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RNAs Regulate Biology

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We both entered the RNA field in the late 1980s, when catalytic RNAs and *in vitro* selection approaches were an exciting new frontier. The first scientific meeting one of us (J.A.D.) attended was the 52nd Cold Spring Harbor Symposium (CSHS) on the Evolution of Catalysts in the spring of 1987. It was a spectacular experience: attendees at the meeting were abuzz with the excitement and novelty of ribozymes and the mechanisms of chemical reactions critical for life. Ample time to mingle with colleagues fostered many conversations about the details and unanticipated connections among the systems we were each studying.

Almost 20 years later, the world of functional RNAs is no less exciting. This year's 71st CSHS on Regulatory RNAs, held May 31–June 5, 2006, in Cold Spring Harbor, NY, discussed novel aspects of RNA biology that we are only beginning to understand. Focused largely, though not exclusively, on RNA interference (RNAi), the meeting brought together a diverse group of scientists broadly interested in understanding how, where, when, and why RNA molecules have evolved to regulate gene expression in a wide variety of ways in cells and viruses. Although the pathways and molecular players involved in RNA-mediated gene regulation are being elucidated at a rapid pace, the chemical and mechanistic underpinnings remain to be worked out. The underlying molecular mechanisms, and the possibilities for tapping these processes for therapeutic purposes, fall squarely into the realm of chemical biology.

Rather than attempt to summarize every topic covered at the meeting (a nearly impossible task!), this review focuses on several of the key themes that emerged from the many presentations and informal discussions that occurred during the course of the symposium.

A central topic was the discovery of new noncoding RNAs (ncRNAs). What's out there? The resounding answer: a lot! ncRNAs are abundant in all three kingdoms of life, as revealed by a myriad of approaches, including direct cloning and sequencing of cellular RNA, computational prediction, and microarray analysis. Several research groups are using sequencing methodology, referred to as 454 (www.454.com), for the "deep sequencing" of many thousands of small RNAs (sRNAs). This technique has enabled the rapid compilation of large numbers of sequences that can be sorted by size, sequence, evolutionary conservation, and location within a genome.

As a result, new classes of small ncRNAs are rapidly being identified, although the functions for these molecules remain mysterious. What is clear so far is that size matters. In plants, for example, David Baulcombe described how four distinct variants of the RNA-cleaving enzyme Dicer each produce double-stranded RNA (dsRNA) products (micro- or small interfering RNAs) of a characteristic length that confer on them the ability to enter particular gene-regulating pathways. Steve Jacobsen and Richard Jorgensen presented genetic studies that similarly emphasized the diversity of RNAi-mediated silencing pathways

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that appear to function in different plants. Greg Hannon (*1*) presented a large family of ~30-mer RNAs, found exclusively in murine testes, that have been dubbed piRNAs because of their propensity to bind Piwi-domain-containing proteins in these germ cells. These 30-mers, which usually have a 5′ uridine residue, are typically encoded in intergenic regions clustered in the genome and are not conserved at the sequence level in other organisms. Phil Zamore presented data suggesting that a similar class of sRNAs, the rasiRNAs (repeat-associated silencing RNAs), might be generated through a previously undescribed pathway. In the nematode *Caenorhabditis elegans*, David Bartel has found that almost one-tenth of the sequences identified so far by the 454 method corresponds to so-called 21U-RNAs. These are 21-mer RNAs that always contain a 5′ uridine residue and are derived from thousands of loci in two broad regions of chromosome IV, dispersed between protein-coding genes and within their introns. No precise function for piRNAs or 21U-RNAs has yet been determined.

