repressed *lin-14* and *lin-28* mRNAs associate with polyribosomes, but do not accumulate more ribosomes than the non-repressed mRNA.

To measure the effect of short RNA on translation termination, we devised a translational readthrough assay. In this assay, short RNA bulged binding sites were placed downstream of an in-frame, bicistronic luciferase reporter in which a stop codon or an encoding codon was placed between the two ORFs (upstream *Firefly* abbreviated as ORF1 in the following paragraphs, downstream *Renilla* abbreviated as ORF2). Addition of targeting siRNA resulted in 10-fold repression of ORF1 and 20-fold repression of ORF2 for the constructs containing UAG and UGA stop codons. Repression was 15-fold for ORF1 and 26-fold for ORF2 for the construct containing UAA stop codon. Therefore, in each case, short RNAs repressing translation cause a 2-fold greater repression of downstream ORF2 versus upstream ORF1. This difference accounts for the relative difference in translational readthrough of each construct, with or without siRNA (Figure 5a).

As controls, we used similar vectors to measure the fold repression on constructs with enhanced translational readthrough between ORF1 and ORF2. In one case, the stop codon between ORF1 and ORF2 was replaced with a codon encoding alanine (data not shown). This construct was repressed 16-fold for ORF1 and 14-fold for ORF2. In a second control, a construct expressing amber suppressor tRNA was added to cells which had been transfected with the UAG-containing vector described above. Suppressor tRNA caused 8-fold enhanced readthrough (Figure 5b), and after addition of siRNA, repression
was 11-fold for ORF1 and 11-fold for ORF2. Taken together, these two experiments indicate that under conditions of high readthrough of the codon between ORF1 and ORF2, there was the same level of repression by short RNA of ORF2 compared to ORF1.

The ribosome drop-off model for microRNA translational repression makes a few predictions about the outcome of the translational readthrough experiments described above. First, the theory suggests that additional distance traversed by ribosome enhances the likelihood of ribosome drop-off, so a longer ORF should be repressed to a greater degree than a shorter ORF. Therefore, in the readthrough constructs, downstream ORF2 should be repressed to a greater degree than the upstream ORF1. However, there are at least two ways in which this result does not necessarily argue against the drop-off model. The expectation of enhanced repression with greater length assumes that drop-off events are not complete after translation of ORF1. However, it is possible that ribosomes susceptible to drop-off have done so after completion of ORF1. If enough drop-off events occur within ORF1 to reach a state of complete silencing, the additional distance in ORF2 would not be traversed by ribosomes and therefore, this additional distance would not increase translational repression. In the case of this bicistronic reporter, the upstream distance is 2 kb, which may be long enough to ensure maximal repression under these conditions. Additionally, in the readthrough constructs, because translation does not terminate efficiently at the end of ORF1, we assume that little unfused ORF1 protein product is produced. If so, most of the ORF1 product would be fused to ORF2 and therefore, repression of the two ORFs would be equal. When ORF1 and ORF2 are separated by a stop codon, however, the majority of ORF1 in the cell is unfused. In these
vectors, we detect a greater repression of ORF2, which is fused to ORF1, than ORF1 alone, a result which the drop-off theory anticipates.

The drop-off theory also suggests that a repressed mRNA will produce a distribution of truncated polypeptides biased toward the N-terminal end. Therefore, another prediction of the model is that repression of ORF2 should be greater than ORF1, even when readthrough between the two ORFs is high. In our experiments, we do see a relative reduction of downstream expression versus upstream expression during translational repression when ORF1 and ORF2 are separated by a termination codon (Figure 5a). However, as stated above, increasing the readthrough between ORF1 and ORF2 results in no additional repression of ORF2 versus ORF1 (Figure 5b). Possibly, the truncated polypeptide chains produced by drop-off are not stable. This would explain why repression of ORF1 and ORF2 is the same when readthrough between them is high. In a context where readthrough of an internal codon is very inefficient, however, as is the case for the stop codon-containing constructs, repression downstream of the inefficient codon is enhanced versus repression of the upstream luciferase. We conclude from this experiment that microRNAs act at a distance to enhance the process of termination at a stop codon. This effect could be caused indirectly by decreasing the translation elongation efficiency in a manner that is greater for codons with already-low efficiency. Alternatively, this may directly result from increasing the local concentration of termination factors.

