Meeting report

Diverse roles for RNA in gene regulation

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A report of the Keystone Symposium ‘Diverse roles for RNA in gene regulation’, Breckenridge, USA, 8-15 January 2005.

The recent Keystone Symposium on the role of RNA in gene regulation brought together a diverse and highly interactive group of biochemists, geneticists, crystallographers, and bioinformaticians to discuss the next frontier - how RNA regulates gene output at the transcriptional, translational, and mRNA-stability level. Many new developments concerned the biochemical mechanism of RNA interference (RNAi) and the endogenous biological processes regulated by microRNAs (miRNAs) and small interfering RNAs (siRNAs). There was, however, also a strong appreciation of previously unannotated RNA genes lurking in the genome and other novel mechanisms by which RNA regulates gene expression. Participants also heard about the fruition of efforts to exploit RNAi for genome-wide functional studies and progress in the use of the technology to develop human therapeutics.

MicroRNAs come of age

The most hotly pursued class of non-coding RNA (ncRNA) in recent years has been the miRNAs. Although research still continues on miRNA discovery, the field has largely made the transition to studying the endogenous biological functions of miRNAs. In particular, speakers discussed computational or experimental approaches to identifying the specific targets of miRNA regulation, as well as genetic approaches to identifying mutant phenotypes associated with altered miRNA activity.

David Bartel (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, USA) presented TargetScanS, his group’s animal miRNA-target-finding algorithm. It predicts potential targets of miRNAs by searching for 3′ untranslated region (UTR) sequences with highly and specifically conserved Watson-Crick matches to positions 2-7 or 2-8 (the ‘seed’) of the query miRNA; increased confidence is assigned to target sites with an adenosine at position 1 of the miRNA interaction site. Bartel predicted that at least one third of the genes in the human genome are miRNA targets. Lee Lim (Rosetta Inpharmatics, Seattle, USA) challenged the dogma that the main function of animal miRNAs is to inhibit productive translation without causing mRNA degradation. Lim found that miR-1 and miR-124 in humans can indeed downregulate the transcript levels of approximately 100-200 genes via 3′ UTR seed matches. He speculated that *mir-1* and *mir-124*, which are restricted to muscle/heart and the nervous system, respectively, may have rather global effects on gene expression that help maintain the identity of these tissues.

Although many animal miRNA targets are regulated by pairing of the miRNA to the 3′ UTR of the target mRNA, Tom Tuschl (Rockefeller University, New York, USA) showed that several virus-encoded miRNAs are completely antisense to viral mRNAs and are likely to be targeting them for degradation. But miRNAs from any individual virus exhibit little sequence similarity to miRNAs from animals, plants, or other viruses, leading Tuschl to postulate that viral miRNAs may have independently evolved multiple times.

Jim Carrington (Oregon State University, Corvallis, USA) added another twist to the story by describing an unexpectedly complex regulatory pathway involving small RNAs in plants. Certain plant miRNAs guide the cleavage of particular ncRNAs, thereby defining new 5′ ends to these transcripts. The miRNA-defined 5′ end sets the frame for processive cleavage of the (presumably double-stranded) transcript at 21-nucleotide intervals by the RNase Dicer, which generates multiple siRNA molecules. At least some of these function as *trans*-acting siRNAs that are complementary to, and mediate cleavage of, mRNA targets different from those of the original miRNAs.

The first miRNA discovered, *lin-4* in *Caenorhabditis elegans*, was found by virtue of the severe cell-lineage defect in mutant nematodes. Victor Ambros (Dartmouth College, Hanover, USA), whose lab discovered the *lin-4* RNA, continued the discussion of the biological roles of miRNAs by describing several worm and fly miRNA knockouts. He suggested that many animal miRNAs may work redundantly in genetic circuits, as only the triple knockout of the *let-7*-related miRNAs *mir-48*, *mir-84* and *mir-241* revealed a heterochronic cell-lineage phenotype (in which the normal timing of developmental events is perturbed). Ambros also described how expression of *mir-1* in mesodermal derivatives has been conserved between invertebrates and vertebrates, and that *Drosophila* lacking *mir-1* display incredible defects in muscle morphogenesis, such as alterations in myoblast fusion and distortions in muscle morphology. Ronald Plasterk (Hubrecht Laboratory, Utrecht, The Netherlands) described the use of locked nucleic acid (LNA) probes to reveal beautiful and diverse expression patterns of zebrafish miRNAs in developing tissues and differentiated organs. This technique has great potential to guide biological studies of miRNA function by indicating tissue-specific sites of regulation by individual miRNAs.