ncRNAs are not always small. RNAs produced by RNA polymerase II, the same enzyme that transcribes precursor messenger RNAs (mRNAs) in the nucleus, are sometimes retained in the nucleus rather than exported to sites of protein synthesis in the cytoplasm. Two fascinating examples discussed by David Spector include the 9-kb CTN-RNA and another 7-kb RNA, found in murine and human cells. The CTN-RNA appears to be a long version of an mRNA encoding an amino acid transporter, raising the possibility that CTN-RNA is a storage form of the message that is on hand for rapid processing and export in the event of amino acid starvation. The 7-kb-long RNA is also found in the nucleus, in this case in neurons at sites of preliminary mRNA processing, and its abundance intriguingly correlates with the numbers of neural synapses formed in cell culture. Other intriguing connections among RNA editing, transport, and RNAi were touched upon in talks by Brenda Bass and Gordon Carmichael, highlighting the extensive interplay among these seemingly distinct processes. Tom Gingeras and Mike Snyder described results from the ENCODE (Encyclopedia of DNA Elements) project aimed at characterizing all the transcripts produced from 1% of the human genome. Findings from their labs and others suggest that much more of the genome is transcribed than previously known, at least at a low level, and more than half of all coding genes have very distal, alternative, tissue-specific transcription start sites. Nick Proudfoot presented evidence that aberrant, intergenic, and genic globin locus transcripts are subjected to RNAi mechanisms, whereas regular globin transcripts are made from genes arranged in transcription-dependent loop structures.

ncRNAs are not unique to eukaryotes: bacteria also contain small regulatory RNAs (so far at least 80 have been identified in *Escherichia coli*), and use structured RNAs called riboswitches (as described by Wade Winkler, Tina Henkin, and Eduardo Groisman) to control gene expression in response to a wide array of small molecules, including magnesium ions (*2*). Gigi Storz shared new data indicating that small bacterial RNAs can mediate their effects by binding to the 3′ untranslated region of the gene, reminiscent of many examples of translational control in eukaryotes (*3*). Molecular structures of riboswitches are appearing at a rapid clip, including recent crystal structures of the guanine (*4*) and *S*-adenosylmethionine-binding riboswitches (*5*) presented by Robert Batey and a structure of the *E. coli* thiamine pyrophosphate-binding riboswitch presented on a poster from Dinshaw Patel's lab (*6*). The remarkable T-box RNA described by Tina Henkin recognizes specific transfer RNAs (tRNAs) and changes structure depending on whether the tRNA is charged with its cognate amino acids; thus, expression of bacterial genes in response to cells' nutritional status is controlled (*7*). One wonders whether such relatively small RNAs, linked together, could function as a primitive ribosome if supplied with an mRNA template, food for thought about the evolutionary origins of RNA-catalyzed protein biosynthesis.

Many of the identified sRNAs whose functions are known are microRNAs (miRNAs), and hence, numerous labs have used genetic and biochemical approaches to set about finding their molecular partners in different organisms. Scientists from the Ruvkun and Carthew labs described negative regulators of RNAi in nematodes and fruit flies; that cells might control RNAi in response to environmental stimuli was suggested. Craig Hunter presented evidence for a transmembrane transporter of dsRNA required for the spreading of RNA silencing between cells in nematodes (*8*, *9*).

Crystallographic structure determinations of RNAi pathway components, presented by the Barford (*10*), Joshua-Tor (*11*), and Doudna (*12*) and Patel labs (*13*, *14*), show how siRNAs are likely to bind within complexes containing Dicer and the mRNA target-cleaving endonuclease Argonaute 2. These structures are guiding biochemical investigation of the molecular mechanisms that underlie the individual steps in RNAi.

How miRNAs work *in vivo* remains a subject of much discussion and debate. Although evidence in some experimental

systems and from computational approaches suggests that miRNAs can fine-tune gene regulation, data from the Hobert lab show that a miRNA in the nematode nervous system is a clear switch that determines left- versus right-brain functional asymmetry (*15*, *16*). These studies are consistent with early genetic observations in the field and suggest the possibility that such on–off control of gene expression by miRNAs will be more generally observed. Although miRNA tissue localization can be exquisitely specific, as shown in the zebrafish by Ron Plasterk, just a quarter of the miRNAs for which chemically stable antisense oligonucleotides were used to block function had a discernible phenotype. That said, Frank Slack shared compelling evidence for the involvement of the let-7 miRNA family in regulating the expression of important oncogenes implicated in lung cancer. Alex Schier showed an example of another way to affect gene expression, whereby a miRNA promotes deadenylation of maternal mRNAs during early embryogenesis (*17*, *18*). In a related story, Jim Dahlberg and Elsebet Lund reported that miRNA biogenesis is controlled during *Xenopus laevis* egg maturation and that miRNA-dependent deadenylation can occur in the absence of translation.