Inhibition at a post-initiation stage has been observed in a variety of systems in which 3’ UTRs regulate translation. In fact, silencing of \textit{lin-14} and \textit{lin-28} mRNAs by the \textit{lin-4} microRNA was originally interpreted as repression after translation initiation (Olsen...
and Ambros, 1999; Seggerson et al., 2002). More recently, unlocalized nanos mRNA was shown to be regulated by its 3’ UTR at a step after translation initiation (Clark et al., 2000). Similarly, IL-1 beta mRNA in human monocytes, ribulose 1,5-bisphosphate carboxylase mRNA in amaranth seedlings, and CKB mRNA in rat brain are associated with polyribosomes during translational repression and hence are thought to be regulated after the initiation of translation (Berry et al., 1990; Ch'ng et al., 1990; Kaspar and Gehrke, 1994; Shen et al., 2003). These situations may share a common mechanism with translational repression by siRNA and microRNA.

During the preparation of this manuscript, a report showed that let-7 or tethered Argonaute proteins can repress translation of a designed target mRNA (Pillai et al., 2005). This inhibition was proposed to occur at the stage of translation initiation. Therefore, our data and those obtained by Pillai et al. 2005 appear to support two different mechanisms for translational repression by microRNAs. The differences in the data are as follows. The first difference is that we observe that repressed mRNAs are associated with translating ribosomes whereas Pillai et al could detect a decrease in the ribosome loading of mRNAs during microRNA repression. Pillai et al. interpret this difference to reflect a defect in translation initiation caused by microRNAs. However, it is equally possible that the decreased loading reflects an increase in activity ribosome-drop off during translational repression. Additionally, the ribosome drop-off model predicts a labile association between ribosomes and repressed mRNA, so it is possible that this association was lost in the cell-lysis conditions used by Pillai et al. Both Pillai and our results describe a change in the extraction properties of polysome bound mRNA after inhibition by miRNAs (Figure 8).
A second difference between the two results emerges from the use of IRES to functionally address the nature of the translational silencing. Pillai et al. transfect cells with monocistronic, in vitro transcribed mRNAs and observe 10-fold repression of an mRNA containing a cap but no repression for a similar mRNA lacking a cap and containing an IRES. They conclude from this experiment that microRNAs repress a step in translation during translation initiation. The rationale behind using cap-less mRNAs is to ensure that the IRES directs a truly cap-independent translational repression. However, a loss of cap may cause effects on other aspects of translation. In essence, Pillai et al. are interpreting a negative result, no inhibition. In this regard, the control of miRNA repression of the transfected capped mRNA is important in interpretation of these results. However, Pillai et al. do not show that microRNA repression by this assay of transfection of capped RNA is a translational repression phenomenon. Possibly, they have recapitulated an mRNA-degradative microRNA phenomenon. If this were the case, the structured IRES may cause an inability for a 5’-3’ exonuclease to degrade the mRNA in a microRNA-dependent fashion. Finally, a cap-less mRNA may not accurately represent the behavior of capped mRNA in an unknown manner. In our IRES experiments, we use bicistronic plasmid reporters in which viral IRESes are inserted between two open reading frames. Within the cell, these plasmids express bicistronic messenger RNA which is capped. In these experiments, inhibition of cap-dependent translation initiation using the drug hippuristanol, which targets eIF4A, results in 26-fold downregulation of upstream activity but only 2-fold downregulation of translation activity downstream of the IRES, suggesting that the IRESes are active in this context. Both upstream cap-dependent and downstream cap-independent translation is repressible
by microRNA, either for the HCV and the CPV IRES, the latter of which requires no
initiation factors for translation. These results suggest that in the context of mRNA
which contains a cap and undergoes essentially normal mRNA maturation and export,
translational repression by microRNA does not affect translation initiation.

