Regulatory RNAs and beyond

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The dynamic regulation of biological processes by RNA has emerged as a key field in recent years, and was the topic of the 62nd Mosbacher Colloquium of the German Society for Biochemistry and Molecular Biology (GBM). The 2011 Colloquium, held in April in the romantic Neckar-river region, was also a celebration of the tenth anniversary of the RNA Biochemistry study group within the GBM, which acts as platform for RNA biologists and chemists within Germany and in other European countries.

Numerous non-coding RNAs (ncRNAs) have been discovered in prokaryotes. Most do not exceed 200 nucleotides in length and have thus been termed small RNAs (sRNAs). Many act by an antisense mechanism and bind to their target—primarily messenger RNAs (mRNAs)—through short stretches of Watson–Crick (WC) base pairs, often interspersed with non-WC base pairs or mismatches, which makes reliable prediction difficult. In many such bacterial *trans*-regulations, the sRNA–target interactions are mediated by the Sm-like protein Hfq. The hexameric Hfq binds to many different RNAs (A-rich single-stranded RNAs on its proximal surface and U-rich single-stranded RNAs on its distal surface) with high affinity in the sub-to-mid-nanomolar K_d range. This tight binding of Hfq raises the question of how sRNAs that are rapidly induced in response to environmental cues get immediate access to Hfq, considering that pre-bound sRNAs dissociate slowly—their half-lives are longer than 1 h.

Gerhart Wagner (U. Uppsala, Sweden) provided data supporting a model of active cycling of sRNAs on the surface of the hexameric Hfq: a newly synthesized sRNA can bind to pre-existing RNA–Hfq complexes and induce rearrangements of the two RNAs on the six binding interfaces of Hfq to drive the dissociation of one of the RNAs. This mechanism enables the rapid, dynamic equilibration of cellular sRNA and Hfq

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pools, in line with the timescale—1–2 min—of sRNA regulation *in vivo*. Gisela Storz (National Institutes of Health, Bethesda, USA) reported on the *Escherichia coli* sRNA Spot42, which has a broad role in catabolite repression, representative of regulation within prokaryotic gene networks. Spot42 acts in a ‘multi-output, feed-forward loop’, modulating the global dynamics of sugar consumption by reducing leaky expressions of non-essential genes in the presence of glucose, thereby committing cells to glucose consumption under fluctuating environmental conditions. Jörg Vogel (U. Würzburg, Germany) addressed the

different mechanisms by which sRNAs act to regulate mRNAs. Although many sRNAs inhibit 30S ribosomal access to the Shine–Dalgarno sequence, some act upstream from it, or even base-pair within the coding region—for example, MicC—and trigger mRNA decay by RNase E. In this way, the sRNA RybB regulates almost all the key mRNAs encoding outer-membrane proteins in *Salmonella*. The conserved 5′-terminal 16-nucleotides of RybB base pair with the respective target mRNAs at various

locations between –50 and +50 nucleotides. RNase E recruitment is mediated by Hfq that binds to RybB. RybB, similarly to other Hfq-associated sRNA, binds to its targets by seed pairing of ≥ 7 base pairs and includes a 3′-anchor A-residue in the mRNA, which is reminiscent of eukaryotic microRNA (miRNA)–3′-untranslated region (UTR) interactions. The evolution of sRNAs seems to have taken a distinct twist in the photosynthetic cyanobacteria. Wolfgang Hess (U. Freiburg, Germany) described the discovery of a new, prevalent class of internal *cis*-antisense RNAs, located within open reading frames. In the model cyanobacterium strain *Synechocystis* PCC6803, antisense transcripts of protein-coding genes (≥ 800) outnumber the *trans*-encoded ncRNAs (≥ 300). An example is the *cis*-antisense RNA IsrR, which downregulates the light-harvesting protein IsiA under conditions of iron limitation by inducing co-degradation of IsrR–*isiA*-mRNA complexes.

The application of high-throughput sequencing in transcriptome studies requires the use of strategies to process and extract biologically meaningful information from large amounts of data, as well as to identify technical biases. As pointed out by Peter Stadler (U. Leipzig, Germany), RNA maturation—editing, chemical modification and processing events—and the influence of RNA structures leave traces in RNA-sequencing data, representing a hidden layer of biologically relevant information. As an example, 1-methyladenosine (A58) modification in transfer RNAs (tRNAs) blocks reverse transcription during first-strand complementary DNA (cDNA) synthesis and results in shorter cDNAs, the 5'-ends of which map to tRNA nucleotide 59. Other RNA modifications might increase the incorporation of non-templated nucleotides during cDNA first-strand synthesis. Another finding is the presence of CCA tails at the 3'-end of many non-tRNA transcripts in mammalian transcriptomes, including a subset of mature miRNAs.

