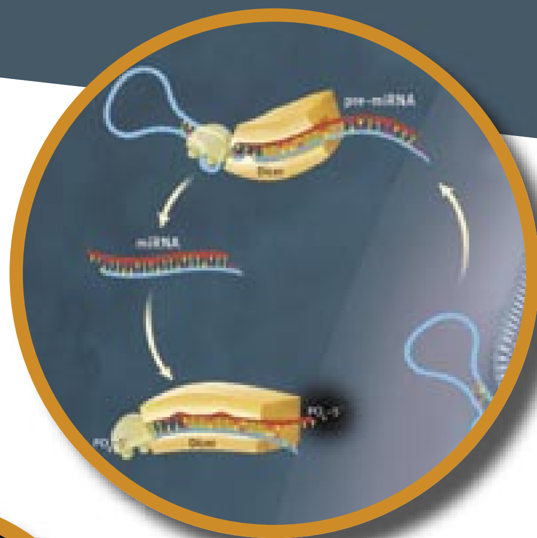
RNAi:
microRNA packs a wallop

MicroRNAs play a hitherto unsuspected, integral role in regulating gene expression. But their short length, sequence similarities, multiplicity of mRNA targets, and unpredictable pairing to those targets make studying them tricky. The good news: Methods for analyzing more familiar RNA species have been adapted for investigation of these extraordinary molecules and improved technologies are constantly becoming available. **By Bruce Goldman**



Wait! Stop. Don't toss out that small-RNA fraction just yet. It took researchers a while to break that habit. Tiny pieces of RNA were long thought to be mere degradation fragments of more important longer molecules.

But it's now obvious that the famous formulation enshrined as the Central Dogma of Molecular Biology – "DNA makes RNA makes proteins" – is only partly true. Small noncoding RNAs called microRNAs (miRNAs), typically about 21 or 22 nucleotides in length, exert a powerful posttranscriptional regulatory influence. By binding to untranslated regions of messenger RNAs (mRNAs), miRNAs stall ribosomal output of the proteins encoded by the target mRNA, fine-tuning translation and, on occasion, marking the target mRNA for degradation. Gregory Hannon at **Cold Spring Harbor Laboratory** and his colleagues recently reported findings implicating miRNA in DNA methylation, too.

From being regarded as an oddity, miRNA has proceeded to the cutting edge of biomedical research. After a sluggish start following isolation of the first miRNA, from *C. elegans* in 1993, the number of human miRNAs logged on the Sanger Institute's miRBase Sequences Database (a central repository of validated miRNAs in the public domain) has exploded in the past few years, and has now reached almost 500.

"Smart scientists that we are, we managed to miss this entire class of incredibly important regulatory molecules until the 21st century," says Eric Lader, associate director of research and development at **Qiagen**, a Germany-based supplier of nucleic acid sample-preparation materials and polymerase chain reaction (PCR) kits.

But researchers are making up for lost time. While only four papers on miRNA were published as recently as 2001, last year the number exceeded 600. "The likely impact of miRNAs in mammalian biology is enormous," says Carl Novina, of the **Dana-Farber Cancer Institute**. "They play roles in differentiation and development, chromosomal segregation and division, cell type-specific functioning, metabolism, and apoptosis." Alterations of miRNA expression, Novina continues, have been correlated with numerous cancers and, at least in mice, with diabetes, spurring the investigation of miRNA as a potential diagnostic and therapeutic tool.

One reason for the field's accelerating advance: The tools were already mostly in place. "Over the last 20 years, an extraordinarily large number of really smart molecular biologists worked out every technique you could ever desire to study small RNAs while somehow scrupulously avoiding discovering too much about them, so that people of my generation would have something to do," marvels Philip Zamore, professor of biomedical sciences and biochemistry at the **University of Massachusetts School of Medicine** in Worcester.

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"We managed to miss this entire class of incredibly important regulatory molecules until the 21st century."

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Those tools did require some modification. Early RNA methodologies weren't optimized for ultrashort species such as miRNA, which got tossed out with the buffer solution. Nowadays, companies selling sample-preparation tools, such as Qiagen and **Ambion** (acquired last year by **Applied Biosystems**), offer kits adapted for isolating small RNAs.

Once you've found a given miRNA, figuring out which mRNAs it targets is nontrivial, at least in animals, as only six or seven of the 21 or 22 nucleotides in an miRNA sequence (the seed region) bind to their mRNA targets in a strict antisense fashion. This "fuzzy pairing," with the seed sequence dictating much, but not all, of binding strength, makes it hard to predict mRNA targets computationally—all the more so with the recent discovery that miRNA transcripts get "edited": Enzymatic nucleotide substitutions differentiate them from their DNA sense-strand progenitors.

