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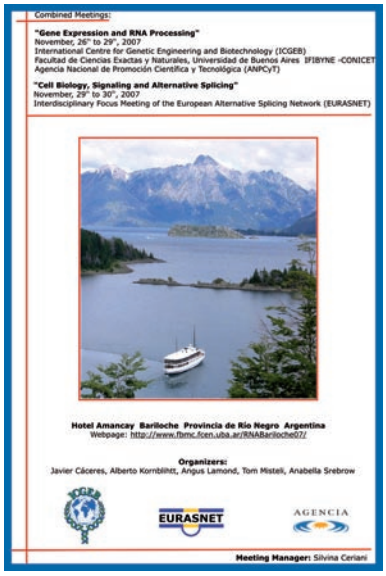
meeting report

El Sur también existe: processing RNA in the Argentine Patagonia

Meetings on 'Cell Biology, Signaling and Alternative Splicing' and 'Gene Expression and RNA Processing'

Javier Martinez[1+] *& Karla M. Neugebauer*[2++]

[1]Institute of Molecular Biotechnology of the Austrian Academy of Science, Vienna, Austria, and [2]Max Plank Institute of Molecular Cell Biology and Genetics, Dresden, Germany



The meetings on 'Cell Biology, Signaling and Alternative Splicing' and 'Gene Expression and RNA Processing' took place between 26 and 30 November 2007, in Bariloche, Argentina, and were organized by A. Kornblihtt, A. Srebrow, J. Caceres, A. Lamond and T. Misteli. Support was provided by the International Centre for Genetic Engineering and Biotechnology (ICGEB) and by the European Network on Alternative Splicing (EURASNET).

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[1]Institute of Molecular Biotechnology of the Austrian Academy of Science, Dr. Bohr-Gasse 3, A-1030 Vienna, Austria
[2]Max Plank Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany
[+]Corresponding author. Tel: +43 (1) 79044 4840; Fax: +43 (1) 79044 110; E-mail: martinez@imp.univie.ac.at
[++]Corresponding author. Tel +49 351 210 2589; Fax +49 351 210 1209; E-mail: neugebau@mpi-cbg.de

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"El Sur tambien existe"

However down here,
down close to the roots,
is where memory
forgets no memories.
And there are those who struggle
and there are those who try hard
and this is how together they manage
what seemed impossible.
The whole world knows
that the South,
the South also exists.

Mario Benedetti (translated by J. Martinez)

The heart of science also beats in the southern hemisphere, and in the last days of November 2007, in the middle of the southern spring, the RNA community met in Bariloche, in the beautiful Argentine Patagonia. Transcription and RNA processing were the focus of the combined meetings 'Gene Expression and RNA Processing' and 'Cell Biology, Signaling and Alternative Splicing'. The organizers chose a lovely site, as well as an eclectic group of speakers and participants. The result was a stimulating mixture of science, lively poster sessions with students and postdocs, and hours of informal discussion. Here, from the 52 talks presented, we strive to highlight selected emergent themes and apologize to those speakers whose topics we have been unable to include.

The role of RNA in diverse biological activities

The meeting started with a Keynote Lecture from T. Cech (Boulder, CO, USA), whose talk on telomerase foreshadowed many of the subsequent topics. Telomerase, the enzyme that extends chromosome ends and thereby maintains chromosome integrity over infinite cycles of DNA replication and cell division, uses an RNA primer provided by the telomerase RNA—Tlc1 in budding yeast and Ter1 in fission yeast (Zappulla & Cech, 2006). Similar to spliceosomal small nuclear RNAs (snRNAs), this small RNA (~1,200 nucleotides in both

yeasts and 450 nucleotides in human) is modified with a tri-methyl-guanosine cap and assembled into a ribonucleoprotein (RNP) complex, comprised in yeast of proteins shared by spliceosomal RNPs, for example, Sm proteins. Cech described how the extended stem–loops formed by Tlc1 provide binding sites for the protein components of Tlc RNP. A shortened yeast Tlc1—mini-T—leads to a growth disadvantage linked to reduced RNA stability, suggesting that the length of Tlc1 RNA maintains high levels of expression. However, length *per se* is dispensable for function, as mini-T can substitute for wild-type Tlc1 provided that the RNP proteins—Ku, Est1, Est2 and Sm proteins—can independently bind to the RNA. This underscores the potential for small RNAs to have structural roles in biology by tethering several components of a complex together in the absence of protein–protein interaction domains.