Possibly, translational repression by miRNAs has different consequences
depending on the cell type, target mRNA or miRNA sequence. Notably, 3’ UTR
regulation of oskar mRNA translation can occur at two different stages. BRE elements in
the 3’ UTR of oskar mRNA repress initiation through interactions with Bruno and Cup
proteins, but at a later time in development, the BRE causes a post-initiation repression
where repressed mRNAs cosediment with puromycin-sensitive polyribosomes (Braat et
al., 2004). The general observation that most mammalian microRNAs co-sediment with
polyribosomes suggests that most endogenous targets of miRNAs are indeed regulated
after translational initiation (Kim et al., 2004; Nelson et al., 2004).

No mechanism has been established in eukaryotic cells for ribosome drop-off
within an open reading frame. However, ribosome drop-off is known to occur in vivo
and in vitro in prokaryotic translation in which peptidyl-tRNA dissociates from the
ribosome at significant rates before reaching the stop codon (Jorgensen and Kurland,
1990; Manley, 1978). The mechanism of early peptidyl-tRNA release is not well
understood, but the process requires ribosome recycling factor RRF, elongation factor
EF-G, release factors RF3 and initiation factors IF1 and IF2 (Dincbas et al., 1999;
Heurgue-Hamard et al., 1998; Karimi et al., 1998; Singh and Varshney, 2004).
Additionally, ribosome drop-off by microRNAs could trigger nonsense-mediated decay
(NMD), an mRNA degradation process initiated by a premature termination codon (PTC)
located upstream of the final intron in aberrant mRNAs (Neu-Yilik et al., 2004). Indeed, a connection between short RNAs and NMD has been established in *C. elegans*, in which NMD factors *smg*-2, -5, and -6 are required for RNA interference (Domeier et al., 2000). Possibly, NMD could explain why microRNAs cause localization of target mRNAs in or near P-granules and cause a variable amount of mRNA degradation (Bagga et al., 2005; Liu et al., 2005; Pillai et al., 2005; Sen and Blau, 2005). Alternatively, transiently paused ribosomes with an increased probability for termination could facilitate nuclease accessibility within the mRNA, which could account for the appearance of long mRNA degradation products during *lin-4* and *let-7* silencing in worms (Bagga et al., 2005). Future studies will be necessary to address which stages of translational elongation or termination are influenced by short RNAs as well as which trans acting factors are involved in short RNA-mediated ribosome drop-off.
Methods and Materials

Transfections and cell culture

Cells were transfected with lipofectamine 2000 (invitrogen) according to the manufacturer’s instructions. The media was replaced 4 hours post-transfection. 293T cells were grown in DMEM supplemented with 10% IFS, penicillin/streptomycin and L-glutamine.

Polyribosome fractionation

At 20h post-transfection, one 60 cm plate of 293T cells per gradient were immediately placed on ice and washed twice with cold PBS containing 100 uM cycloheximide (sigma), and lysed by addition of 500 uL of lysis buffer (110mM potassium acetate, 2 mM magnesium acetate, 10 mM Hepes pH 7.5, 50 mM potassium chloride, 10 mM magnesium chloride, 2 mM DTT, 1% NP-40 and 1% deoxycholate, supplemented with 1x Complete-mini protease inhibitors, 500 U/ml RNasin, and 100 uM cycloheximide), scraped and collected in microfuge tubes. The cell mixture was homogenized 8 times with a 26 gauge needle at 4 degrees and centrifuged for 10 minutes at 4800rpm. Extracts were layered onto 11 ml 15-50% linear sucrose gradients that were prepared by horizontal diffusion and centrifuged in an SW-41Ti rotor at 40,000 rpm for 90 minutes. Gradients were fractionated by upward displacement with 60% sucrose on an ISCO fraction-collector, and absorbance at 254 nm was monitored continuously. The addition of cycloheximide to PBS washes or extract did not change the polysome profile (data not shown) and was omitted in figure 6. Fractions were supplemented with SDS to 0.5% and
treated with proteinase K at 37 degrees for 1 hour. RNA was extracted with acid phenol (Ambion) and precipitated with isopropanol, washed with 75% Ethanol, and resuspended in DNase I reaction buffer (DNase-free, Ambion). DNase was removed with anti-DNase beads and RNA was analyzed by RPA or Northern analysis. Probes for RPA of *Renilla* and *Firefly* luciferase mRNAs were used as described previously and the Northern probe for *Renilla* mRNA consisted of a HindIII/XbaI fragment encompassing the open reading frame (Doench and Sharp, 2004). For polysome run-on analysis in figure 6, 1 uM of Hipperistanol or DMSO was added to cells for 4 or 5 minutes at 37 degrees. Cells were harvested and fractionated as described above. Northern analysis was performed using a probe spanning the open reading frame of *Renilla* luciferase.