Finding New miRNAs

The sequencing of the human genome and partial elucidation of miRNAs' structural features made it possible to do computational searches for nucleotide stretches that could fold into characteristic hairpin structures and are conserved across species lines. Unfortunately, the vast majority of computational hits were false positives.

Rosetta Genomics, an Israeli company that presciently began life with an miRNA focus in 2000, created a sophisticated algorithm that located some 11 million hairpin structures, eliminated most of them (those that are thermodynamically unstable, structurally suspect, or located on protein-coding sequences or in repetitive elements), and then filed patents on thousands of candidate miRNAs. Ronit Aharonov, executive vice president for intellectual property and computational biology, says Rosetta has internally validated many miRNAs not yet listed on the Sanger database. In a highly cited 2005 article, Rosetta Genomics scientists claimed that a significant number of these novel miRNAs are conserved among primates but not other mammals, and may therefore have played a major evolutionary role in the higher complexity of primates, including humans.

Massively parallel oligonucleotide sequencing now appears to be outstripping computational approaches for discovering novel miRNAs and is even unveiling entirely new realms of small RNAs. In the past year alone, groups led by David Bartel of **Whitehead Institute at MIT** and by Hannon at Cold Spring Harbor have used this methodology to discover two small RNA classes, each consisting of tens of thousands of members, whose defining features differ considerably from those of miRNA and from one another's.

454 Life Sciences, previously a Curagen subsidiary, was selling its sequencing instrument along with reagents via a marketing agreement with **Roche Diagnostics**, who has now purchased the company outright. Each sequencing run yields between 200,000 and 400,000 readouts of 100 to 250 nucleotides. A newer machine by **Solexa** (now owned by **Illumina**) can deliver up to 20 million short readouts of about 27 nucleotides. Although Solexa's device may truncate slightly longer small-RNA sequences—some species run 30 bases—Hannon, who has used both companies' instruments, says it usually provides enough information to identify a novel miRNA.

While the huge number of sequence readouts per run makes the per sequence cost ultracheap, a single run is still quite expensive. "You don't need a computer to figure out 99.9 percent of all the miRNAs in a human being," comments Zamore. "You just need a big budget."

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There is a feeling among those in the field that miRNA discovery, per se, is hitting the point of vanishing returns. "The numbers of new miRNAs we're detecting are diminishing," confirms Novina.

What's Different?

Meanwhile, expression profiling of miRNA molecules, once they're found, is a growth area. Microarray techniques for profiling RNA expression, which were quite advanced by the time the study of miRNA began, merely had to be adapted for use with smaller species. Planar arrays are ideal for comparing small numbers of samples for patterns of over- or underexpression of large numbers of miRNAs.

Ambion was one of the first companies to focus on the miRNA tools market. This company and its diagnostics-and-services spinoff, **Asuragen**, have licensed Rosetta Genomics' candidate miRNAs for their microarrays, which feature capture probes representing 13,349 sequences: several hundred of them from the public domain but the vast majority from Rosetta. Scott Hunicke-Smith, vice president and general manager of Asuragen's service group, says, "You can update a database every month. But you can't update content on an array cost-effectively every month. We think most of the near-term future releases of the Sanger database will probably already be represented by these sequences." Based on its own preliminary initial validation studies (looking for expression in a small number of normal human tissues), Asuragen has already identified three to five times the number of miRNAs that show up in the Sanger database. Hunicke-Smith thinks this multiple could go to as high as 10 when more tissues are examined.

Invitrogen is another big player in the miRNA-microarray space. Invitrogen's microarray methodology makes it easy to detect differences in miRNA expression between two samples—for example tumor versus normal tissue—whose respective miRNA fractions fluoresce simultaneously at different frequencies, thus allowing comparison of samples processed under identical conditions. Invitrogen sells the microarray itself, the labeling kit, and a real-time PCR kit for validating array data, says Invitrogen's research and development lead, Christopher Adams. In May 2006 Invitrogen launched a kit for specifically amplifying small RNAs. "A lot of people in the stem cell field don't have a lot of cellular material available—in some cases as few as 100 cells," Adams notes. "Evaluating miRNA expression in those circumstances can be a real challenge." The ultimate goal, he says, is to profile a single cell without any amplification or other manipulation.

For some academic laboratories, an alternative to buying arrays is to buy the probes and have them spotted onto arrays at their institutions' own facilities. One leading probe supplier is **Exiqon**, a Danish company that manufactures "locked nucleic acids" (LNAs). Unlike RNA, whose ribose backbone is quite flexible, continued ›

Exiqon's synthetic oligonucleotide is chemically constrained, locking the probe's bases into a hybridization-friendly conformation. Whereas miRNAs' short size makes optimizing probe-melting temperatures tricky, individual LNAs' compositions can be modulated to give them very similar melting temperatures.