Nowhere are the structural and functional roles of small RNAs more highly appreciated by its students than in the spliceosome. Similar to Tlc1, the spliceosomal snRNAs hang onto a diverse constellation of shared and snRNP-specific proteins that are important for the assembly and stability of snRNPs as well as the assembly and function of the spliceosome, which attains the size and complexity of the ribosome at the pinnacle of its assembly cycle. The characterization of these dynamic RNPs has become possible in recent years through mass spectrometry (Trinkle-Mulcahy & Lamond, 2007), and structural studies have begun (Behzadnia *et al*, 2007; Jurica & Moore, 2003). A. Lamond (Dundee, UK) presented a new integrative approach that combines quantitative mass spectrometry for high-throughput protein identification with quantitative measurements of protein localization, dynamics and protein-interaction partners. Lamond used stable isotope labelling to measure the distribution of 3,000 cell proteins between the cytoplasmic, nucleoplasmic and nucleolar compartments, which illustrated the power of this 'spatial proteomics' technique.

The isolation of a minimal spliceosomal complex that is able to carry out catalysis has previously eluded splicing biochemists. However, R. Lührmann (Göttingen, Germany) described a masterful piece of biochemistry that has led to the achievement of this goal (Bessonov *et al*, 2008). For the first time, spliceosomal complexes affinity-purified on truncated pre-mRNA substrates were shown to catalyse exon ligation in the absence of added factors. A salt-stable core complex was thereby identified and characterized, showing that splicing intermediates, together with the U2, U5 and U6 snRNAs and a surprisingly small subset of spliceosomal proteins, constitutes the catalytic centre. All of the spliceosomal snRNPs are substantially remodelled during the assembly and function of the spliceosome; therefore, in contrast to the telomerase RNP, the protein components of snRNPs and the spliceosome seem to position the snRNAs and pre-mRNA instead. Furthermore, unlike the ribosome, this dynamic behaviour of the spliceosome requires that snRNPs be reassembled to function in subsequent rounds of splicing.

New aspects of spliceosomal snRNP biogenesis also arose at the meeting. G. Dreyfuss (Philadelphia, PA, USA) focused on the role of the survival of motor neurons (SMN) complex, which guides the formation of a heteroheptameric ring of Sm proteins on the major U1, U2, U4 and U5, as well as the minor U11, U12, U4atac and U6atac spliceosomal snRNAs. Having established an assay to measure this activity of the SMN complex (Wan *et al*, 2005), Dreyfuss showed that the capacity of snRNP assembly is reduced in SMN-deficient cells. SMN genes are mutated in patients with spinal muscular atrophy (SMA) and it has been a mystery as to how motor neurons are selectively affected by a deficiency in the ubiquitously expressed SMN. By using a SMA mouse model, the Dreyfuss group has now identified tissue-specific changes in the level of each snRNA—that is, an altered snRNP repertoire—and, probably as a result, numerous tissue-specific splicing abnormalities. Dreyfuss suggested that SMA is a general splicing disease and is not restricted to motor neurons. With the aim of repairing the SMA defect, A. Krainer (Cold Spring Harbor, NY, USA) has used an antisense-oligonucleotide (ASO)-tiling method to screen systematically for intronic regulatory elements in SMN transcripts. Targeting of identified regions with ASOs in human *SMN2*-transgenic mice strongly increased the inclusion of human *SMN2* exon 7 in transgenic animals, showing the therapeutic potential of ASOs. From the transcriptional angle, K. Neugebauer (Dresden, Germany) showed that levels of the U6 snRNA—an RNA polymerase III transcript—are determined by RNA polymerase II (Pol II) activity. Pol II accumulates upstream of human *U6* genes, where its activity is linked to histone modifications such as acetylation (Listerman *et al*, 2007). Together, these data indicate that cellular levels of all protein and RNA components of the spliceosome are ultimately controlled by Pol II, and that their stability is determined by the rate of snRNP assembly.