**IRES experiments**

The HCV plasmid was constructed by PCR cloning HCV IRES into the NheI site of pRL x 6 and inserting the *Firefly* luciferase ORF from pGL3 (HindIII, XbaI) into the resulting vector (HindIII, SpeI). CrPV IRES sequence was cloned by splint ligation of two synthetic 100-mers, and PCR cloning into the 5’UTR of pRL-TKx6 and to the resulting construct the *Firefly* luciferase ORF was then inserted similarly. The resulting constructs were verified by DNA sequencing. Bicistronic luciferase vectors with microRNA binding sites in the 3’ UTR of the downstream cistron were constructed by inserting the Ppluc ORF from pGL3 (HindIII and XbaI) into HindIII and NheI sites in pRL-TK x 6 described previously (Doench et al., 2003). 0.8 ug of reporter plasmid was co-transfected with 5 nM of targeting CXCR4 or control siRNA (Invitrogen). 4 hours post-transfection,
the media was replaced with media containing either DMSO or 1 uM Hipperistanol and luciferase assays were performed 10 hours later.

**Translational readthrough assay**

The stop codon of PpLuc ORF of pGL3 was removed by PCR and each of the stop codons (UAG, UAA, UGA) was inserted. These products were inserted into the bicistronic HCV IRES construct described above, removing the IRES. The resulting constructs contained *Firefly* ORF fused in-frame to *Renilla* with 18 bp distance between the stop codon and the first ATG within *Renilla* ORF. All constructs were sequenced to confirm the indicated structure. 293T cells were transfected with each of these constructs, with 5 nM targeting CXCR4 or control siRNA (Invitrogen), and luciferase assays were performed 8 hours later. Readthrough was calculated as a ratio of downstream *Renilla* expression to the sum of upstream *Firefly* plus downstream *Renilla* (*Renilla / (Firefly + Renilla)*) (Grentzmann et al., 1998). In experiments with suppressor tRNA, 0.08 ug serine amber suppressor tRNA plasmid was co-transfected with 0.72 ug readthrough reporter and 5 nM siRNA.

**Metabolic labeling and immunoprecipitation**

4x10^5 293T cells were co-transfected with 2.4 ug N-terminally flag tagged firefly luciferase cloned into pcDNA3.1 containing 6 bulged binding sites complementary to CXCR4 siRNA in its 3’ UTR, and either 25 nM targeting CXCR4 siRNA or control siRNA (Invitrogen). 20h post-transfection, the cells were starved in Met-, Cys- media for 20 min and pulse labeled with 100 uCi 35-S methionine ExpreSS labeling media (NEN)
for 3 minutes at 37 degrees. Cells were then washed with cold PBS, lysed in RIPA buffer and centrifuged for 10 minutes at 10,000 rpm at 4 degrees. Extracts were normalized by scintillation counting and were pre-cleared by treatment with 1/10 v Sepharose G for 1 hour and the supernatants from this pre-clear were then incubated with anti-FLAG M2 beads for 1.5 h. These beads were washed three times with lysis buffer and eluted with 1x sample buffer lacking DTT. The eluates were separated on a 4-20% Tris-glycine polyacrylamide gel, dried and visualized by autoradiography.