When a researcher doesn't need to look at the entire universe of known miRNAs, but rather wants to select a set of them for, say, exploring a diagnostic application, **Luminex** offers a new bead-based technology that can multiplex up to a thousand samples—less costly per run than microarrays—testing each against probes representing 320 miRNAs from the Sanger database. "The number of miRNAs that will turn out to be relevant for a particular condition may be 20, or 10, or five," says Keld Sorensen, director of research and development. "We expect scientists profiling miRNA for a particular medical condition to want to run large numbers of samples. If you have a thousand samples, you're going to want a multiplex system."

Unlike sequencing methodologies, hybridization techniques such as Luminex's confront the dual challenge posed by miRNAs' short sequences and the fact that they often cluster in families whose members may differ by as little as a single nucleotide. That doesn't leave much room for discrimination. To tell them apart, Luminex turned to Exiqon's capture probes, Sorensen says.

Time and Place

An miRNA's role in development practically guarantees that it will often exhibit transient or highly tissue-specific expression. Chemistries that increase the stringency of binding, as LNAs do, enhance in situ analysis, performed to locate cells or tissues where a particular miRNA is being expressed at a particular time in the developmental cycle.

If locating miRNAs is getting easier, finding out what they do there is still difficult. Attempts to learn about miRNA function usually boil down to painstaking, labor-intensive experimentation, says Bartel of Whitehead Institute. "You can use antisense reagents that will inhibit the miRNA," he says. "But because each miRNA has hundreds of targets, you'll still be at a bit of a loss as to what's the real mechanism for any effect you see." Also, while miRNA function can be transiently knocked down with standard or modified antisense or RNAi approaches, cell division dilutes such agents. Furthermore, different cell types vary in their susceptibility to transfection with these substances. "There's no good, universal, or conventional way of transfecting multiple, different cell lines," says Robert Setterquist, senior manager of research and development at Ambion. "Each of them has to be optimized. And once you get that worked out, you still may not be able to get good miRNA inhibition."

How Do miRNAs Work?

As the number of relevant miRNAs in humans approaches saturation, and as ever more of them are subjected to expression profiling and characterization within particular tissues or during various developmental stages and disease states, attention is turning to the thorny matter of target identification.

Typical computational approaches grossly overpredict the number of targets, as a random search of seven-nucleotide sequences will yield more than 500 hits, says Deepak Srivastava, director of the **Gladstone Institute of Cardiovascular Disease** in San Francisco. Srivastava hopes to soon make public a more sophisticated computational algorithm. "Only around 20 or so miRNA targets have been validated at the level of protein regulation. Virtually all of them have something in common: The target site in the mRNA is located in a stretch of RNA that is physically accessible to the miRNA." Srivastava's algorithm, in addition to searching the genome for spots amenable to binding to miRNA seed regions, also screens out DNA stretches whose transcripts would be likely to form secondary RNA structures rendering them inaccessible to a corresponding miRNA. Srivastava says this appears to significantly increase specificity, although perhaps at the expense of sensitivity.

Says Bartel, "Learning the biological function of particular miRNA/ target interactions is certainly possible. But it's a lot of work. I don't see any way around it."

Complicating things, there can be several miRNA-binding sites on a single mRNA transcript. In a study published in early 2007, Bartel's group picked a target mRNA with not one but seven predicted binding sites for miRNA, all of which sat on a section of the molecule whose deletion, via a translocation in the corresponding gene, was known to be associated with oncogenesis in humans. To see if the loss of translational regulation by miRNA was indeed responsible, Bartel and his colleagues created point mutations in each of the mRNA's seven miRNA-target sites – and observed the same tumor phenotype. "You get the same results as if you had that translocation," he says. "That tells us that at least part of what's going on in those human tumors is the loss of suppression of that gene."

Bartel, who has long been out in front of the pack, says he's accustomed to inventing his own techniques, but "companies are making it a lot easier for people who haven't been working on miRNAs for the last six years."

A strong motive in any business, naturally, is to make money. But there are other incentives in play, not least intellectual ones. "When you find out some miRNA is 100-fold downregulated in an early cancer cell," says Lader of Qiagen, "and you know one miRNA could affect the expression of 100 genes, the allure of figuring out what's going on is powerful."

Bruce Goldman, a freelance science writer, lives in San Francisco.

Featured Participants

454 Life Sciences
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Ambion
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Applied Biosystems
www.appliedbiosystems.com

Asuragen
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Cold Spring Harbor Laboratory
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Exiqon
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