Small RNAs are full of big surprises

Being so small, microRNAs (miRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs) and even tRNAs squeezed into the meeting to illuminate their wide involvement in regulating the expression of the genome. So far, miRNAs have been shown to repress the expression of target mRNAs (Fig 1A). J. Steitz (New Haven, CT, USA) surprised everyone by reporting that miRNAs are also able to activate protein translation (Vasudevan *et al*, 2007), in this case, tumour necrosis factor-α (TNFα) mRNA. On cell-cycle arrest by serum starvation, miRNA-369-3 targets an A+U-rich element in the TNFα 3' UTR and activates translation through the recruitment of miRNA-associated proteins such as Argonaute and Fragile X mental retardation-related protein 1 (FXR1; Fig 1B). Two additional miRNAs showed a similar divergence in activity on cell-cycle arrest; therefore, it seems that cell-cycle stages might dictate the effect of miRNAs. We await some mechanistic insights into this unexpected type of regulation.

J.E. Dahlberg (Madison, WI, USA) described the accumulation of miRNA-427 to high levels in early *Xenopus* embryos, which was coincident with the onset of zygotic transcription. His group found that miRNA-427 triggers deadenylation and destabilization of the maternally encoded A1 and B2 cyclin mRNAs. In addition to the sequences in the mRNA 3' UTRs that are complementary to the 'seed' sequence of the miRNA, approximately 20 other nucleotides are necessary for the deadenylation activity; however, participation of the miRNA in translation is not required. Curiously, another miRNA, let-7, can substitute for miRNA-427 in the deadenylation reaction provided that the 3' UTR contains sequences complementary to the seed of that miRNA. Therefore, the identity of the miRNA that associates with the target mRNA is not crucial. In contrast to its effects on the cyclin mRNAs, miRNA-427 can also inhibit the translation of distinct mRNAs without causing deadenylation and destabilization.

M. Rosbash (Waltham, MA, USA) spoke about miRNAs and circadian rhythms in *Drosophila*. Transcriptional regulation is known to have a role in circadian timekeeping in animal systems, but the importance of translational regulation by miRNAs is only beginning to emerge (O'Neill & Hastings, 2007). The work presented indicates that there are circadian oscillations in the levels of specific miRNAs in the heads of *Drosophila*, and the phenomenon might be related