**Subcellular fractionation**

Free and membrane associated cytoplasm fractions were isolated by differential detergent solubility and centrifugation essentially as described previously (Lerner et al. 2003). Briefly, 2x10^6 293T cells were washed twice with cold PBS, and collected in 500 ul lysis buffer (150 mM potassium acetate, 20mM Hepes pH 7.5, 2.5 mM magnesium acetate, 2 mM DTT, 200 U/ml Superasin (ambion) and Complete-mini (Roche)). Digitonin (Sigma) was added to 200 ug/ml, the cells were placed on ice for 5 minutes and centrifuged at 4 degrees for 5 minutes at 500g. The supernatant was further centrifuged for 10 minutes at 7500g and this supernatant contained free cytoplasm. The post-digitonin pellet was washed once with lysis buffer containing no detergent, extracted with lysis buffer containing 1% deoxycholate and 1% NP-40 and centrifuged at 4 degrees for 5 minutes at 500g to give a supernatant that contained the membrane-bound cytoplasmic fraction.
Acknowledgments

Suppressor tRNA plasmid was a kind gift from Dr. U. Rajbhandary. HCV IRES sequences were a gift from Dr. S. Lemon. Hippuristanol was a kind gift of Dr. Junichi Tanaka (University of Ryukyus, Okinawa). C.P.P. acknowledges Dr. D. Housman, Dr. F. Goldberg, Dr. U. Rajbhandary and Sharp lab members for helpful conversations. This work was supported by United States Public Health Service RO1-GM34277 from the National Institutes of Health, PO1-CA42063 and U19 AI056900 from the National Cancer Institute to PAS and partially by Cancer Center Support (core) grant P30-CA14051 from the National Cancer Institute and an NCIC grant (#014313) to J.P. C.P.P was funded in part by an NSF graduate research fellowship. J.P. is a Canadian Institutes of Health Research (CIHR) Senior Scientist.
Figure 1
control siRNA

Figure 2
Figure 3
Figure 4
**Figure 5**

A

![Diagram A](image)

- **UAG**
  - - siRNA
  - + siRNA

- **UAA**
  - - siRNA
  - + siRNA

- **UGA**
  - - siRNA
  - + siRNA

B

![Diagram B](image)

- **untreated**
  - - siRNA
  - + siRNA

- **su(tRNA)**
  - - siRNA
  - + siRNA
Figure 7

ribosome drop-off
A

<table>
<thead>
<tr>
<th>Digitonin</th>
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<tr>
<td>1%NP40, 1%DOC</td>
<td>Membrane associated</td>
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<tr>
<td>↓</td>
<td></td>
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<tr>
<td>Pellet</td>
<td>Nuclei</td>
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B

<table>
<thead>
<tr>
<th>siRNA</th>
<th>M</th>
<th>F</th>
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<tbody>
<tr>
<td>-</td>
<td>+</td>
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<tr>
<td>-</td>
<td>+</td>
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<table>
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1 2 3 4 5

C

<table>
<thead>
<tr>
<th>active</th>
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<td>w1 F w2 M N</td>
<td>w1 F w2 M N</td>
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Pp ctrl

Rr x 6

Figure 8
Figure Legends

Figure 1. Repressed mRNA associates with polyribosomes

(A) 293T cells were transfected with pGL3 control and pRL-TK containing 6 binding sites in its 3’UTR complimentary to CXCR4 siRNA, and with either targeting CXCR4 or control siRNA (Invitrogen). (B) Schematic illustrating the interaction between CXCR4 siRNA and target mRNA sequence. (C) Protein and total RNA analysis of cells 20h post-transfection. Luciferase assays were performed and the fold repression by siRNA calculated by normalizing Renilla to Firefly expression and dividing the ratio obtained from cells treated with control siRNA into the ratio obtained from cells treated with targeting CXCR4 siRNA. RNA analysis by RPA was performed simultaneously on total RNA prepared using Trizol and quantitated as in the luciferase assay. Error bars represent standard deviations. (D) Extracts prepared from transfected cells were separated on 15-50% sucrose gradients. The absorbance profile is shown (top panel) with polysomal fractions indicated. 11 fractions were collected and analyzed by RPA for abundance of Renilla (bottom band, indicated by bar) and Firefly (top band) mRNA simultaneously for each siRNA treatment (separate panels). Signal from each fraction was quantitated and normalized to the total intensity across all fractions for Renilla reporter and Firefly control (separate panels).