The genetics and mechanisms of RNAi

In the principal mechanism of gene silencing by RNAi, the RNA-induced silencing complex (RISC) cleaves target mRNAs that are complementary to the guide siRNA or miRNA within RISC. Studies examining this key event in greater detail were presented at the meeting, and the biogenesis of siRNAs/miRNAs and the steps that lead to the formation of RISC were also topics of great interest. New approaches to identifying other factors with roles in the RNAi pathway were also unveiled.

Although it has been known for some time that Dicer generates siRNAs and miRNAs, the lingering question of what enzyme cleaves the mRNAs complementary to siRNAs and miRNAs was answered only recently. Greg Hannon (Cold Spring Harbor Laboratory, New York, USA) described studies showing that recombinant human Argonaute 2 (hAgo2) is sufficient to cleave substrate RNA. Tuschl, John Rossi (Beckman Research Institute of the City of Hope, Duarte, USA), and Elisa Maniataki (University of Pennsylvania, Philadelphia, USA) described many other factors that co-purify with human RISC activity, including Dicer and mammalian homologs of R2D2 and Armitage, proteins known to be involved in RISC maturation in flies. The roles of the novel proteins in RISC maturation and function are under investigation.

Hannon and Tuschl also reported that the 5′ phosphate of siRNAs is necessary for efficient siRNA incorporation into native RISC; but this requirement can be bypassed when highly purified hAgo2 is assayed instead. Hannon's enzymatic data suggest that the 5′ phosphate may actually be the critical determinant of the specificity of substrate cleavage, which occurs 10 base pairs from the 5′ end of the siRNA. Leemor Joshua-Tor (Cold Spring Harbor Laboratory) presented a structural tour of an archaeal Argonaute, where she modeled the binding of an siRNA's 5′ phosphate by the Paz domain and the expected location of catalytic residues in the Argonaute Piwi domain near the tenth base pair of the bound siRNA.

The story of how RISC receives siRNAs continues to unfold, with Phil Zamore (University of Massachusetts, Worcester, USA) proposing that the asymmetric assembly of Dicer and R2D2 on the siRNA duplex may be the initiation step for asymmetric incorporation of single-stranded siRNAs into RISC. He also described a more complicated assembly mechanism whereby *Drosophila* Ago2 cleaves the passenger siRNA strand (the strand not incorporated into RISC) before or during the unwinding of the siRNA duplex. Adding to this discussion, Rossi noted that the minimal Dicer substrates of 27-nucleotide dsRNAs are more powerful drivers of siRNA-RISC formation than the standard 21-nucleotide siRNAs, suggesting that Dicer activity is directly coupled to RISC assembly. The hunt is now on for an 'unwindase' enzyme that separates the siRNA duplex into the single-stranded siRNA incorporated into RISC.

Studies of RISC assembly were complemented by the genetic screens from Richard Carthew's lab (Northwestern University, Evanston, USA), which had previously revealed the functional partition of Dicer-1 and Dicer-2 in flies. Carthew described new mutants that are deficient ('RISC-free') or display enhanced ('high-RISC') RNAi activity, and discussed how these are being used to define biochemical intermediates of RISC assembly. In the quest to find more players in the pathway, Gary Ruvkun (Harvard Medical School, Boston, USA) and Craig Mello (University of Massachusetts) reported the use of genome-wide RNAi and proteomics, respectively, to identify novel factors involved in RNAi in *C. elegans*. These include groups of Dicer-interacting proteins and chromatin-regulating genes. Mello also touched upon a possible genetic link between heritable forms of gene silencing triggered in the germline by RNAi and the germ-cell-segregating P-granule bodies in worms because mutations that disrupt P-granule formation also appears to impair RNAi and compromise siRNA accumulation.

Regulating chromatin by RNA

While RNAi is traditionally thought of as a cytoplasmic phenomenon, small RNAs in protozoans, fungi, and plants also function in the nucleus to induce modifications to histones or DNA. This results in the packaging of DNA into transcriptionally silent heterochromatin in plants and fungi, and in the phenomenon of DNA elimination in protozoans. These RNA-based mechanisms are collectively

referred to as transcriptional gene silencing. Understanding how RNA feeds back to chromatin regulation may be integral to the question of how cells can distinguish euchromatin from silenced heterochromatin, which includes centromeres and transposable elements.