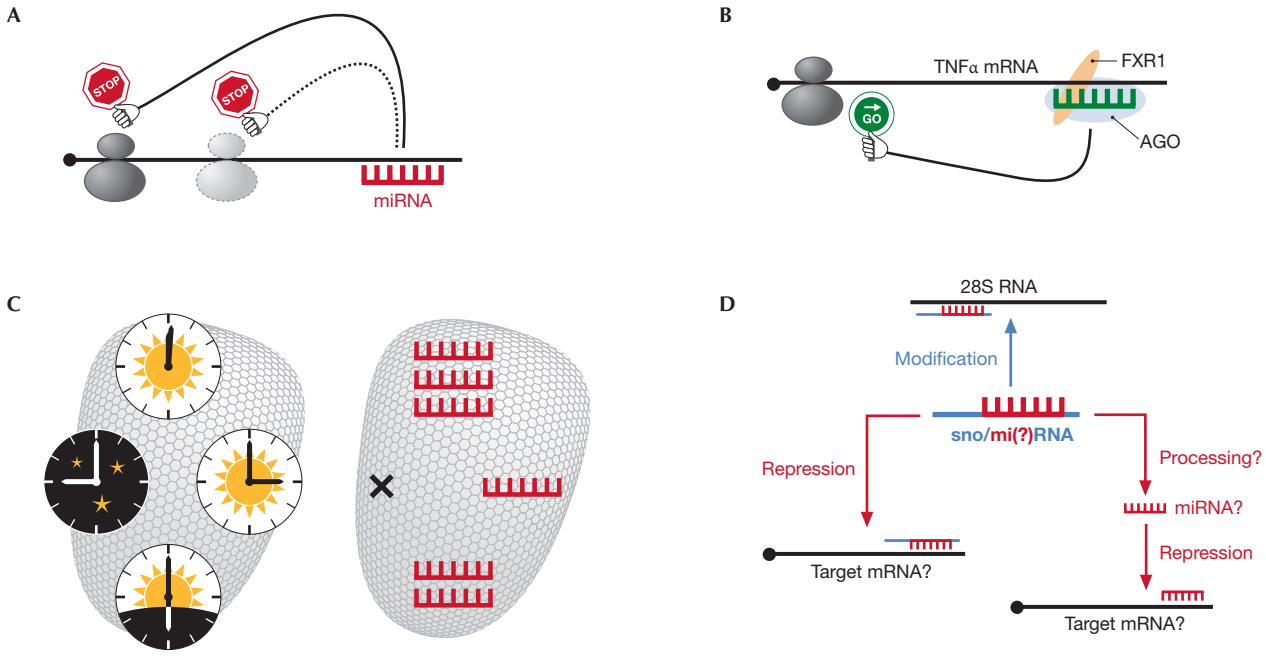

Fig 1 | The biology of small RNAs. **(A)** Translational repression—a known effect and several mechanisms. Translational inhibition takes place at the initiation or elongation phases, followed by miRNA-dependent degradation. **(B)** Translational activation—a new effect and a mysterious mechanism. Translational activation occurs under cell-cycle arrest conditions. **(C)** A full-day job for miRNAs. miRNA expression varies by the time of the day: the fly eye on the left shows the time, whereas in the fly eye shown on the right side, miRNA expression peaks at noon, is reduced at 3:00 pm, increases again by 6:00 pm and is at a minimum at 9:00 pm. Similarly, miRNA expression in fly heads varies with circadian rhythm. **(D)** SnoRNA and miRNA: two for one? The potential for a single small RNA to act as a 'guide RNA' directing nucleotide modifications, while simultaneously functioning as an miRNA to regulate mRNA expression, is shown. AGO, Argonaute; FXR1, fragile X mental retardation-related protein 1; miRNA, microRNA; snoRNA, small nucleolar RNA; TNFα, tumour necrosis factor α.

to the clock neuron specificity of some of these miRNAs (Fig 1C). In addition, Rosbash identified potential target mRNAs of the circadian miRNA system and these include both central clock mRNAs involved in timekeeping, as well as mRNAs involved in circadian output functions.

The final talks on small RNAs were dubbed the 'three Javier's session' by the chair, T. Misteli (Bethesda, MD, USA). There has been substantial progress in identifying plant miRNAs and the biological processes that they regulate; however, little is known about how the miRNAs themselves are regulated. The miRNAs are generally encoded in gene families, with similar or identical sequences. By using the miR319/159 family of miRNAs as a model, J. Palatnik (Rosario, Argentina) presented data showing that small variations in the sequences of mature miRNA and differences in expression can have a profound impact on target selection *in vivo*. Studies on miRNA promoters revealed that miRNA family members could have different regulatory sequences. Mutagenesis studies identified specific boxes responsible for the tissue-specific expression of one particular miRNA and the search is on for interacting factors. In turn, J. Caceres (Edinburgh, UK) expanded on previous findings from his group, showing that heterogeneous nuclear RNP (hnRNP) A1 is required for the processing of miR-18a (Guil & Caceres, 2007). New data suggest that auxiliary factors are generally required for the processing of specific miRNAs, revealing an additional level of regulatory complexity. Caceres also addressed translational regulation by auxiliary factors—not miRNAs—and showed that the serine-arginine (SR) protein SF2/ASF promotes translation initiation of bound mRNAs by suppressing the activity of eIF4E-binding protein (4E-BP), a competitive inhibitor of cap-dependent translation. This activity is mediated by interactions of SF2/ASF with components of the mammalian target of rapamycin (TOR) pathway. These findings suggest a model in which SF2/ASF functions as an adaptor protein that recruits signalling molecules responsible for regulation (Michlewski *et al*, 2008).