Figure 2. Ribosomes associated with repressed mRNA exhibit peptidyl transferase activity.

Cells were transfected with Renilla reporter and control or CXCR4 targeting siRNA as in Figure 1. and 20 hours later were incubated with or without 100 ug/ml puromycin for 3
minutes at 37 degrees before lysis and fractionation. The distribution of *Renilla* mRNA was detected by RPA. This experiment was repeated two times.

Figure 3. IRES-dependent translation can be repressed by short RNA.

(A) Bicistronic luciferase vectors containing the Hepatitis C Virus (HCV) and Cricket Paralysis Virus Intergenic Region (CrPV-IGR) IRESes and short RNA binding sites in the 3’ UTR of the downstream cistron were transfected into 293T cells for 4 hours and then cells were treated with either DMSO (dark gray) or 1 μM eIF4A inhibitor hipperistanol (light gray) for 10 hours. Luciferase assays were performed and the relative amount of upstream *Firefly* and downstream *Renilla* activities is shown, normalized to the activity of each cistron in the absence of drug for each IRES construct. Results are presented as the average of three experiments with standard deviations indicated. (B) HCV or CrPV-IGR plasmids were transfected with targeting 5 nM CXCR4 or control siRNA (Invitrogen) for 4 hours then administered DMSO or 1 μM hipperistanol for 10 h. Luciferase assays are normalized to the control siRNA treatment, for both DMSO and drug treatments, for each cistron. All experiments were done in triplicate and error bars represent standard deviations.

Figure 4. Repression occurs prior to synthesis of full-length polypeptide.

293T cells were co-transfected with 2xFlag PpLuc pcDNA3.1 containing 6 bulged binding sites complementary to CXCR4 siRNA and either targeting CXCR4 siRNA or control siRNA (Invitrogen). 20h post-transfection, cells were starved in Met- media for 20 min and then pulse labeled with 100 uCi S35 methionine for 3 minutes at 37 degrees. The
cells were then harvested and immunoprecipitated on anti-FLAG M2 beads. The eluates were separated on a 4-20% Tris-glycine polyacrylamide gel and visualized by autoradiography. The bar marks the migration rate of full-length polypeptide, and the faster migrating label was shown by pulse-chase experiments (data not shown) to be transient and thus probably nascent polypeptide chains.

Figure 5. Silencing by short RNAs increases termination at stop-codons.
(A) In-frame bicistronic luciferase vector with each of the three possible stop codons were transfected into 293T cells with either the targeting CXCR4 or control siRNA (Invitrogen). Luciferase assays were performed 24 hours later, and readthrough was calculated as the ratio between downstream Renilla activity and the sum of downstream Renilla plus upstream Firefly activities. Error bars represent standard deviations. (B) Cotransfection of a plasmid encoding an amber, serine suppressor tRNA enhances translational readthrough of the bicistronic construct containing an amber stop codon and dramatically reduces the microRNA-dependent reduction in translational readthrough. Error bars represent standard deviations.

Figure 6. Drop-off of ribosomes associated with repressed mRNAs
Cells were co-transfected with the Renilla luciferase vector containing 6 CXCR4 binding sites and driven by the CMV promoter and either the targeting CXCR4 siRNA or control siRNA (Invitrogen). 24 hours later, cells were either treated for 4 or 5 minutes with the eIF4A inhibitor hipperanistol (1 uM) or DMSO at 37 degrees before cell lysis and polyribosome separation. 10 fractions were collected and RNA was harvested and
subjected to Northern analysis. Quantitations are shown below comparing control (light gray), 4 minute treatment (dark grey) and 5 minute treatment (black) for active mRNAs treated with control siRNA (bottom right) or repressed mRNAs treated with targeting CXCR4 siRNA (bottom left), normalizing to the total amount of signal across all fractions.

Figure 7. Short RNAs repress translation after initiation in human cells. Short RNA complexes associated with Argonaute proteins bind with mismatches to the 3’ UTR of a target gene and act at a distance to cause drop-off of translating ribosomes at multiple sites within the open-reading frame. A very small increase in drop-off frequency at multiple sites would significantly decrease the synthesis of full-length polypeptides. Note that if the initiation rate is sufficiently high, such ribosome drop-off would not significantly decrease ribosome loading.