Shiv Grewal (National Cancer Institute, NIH, Bethesda, USA) reviewed his groundbreaking work on the proteins associated with the RNAi-based heterochromatic silencing of the mating type (MAT) locus in the fission yeast *Schizosaccharomyces pombe*, which contains centromeric repeats. He proposed that the RNAi machinery acts *in cis* on the MAT locus to induce transcriptional gene silencing and discussed his recently published model of a complex, self-reinforcing feedback pathway of heterochromatin regulation. Mohamed Motamedi (Harvard Medical School) described the biochemical dissection of the RNA-induced transcriptional silencing (RITS) complex in *S. pombe*. Using a novel RNA-chromatin immunoprecipitation technique, he concluded that RITS can bind to the 'aberrant' RNAs that are transcribed from heterochromatic loci. Robin Allshire (University of Edinburgh, UK) proposed that *S. pombe* RNA polymerase II associates with the RNAi machinery and is required for transcriptional gene silencing, providing an additional layer of control to heterochromatin regulation.

Progress reports on dissecting transcriptional gene silencing in multicellular organisms began with the proposal from Rob Martienssen (Cold Spring Harbor Laboratory) that repeat elements in *Arabidopsis*, like centromeric repeats, may need to be in tandem configuration in order to perpetuate the self-reinforcing action of RNAi on heterochromatin. Interestingly, Alan Herr (Sainsbury Laboratory, John Innes Centre, Norwich, UK) described work showing that silenced repeats and transposons in plants are specifically transcribed by a novel RNA polymerase - possibly the recently discovered polymerase IV. His results suggest that plants employ a special polymerase distinct from the canonical RNA polymerases I, II, and III for generating the type of aberrant RNAs that ultimately feed into the chromatin-regulation pathways that silence such loci. RNA polymerase IV seems to be restricted to plants, but several other speakers described intensive efforts to investigate the intersection of RNAi and chromatin regulation in animals. For example, David Looney (University of California at San Diego, USA) and Kazunari Taira (University of Tokyo, Japan) suggested that promoter-specific siRNAs can stimulate the formation of silenced chromatin in human cells.

In addition to diminutive RNAs, large ncRNAs also regulate transcription and gene expression in animals. For example, the *roX1* and *roX2* RNAs are key components of a ribonucleoprotein complex that upregulates gene expression on the single X chromosome in male *Drosophila* to achieve gene-dosage compensation. Mitzi Kuroda (Harvard Medical School) reported that in *Drosophila* carrying both a mutation of a nucleosome-remodeling protein and deletions of the *roX* genes, a synthetic phenotype of chromosome defects is now apparent. In contrast, there are no chromosome defects when only the *roX* genes are deleted. This suggests that there is an interplay between the *roX* RNAs and ATP-dependent chromatin-remodeling machines. Mammalian dosage compensation is controlled by the large ncRNA *Xist*, whose expression is itself antagonized by transcription of the overlapping antisense ncRNA gene *Tsix*. Takashi Sado (National Institute of Genetics, Shizuoka, Japan) and Claire Rougelle (Institut Pasteur, Paris, France) reported the correlation of *Tsix* expression with increased methylation of histone H3 at the *Xist* promoter, which probably suppresses *Xist* transcription and hints at unsuspected links between these ncRNAs and chromatin-modification processes. Renato Paro (University of Heidelberg, Germany) illuminated the role of transcribed ncRNAs in controlling the regulation of Polycomb/Trithorax-regulated enhancers in *Drosophila*. In genes controlled by these enhancers, transcription of the enhancer mediates an epigenetic switch from a Polycomb-bound, repressed state to a Trithorax-bound, transcriptionally active state.