J. Martinez (Vienna, Austria) discussed a new RNA kinase, CLP1, the human homologue of the yeast cleavage and polyadenylation factor I subunit, and its roles in RNA interference (RNAi) and tRNA splicing (Weitzer & Martinez, 2007). In contrast to siRNA and miRNA duplexes generated by the RNaseIII endonuclease Dicer, synthetic siRNAs display a 5' OH group. However, to become incorporated into the RNA-induced silencing complex (RISC) and mediate target RNA cleavage, the guide strand of a siRNA needs to display a phosphate group at the 5' end. Monitoring siRNA phosphorylation, his group applied a classical chromatographic approach that resulted in the identification of the kinase CLP1, a known component of both tRNA splicing and mRNA 3'-end formation machineries. Martinez reported that CLP1 phosphorylates and licenses synthetic siRNAs to assemble into RISC for subsequent target RNA cleavage. More importantly, he showed that CLP1 is the RNA kinase that phosphorylates the 5' end of the 3' exon during human tRNA splicing, allowing the subsequent ligation of both exon halves by an unknown tRNA ligase.

SnoRNAs, known to target specific RNA modifications to complementary RNA species, were recently shown to regulate alternative splicing (Kishore & Stamm, 2006). Lamond presented new data on 'ribonomics', and identified RNA species that stably co-purify with nucleoli. Three new species of Box C/D snoRNAs were discovered: each is encoded within different introns of an uncharacterized open reading frame, and all are closely related and have the same guide sequence that targets the same physiological 2'-O-methyl ribose site on 28S rRNA. Notably, these three snoRNAs contain an internal region that is complementary to various cellular mRNA targets (Fig 1D). Lamond's group modified these internal snoRNA sequences to make them complementary to the green fluorescent protein instead, and showed that a snoRNA expression vector system can be used to specifically knockdown the expression of cellular proteins.

Alternative lifestyles of mRNA

Alternative pre-mRNA splicing generates thousands of different mRNA isoforms in metazoan organisms. The potential for regulation is accompanied by the potential for error, as emphasized by the prevalence of human diseases related to splicing (Caceres *et al*, 2007). K. Hertel (Irvine, CA, USA) described experiments designed to examine the error rates of splicing. His group's findings on constitutive transcripts argue that the spliceosome recognizes and removes introns with an astonishingly high degree of accuracy that is limited by the quality of pre-mRNAs generated by RNA Pol II. Conversely, analysis of alternative transcripts suggests that the high levels of alternative splicing observed in the human genome are the consequence of sub-optimal splicing signals. Accordingly, talks on alternative splicing highlighted the role of *trans*-acting factors in specifying which splice sites should be used.

Many current efforts are geared towards the identification of the pre-mRNA targets of known alternative splicing factors; however, a lack of global experimental approaches has held the field back. C. Smith (Cambridge, UK) addressed this by comparing quantitative proteomics and two different microarray platforms for the identification of alternative splicing events altered by knockdown of polypyrimidine tract-binding protein (PTB) and nPTB in HeLa cells. The double knockdown was essential because nPTB is switched off by a PTB-induced exon-skipping event that leads to nonsense-mediated decay (Spellman *et al*, 2007). Surprisingly, there was only a small degree of overlap between the events identified by the proteomic and array approaches, showing that both can provide insights into how regulatory factors such as PTB can affect gene expression at several levels. D. Rio's group (Berkeley, CA, USA) has used various methods to analyse alternative splicing factors in *Drosophila* Schneider cells. These include splicing-sensitive microarrays, bioinformatic searches for splicing-factor-binding sites by using systematic evolution of ligands by exponential enrichment (SELEX) motifs, and RNA immunoprecipitation and microarray hybridization (RIP-Chip) with immunopurified nuclear RNP complexes to define binding regions on the associated pre-mRNAs using whole genome-tiling microarrays. They also used RNAi in *Drosophila*, in conjunction with splice-junction microarrays, to shed light on how many alternative splicing events a given factor controls (Blanchette *et al*, 2005). The results are consistent with the model that sequence-specific RNA-protein recognition can occur in a combinatorial fashion to control both unique and common transcript pools that undergo alternative splicing.