Figure 8. Repressed mRNAs co-fractionates with a membrane-bound compartment within the cytoplasm. (A) Transfected 293T cells were fractionated by differential detergent solubility to give a free-soluble cytoplasmic fraction (F) soluble in 200 µg/ml digitonin, a membrane-bound cytoplasmic fraction (M) soluble in 1% NP-40 and 1% DOC, and an insoluble fraction which contained nuclei (N). (B) Western blotting of GAPDH and TRAPalpha shows efficient separation of free-soluble (lane 4 and 5) and membrane-associated (lane 2 and 3) cytoplasmic compartments compared to unfractionated extracts (lane 1), regardless of siRNA treatment (lane 4 versus 5 and 2 versus 3). (C) RPA analysis shows that repressed Renilla mRNA (lower band) is lost
from the free-soluble compartment (lanes 2 and 7, lower band) but not from the
membrane-soluble compartment (lanes 4 and 9, lower band), whereas there is no change
in the distribution of control Firefly mRNA (upper band in lanes 2, 7, 4 and 9). Washes
are shown, as well as material in nuclei (lanes 1, 3, 5, 6, 8, 10).
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Kaspar, R. L., and Gehrke, L. (1994). Peripheral blood mononuclear cells stimulated with C5a or lipopolysaccharide to synthesize equivalent levels of IL-1 beta mRNA show unequal IL-1 beta protein accumulation but similar polyribosome profiles. J Immunol 153, 277-286.


Conclusions
We have developed a cell culture system in which the CXCR4 short RNA causes translational silencing of a heterologous reporter gene. This system has provided, and continues to provide, important insights into the mechanism of microRNA silencing.

We showed for the first time, in Chapter 2, that the nature of interaction between short RNA and target mRNA, not the origin of the short RNA, dictates the outcome of silencing (Doench et al. 2003). Perfect or near-perfect basepairing results in predominately RNAi cleavage of a target mRNA whereas imperfect basepairing results in translational repression, a result that has been confirmed by others (Zeng et al. 2003). Subsequent work has shown that while siRNA and microRNA pathways share protein components in the initiator phase (e.g. Dicer), other components are not shared (e.g. R2D2 versus Pasha). However, because siRNAs and microRNAs are functionally redundant in their action, we hypothesize that the effecter complexes mediating translational repression may possess a redundant protein composition.

In this regard, a significant pair of discoveries have shown that Argonaute 2, the catalytic core of RNAi, is not required for translational repression, yet any of Argonautes 2-4 are sufficient to cause translational repression (Liu et al. 2004; Pillai et al. 2004). It is not known whether all Argonautes function redundantly to cause translational repression in mammals or whether they each possess unique functions other than RNase H cleavage by Argonaute 2. Genetic studies of each Argonaute will be useful for addressing this question, as will in vitro reconstitution of microRNA-dependent translational repression.

P-body localization of mRNAs is a compelling general hypothesis which may prove to be a unifying theory of many diverse types of known post-transcriptional and
translational repression pathways. However, the evidence is not currently strong enough for P-body localization to be declared causative of silencing mediated by microRNAs (Liu et al. 2005; Pillai et al. 2005). Although a compelling notion, the studies upon which the hypothesis is based show that only a fraction of the repressed mRNA is localized to cytoplasmic foci (R. Pillai, personal communication). Such observations suggest that P-body localization is not the only process involved in the silencing, unless the remaining mRNA were associated with P-body components but not located within discrete loci (R. Parker, personal communication). Careful quantitation of the extent of association (for example by immunoprecipitation) between repressed mRNA and P body components (GW184 or Dhh1p, for example) could possibly resolve this issue. On the other hand, since it is known that a variable degree of mRNA degradation is inevitably associated with translational repression by microRNAs, this limited amount of P-body localization could be the product of cleavage products caused either directly or indirectly by translational repression. Additionally, genetic studies of translational repression by microRNAs could also resolve this issue once it is clear which P-body components are necessary for regulation.