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Recently, a new viral-defence mechanism has been found in prokaryotes, named CRISPR/Cas. It is an adaptive, small-RNA-based immune response, and new data about it are reported regularly. Stan Brouns from the van der Oost laboratory (Wageningen U., The Netherlands) and Michael Terns (U. Georgia, USA) presented data on two branches of the CRISPR/Cas system. Brouns described the composition and low-resolution structure of the *E. coli* Cascade complex and the way in which it specifically recognizes double-stranded DNA targets by seed sequence interactions. Terns showed that the CRISPR RNAs of the Cas RAMP module (Cmr) effector complex require a conserved 5'-sequence tag for function and can be engineered to directly cleave new target RNAs. His X-ray structure of the Cas6 processing enzyme in complex with CRISPR RNA shows that the repeat sequence is bound at the 5'-end and wraps around the posterior of Cas6, where cleavage takes place between nucleotides 22 and 23.

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Beyond the *trans*-regulating effector RNAs discussed so far, a variety of *cis*-acting regulatory RNA elements—such as riboswitches, allosteric ribozymes or RNA thermometers—have been identified, predominantly, but not exclusively, in bacteria. By switching their conformation, they are able to integrate many signals mediated by metabolites, second messengers or even physical parameters such as temperature or pH. Ron Breaker (Yale U., USA) introduced a tandem-RNA sensory system that resides in the 5'-UTR of the mRNA for a putative virulence gene in the pathogenic bacterium *Clostridium difficile*. Here, binding of the second messenger c-di-GMP induces a conformational switch that facilitates attack of the 5' splice-site of a self-splicing group I intron by its cofactor GTP. Removal of the intron generates a freely accessible ribosome-binding site and start codon for translation of the protein. By using bioinformatic screens on genome sequences, Breaker estimated that more than 250,000 riboswitch-type regulatory systems are waiting to be discovered.

RNA thermometers, discussed by Franz Narberhaus (Ruhr U., Bochum, Germany), induce heat-shock or virulence genes through gradual, temperature-dependent increases in the accessibility of translation-initiation regions of their mRNAs. Some features of RNA thermometers are known—such as their formation of weak helices including non-WC base pairs or limited apical-loop sizes—but biologically active thermometers are more than weakly base-paired secondary structures. Similarly tantalizing is the *E. coli* *alx* locus, which is preceded by a pH-responsive RNA regulator. Increased transcriptional pausing at high pH induces a conformational change that stimulates *alx* mRNA translation, as reported by Shoshy Altuvia (Hebrew U., Israel). This effect of high pH on pausing can be mimicked by the transcription factor NusA.

Structural insights into ligand-bound aptamer domains were provided by Robert Batey (U. Colorado at Boulder, USA) and Adrian Ferré-D'Amaré (U. Washington, USA). A common feature of all riboswitches

is that the RNA senses almost all functional groups of the ligand, resulting in high binding specificity. Batey pointed out that most riboswitches to some extent populate the conformation of the bound state—even in the absence of ligand—but this conformation is adopted to a larger extent when the ligand is bound. From his analysis of the nature of conformational changes by small-angle X-ray scattering, Ferré-D'Amaré detected a variety of intermediate conformational states that seem to fine-tune the response of the riboswitch to the specific cellular state; it is more of an 'ensemble of behaviours' than a set of distinct, fixed structures. Clearly, riboswitching is not all or nothing, but involves intricate levels of regulation.

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New RNA species are being found to intervene at new levels in gene regulation, including during transcription. The transcription of human protein-encoding genes produces several RNA species in addition to the mRNA in question, including RNAs derived from sequences within, and immediately upstream from, the protein-encoding genes. Torben Heick Jensen (Aarhus U., Denmark) therefore advocated a non-'gene-centric' view of transcription, as many transcription-related RNA species are still being discovered. In his view, they might be degradation products, rather than functional entities. However, although our understanding of most of these transcription-related RNA species remains incomplete, several interesting applications targeting gene promoters, splice sites or the regions beyond the 3'-termini were illustrated by David Corey (U. Texas, Dallas, USA). Aiming small-interfering RNA (siRNA) and miRNA constructs at sequences outside exons can result in an alteration of splice-site selection, activation and repression of transcription. The RNA polymerase (RNAP) itself seems to be central to the production of sRNA fragments. Patrick Cramer (U. München, Germany) and Robert Landick (U. Wisconsin, Madison, USA) highlighted several mechanisms that regulate transcription by acting directly

on RNAP. Landick focused on the regulatory input of proteins affecting transcriptional pausing. Cramer combined stunning structural biology and biochemistry to visualize a mechanism for the generation of short aberrant RNAs: the back-tracking movement of the RNAP. RNA elements of a different kind, which intervene in regulation through their structural features, were discovered by the application of chemical probing to the full-length human immunodeficiency virus (HIV-1) RNA genome. Kevin Weeks (U. North Carolina, Chapel Hill, USA) described how the application of SHAPE chemistry has revealed that highly structured RNA sequences correlate with and are translated into loops and unstructured sequences within HIV-1 proteins, suggesting that an additional layer of information is encoded in RNA.