One particularly vexing question is how miRNAs are able to down-regulate protein synthesis without affecting mRNA levels, a process called translational repression. Several speakers, including Witold Filipowicz, Phil Sharp, and Tim Nilsen, presented biochemical and cell biological studies that attempted to decipher the mechanism(s) of gene regulation by miRNAs (*19*). And, although little consensus exists about what stage of translation (or some other process) is being controlled by miRNA interactions, all would agree that determining where the components are localized would provide important insight and that processing bodies (P bodies) and stress granules figure prominently in these discussions. An interesting talk by Elisa Izaurralde brought some of these points home. She showed that multiple pathways exist for regulation and that simply knowing that a gene is controlled by a miRNA does not reveal how it is controlled. Peter Sarnow's talk about a hepatic miRNA established that miRNAs can function as activators rather than as repressors (*20*). The take-home message is to keep an open mind in examining every new case: the rules are far from established.

Discussed at the meeting was transcriptional silencing, another broad topic that incorporates both small and large RNAs and their cellular effects. Danesh Moazed and Shiv Grewal presented complementary stories on the idea of a self-reinforcing loop of processes responsible for sRNA-mediated gene silencing: transcription makes RNAs, RNA-dependent RNA polymerase amplifies them, Dicer processes, Argonautes bind, and assembled RNA-induced transcriptional silencing complexes act in *cis* at the locus to degrade nascent transcripts (*21*–*24*). Although initially surprising, transcriptional silencing depends on transcription of the locus. Edith Heard spoke about how specific subnuclear zones appear to be established for gene silencing by Xist RNA, and Jeannie Lee discussed how direct physical pairing between the X chromosomes is important for establishing chromosome inactivation (*25*). As we have learned for miRNA-mediated gene regulation, localization is a big factor in explaining how these processes take place. As biochemists, we should note that biology depends on localization; although we often may be able to mimic such effects *in vitro* with high concentrations, we may not always be successful.

With so much of the meeting focused on RNA, it was refreshing to hear several presentations about RNPs, ribonucleoprotein complexes that facilitate the functional association of RNAs with their *in vivo* protein partners. In a session focused on telomerase and cancer, Liz Blackburn presented her lab's recent discovery of "t-stumps", very short telomeres found on the ends of chromosomes in certain cancerous human cells. Data from the Cech lab suggest that yeast telomerase RNA, which includes the template sequence for extending the telomeric stretches at the ends of the chromosomes, is a flexible scaffold for assembling telomerase proteins whose copy number is important for proper telomere maintenance *in vivo*. Carol Greider presented a genetic story indicating that half the amount of telomerase RNA over multiple generations results in a heritable phenotype associated with shortened telomeres (*26*, *27*). An exciting talk on alternative splicing from Bob Darnell provided new insight into how RNP complexes regulate which parts of a pre-mRNA are stitched together to produce tissue-specific messages. Gideon Dreyfuss's dissection of the SMN (survival of motor neurons) complex is beginning to reveal some of the biochemical rules for splicing complex assembly (*28*, *29*).

After a dizzying week thinking about mechanisms in a seemingly overwhelming sea of connections in RNA biology, we will all remember the striking stories that we heard that bring home just how central these processes are to understanding life. Surely one of the most memorable talks about biology was by Michel Georges, who studies why Texel sheep are "exceptionally meaty"; the answer appears to be that these sheep carry a mutation in the untranslated region of the myostatin gene that creates a binding site for an already expressed miRNA (*30*). We are only beginning to appreciate how much of known phenotypic variation can be explained by these novel classes of regulators, the sRNAs.

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