Studies on the CXCR4 reporter experimentally discovered the seed hypothesis of microRNA/mRNA interactions, another general property of miRNAs which has been validated in other systems (Doench and Sharp 2004). Computational studies which independently derived and employed the seed hypothesis have shown that the biology regulated by microRNAs is vast. Indeed, 22% of all mammalian 3’ UTRs contain conserved microRNA binding sites, and microRNA interaction sites account for the majority of conservation in 3’ UTRs, so each microRNA potentially regulates numerous
targets (Lewis et al. 2005; Xie et al. 2005). This observation supports the
“micromanager” model in which each microRNA is responsible for fine-tuning
expression of many genes. On the other hand, genetic data would suggest that the major
function of lin-4 microRNA in C. elegans, timing the L4 to adult transition in
hypodermal cells, requires the regulation of only lin-14. This is concluded because loss-
of-function mutations in lin-14 alone can suppress the heterochronic defect caused by
loss of function mutations in lin-4 (Ambros 1989). However, lin-4 may be an exceptional
microRNA in this degree of specificity. Indeed, loss of function of the majority of
individual microRNAs in C. elegans produces no phenotype (E. Alvarez-Saavedra,
personal communication). Regardless, it will be important to provide genetic evidence in
support of the micromanager model. With growing examples of microRNAs required for
mammalian developmental processes, and better power to predict relevant targets, it will
be interesting to determine, for a given microRNA and a given process, how many targets
are necessary to confer microRNA-dependent phenotypes. Likewise, it will be very
informative to employ similar computational strategies to determine how many C.
elegans genes possess conserved lin-4 regulatory sites, and thus directly compare
computation with genetic data.

Our model system has also provided insights into the mechanism of microRNA
repression (Chapter 3). Like the lin-4 endogenous microRNA in C. elegans, the CXCR4
gineered short RNA in mammalian cells inhibits translation after initiation, pointing to
a broadly conserved mechanism for gene regulation. With the knowledge of the overall
physical model of the repression, ribosome drop-off, it will now be possible to take
directed genetic approaches to identify the genes necessary for translational repression by
microRNAs. In particular, future work should attempt to knockdown the elongation factors, termination factors, and related proteins to see whether any are required for repression. Alternatively, undirected genetic screens could be performed using knockdown libraries, preferably in *Drosophila* S2 cells because of their apparent lower redundancy in RNA interference genes (for example, they have two Argonautes versus four in mammals and one FMRP versus three in mammals). Additionally, it should be much more feasible now to develop in vitro systems which recapitulate translational repression by microRNAs, now that it is known what functions such an assay should reproduce. In addition, the relationship between translational repression and mRNA decay by microRNAs should be investigated further. In particular, genetic and biochemical methods will be useful in determining whether mRNA degradation and translational repression are dependent processes, and if so, which is the primary event caused by microRNA. In general, the stability of a mRNA may be intimately linked to its translational capacity.

Translational regulation has historically been viewed as less important than transcriptional regulation. Many systems in which translational regulation is studied are special cases in which transcriptional regulation is not possible, such as early embryo development before transcription begins, in mature erythrocytes which lack a nucleus, or during mitosis when transcription does not occur. The observation that a large number of mammalian mRNAs are potential targets of microRNAs calls into question the assumption that transcriptional regulation alone dictates most important gene regulatory events throughout the organism. Indeed, it may be the case that multiple levels of
regulation are employed in the regulation of most genes under most circumstances in order to provide the most robust maintenance and rapid control of expression.

In summary, we continue to be surprised at the depth and breadth of biology controlled by molecules containing only 21nt of RNA. Further analysis of gene regulation by short RNAs and the implementation of short RNA based silencing as a research tool and a potential therapeutic will certainly direct our increasing understanding of developmental disorders, cancer and infectious diseases, and enhance our ability to reduce suffering caused by those ailments.
References


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EDUCATION

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Research Assistant, Max Planck Institute for Polymer Research, Germany, 1998
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* denotes co-first authorship
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Poster: "Solvation of Sodium Chloride: An Effective Fragment Study of NaCl(H2O)n." American Chemical Society national meeting in Dallas, TX March 1998.