... more time will be needed to obtain even a representative overview of the many RNA-regulation patterns

When discussing cellular regulation by RNA, miRNAs and the associated siRNAs cannot be omitted. Although the application of their regulatory effect has been a driving force in the promotion of techniques for gene-knockdown, the mechanistic aspects of miRNA function, its role in metabolism and the interplay of mRNA regulation by RISC- and 3'-UTR-binding proteins are still puzzling. Eliza Izaurralde (MPI for Developmental Biology, Tübingen, Germany) showed that the complex of one Argonaute protein (AGO) with the GW182 protein is required for RISC function. As well as conferring binding to AGO through its amino-terminal domain, GW182 harbours additional bipartite silencing domains that are responsible for the RISC-mediated translational repression and degradation of the miRNA targets. They interact with poly-(A)-binding protein 1 (PABPC1) and interfere with its functions in translation and mRNA stabilization, resulting in the recruitment of deadenylation complexes. The nucleic-acid binding specificities of bacterial and eukaryotic AGO proteins are still unclear. Another

twist in the mechanism of double-stranded RNA (dsRNA) interference was unveiled by Brenda Bass (U. Utah, Salt Lake City, USA): inactivating the helicase domains of Dicer in worms and flies affects processing of endo-siRNA, but not of miRNAs. This indicates a compulsory helicase activity to unwind dsRNA for blunt or 5'-overhanging termini before cleavage and siRNA release, but 3'-overhangs of miRNAs are still, and even preferentially, processed. Unwinding enables Dicer to access more-distant internal siRNA sites, and a processive movement along the dsRNA leads to production of several siRNAs from the same dsRNA substrate. During miRNA biogenesis and its regulation, adenosine-deaminase-acting-on-RNA (ADAR) and its catalysed A-to-I editing event might also be active. Only a few cases of mature miRNA editing have been found so far. However, in *C. elegans*, an *adr1* knockout in the Bass laboratory was shown to increase miRNA levels and affect more than 90% of cellular miRNAs, albeit only 10% more than twofold. Apparently, ADAR1 binds to pri-miRNAs in the nucleus and inhibits Drosha processing.

RNA-binding proteins, together with miRNAs, are key regulators of 3'-UTRs. A paradigm for the investigation of this is the ubiquitously expressed RNA-binding protein Pumilio-1 (PUM1), which binds to the 3'-UTR of the tumour suppressor p27 and induces a local change in mRNA structure that favours association with miR-221 and miR-222, thereby suppressing p27 expression. Witold Filipowicz (FMI Basel, Switzerland) reported on HuR, which binds to A/U-rich elements in many 3'-UTRs, oligomerizes along RNA and contacts the RISC. HuR shuttles between the nucleus and cytoplasm and localizes in the cytoplasm under normal conditions, yet transfers to the nucleus under stress conditions, where it blocks siRNA-mediated cleavage in the 3'-UTR, thus regulating the regulators. Michaela Zavolan (U. Basel, Switzerland) used PAR-CLIP as a global approach to identify HuR-binding sites in 3'-UTRs, scrutinizing an important methodological aspect of RNase digestion following co-immunoprecipitation of RNPs: the type of nuclease (micrococcal

nuclease or RNase T1), its cleavage-site preferences and the extent of RNase digestion can alter the sequence that is thought to be the core binding-site of the protein on the basis of deep-sequencing cDNA reads. Using milder RNase digestion can prevent the introduction of such bias. A direct link to interfering with regulation came from Markus Stoffel's group (ETH Zürich, Switzerland). miR-375 and miR-103/107 have specific roles in the carbohydrate metabolism of mammals, regulating glucose homeostasis. The first is crucial for insulin secretion, whereas the latter confers insulin resistance. These functions could be deduced from mice with genomic deletions of the miRNA gene or through miRNA silencing through antagomirs, opening up therapeutic perspectives for metabolic disorders such as diabetes.

In summary, it was agreed by the 350 participants that regulation by RNA offers new and alternative pathways that are likely to represent the rule, rather than be exceptions. The honorary presentations of two long-standing contributors to the RNA field—Eric Westhof (U. Strasbourg, France) and Renée Schröder (U. Vienna, Austria)—emphasized that more time will be needed to obtain even a representative overview of the many RNA-regulation patterns, and to improve our understanding of RNA folding, structure and interaction beyond WC base-pairing, let alone to develop the applications of this knowledge to interfere with, alter or modify regulation. More meetings are sure to come!

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