Other classes of regulatory RNAs and their functions

Of course, eukaryotes are not the only organisms that possess ncRNAs; bacteria seem to have ncRNAs that are just as rich in regulatory potential. Carin Vanderpool (National Cancer Institute, NIH, Bethesda, USA) and Aixia Zhang (National Institute of Child Health and Human Development, NIH, Bethesda, USA) reported that several of the more than 60 small regulatory RNAs in bacteria base-pair with mRNAs to influence their transcription, stability, or translational efficiency. Most of these targets are involved in metabolic or stress pathways, and regulation by small RNAs may allow rapid responses to a changing environment. Some regulatory functions are actually embedded within mRNAs themselves. Ron Breaker (Yale University, New Haven, USA) reviewed his lab's ongoing discovery of riboswitches, portions of mRNA 5′ UTRs which bind ligands that regulate the stability or translation of the mRNA in an allosteric fashion. Riboswitches are present in more than 2% of *Bacillus subtilis* genes, particularly those involved in metabolite transport or biosynthesis. Some riboswitches even appear to be ribozymes that possess self-cleaving catalytic activity; an example that extends the capabilities of regulatory RNAs beyond simply target recognition.

A significant challenge for the study of ncRNAs still lies simply in the identification of new entities. The discovery of ncRNAs has historically been rather serendipitous; but their unbiased, systematic identification in genomes may be possible in this post-genomic age. The remarkable extent of 'non-genic' transcription was discussed by Tom Gingeras (Affymetrix, Santa Clara, USA). Using tiling microarrays that

systematically cover 10 human chromosomes at 5-nucleotide resolution, his group has found that around 30% of the human genome is transcribed at an experimentally detectable level. Discrete, novel gene structures were then revealed by large-scale hybridization of RACE (rapid amplification of cDNA ends) reaction products to these microarrays. On the computational side, Todd Lowe and Gill Bejerano (University of California, Santa Cruz, USA) reported the identification of a tremendous number of non-genic sequences in human, mouse and rat that are under strong selective constraint, and at least some of which may represent functional ncRNAs. Lowe's systemic search for ncRNAs is being conducted by filtering hidden Markov model-based sequence alignments through algorithms like QRNA, RNAfold, and SLAM to arrive at promising candidate ncRNAs. Bejerano's approach, on the other hand, has been to examine large, ultraconserved elements of more than 200 base-pairs that are not exons and are currently unannotated in vertebrate genomes. Bejerano has been scanning these elements for their potential to form distinct RNA secondary structures, akin to those in rRNA genes, which are also ultraconserved.

RNAi for functional discovery and therapeutics

RNAi has been widely applied to genome-wide forward genetic analysis, both in traditional model organisms and in vertebrates not previously amenable to genetic analysis. Roderick Beijersbergen (Netherlands Cancer Institute, Amsterdam, The Netherlands) described 24,000 small hairpin RNAs (shRNA) retroviral vectors targeting 8,000 human genes, which have been used in several cancer-related screens. Recently, his group has performed highly parallel screens using a 'barcode' strategy in which experimental and control cell populations are infected *en masse* with the entire set of shRNAs. Following appropriate incubation and selection, they can quantify the overall distribution of shRNA inserts using microarrays to identify ones that are significantly over-represented.

Norbert Perrimon (Harvard Medical School, Boston, USA) reviewed his lab's systematic RNAi screens in *Drosophila* using a library of 16,000 dsRNAs that are transfected into various cultured cells. So far, they have performed about 50 genome-wide screens of cell biology, signal transduction and host-pathogen interactions, including both quantitative assays in plate-reader format and qualitative assays using automated microscopy. He also described promising efforts to miniaturize RNAi screening using microarrays.

Finally, progress towards unlocking the therapeutic potential of RNAi was reported at the meeting. Yuriy Federov (Dharmacon, Lafayette, USA) discussed proprietary chemical modifications that appear to increase siRNA specificity and decrease cytoxicity due to off-target effects. David Bumcrot (Alnylam, Cambridge, USA) described how cholesterol levels in mice could be lowered by simple intravenous delivery of cholesterol-modified siRNAs that target the expression of apolipoprotein B. Although clinical applications involving RNAi are still years away, these efforts are providing the first solid proof-of-principle that RNAi technologies may be viable agents for gene therapy.

Despite the incredible progress in recent years, key questions remain unanswered. How many other ncRNAs exist? What are the endogenous functions of these novel ncRNAs, including most miRNAs? What is the mechanism of miRNA-mediated translational inhibition and transcriptional gene silencing? And can the tantalizing therapeutic potential of RNAi be realized in the near future? We look forward to hearing about progress on these questions at a future Keystone Symposium.