Complementary to searches for targets of regulators, B. Graveley (Farmington, CT, USA) described his group's search for *trans*-acting factors that regulate the splicing of a specific pre-mRNA. Down syndrome cell-adhesion molecule (DSCAM) is the champion of alternative splicing, with the potential to generate 38,016 distinct isoforms from 95 variable exons that are organized into four clusters of mutually exclusive exons. The exon 6 cluster contains 48 mutually exclusive exons; previous results have shown that this is a result of competitive RNA base-pairing interactions between a single docking site with one of the selector sequences, which are located immediately upstream of each variable exon. To identify RNA-binding proteins involved in this mechanism, Graveley's group has performed an RNAi screen in which 400 different RNA-binding or splicing proteins were depleted and the alternative splicing outcome analysed by microarray. This approach identified the RNA-binding protein HRP36 as a crucial component; in the absence of HRP36, multiple exon 6 variants are included in the mRNA. HRP36 binds throughout the exon 6 cluster and seems to prevent SR proteins from activating ectopic exon inclusion (Olson *et al*, 2007).

How do cellular signalling pathways influence alternative splicing? K. Lynch (Dallas, TX, USA) focused on how networks of regulated splicing are controlled in response to T-cell stimulation. Her group developed an *in vitro* assay to recapitulate signal-induced alternative splicing of CD45 pre-mRNA and was able to show that PTB-associated splicing factor (PSF) is specifically recruited on stimulation, leading to hyper-repression of the exon. A global investigation by microarray has provided additional examples of signal-induced splicing regulation in T cells, in which signal-regulated exons were found to share sequence elements bound by an overlapping set of factors (Ip *et al*, 2007). B. Chabot (Sherbrooke, Canada) implicated protein kinase C (PKC) in the splicing repression of the 5' splice site of the pro-apoptotic BCL-x isoform. His group has identified a region in the BCL-x pre-mRNA that confers responsiveness to PKC inhibitors in human embryonic kidney 293 cells. The action of several anticancer drugs, which require the same *cis*-acting element, suggests that this region represents a converging platform for various signals. S. Stamm (Lexington, KY, USA) reported that at least ten splicing factors have a protein phosphatase 1 (PP1)-binding site in their RRM. This finding links well-established signalling pathways that regulate the activity of PP1 to the spliceosome.

Roles for chromatin and transcription

Pre-mRNA splicing begins co-transcriptionally on chromatin and this feature influences the alternative splicing outcome; for example, conditions that alter transcription elongation rates and pausing regulate exon inclusion (Batsche *et al*, 2006; de la Mata *et al*, 2003; Roberts *et al*, 1998). This suggests that the kinetics of splice-site synthesis and the time for interaction with splicing factors before synthesis of the next splice site have a role in the combinatorial control of alternative splicing. The development of methods for measuring the rates of Pol II movement *in vivo* was discussed by R. Singer (Bronx, NY, USA), who showed that the speed of the polymerase can be much faster—more than 4 kb per minute—than previously believed and that pausing is surprisingly frequent (Darzacq *et al*, 2007). Work from the group of D. Aubeouf (Paris, France) on two oestradiol-induced genes emphasized heterogeneity in the expression of co-regulated genes; efficient co-transcriptional splicing of cyclin D1 mRNA correlates with high serine 5 phosphorylation levels in the Pol II C-terminal domain, whereas the production of PS2 mRNA is sub-optimal due to inefficient co-transcriptional splicing. F. Pagani (Trieste, Italy) and N. Proudfoot (Oxford, UK) detected a failure in alternative splicing

on insertion of a hammerhead ribozyme into introns between regulatory elements and splice sites. Therefore, although the nascent RNP can hold onto constitutive exons and splice them without intron continuity, regulatory elements must be presented in the correct spatial context (Dye *et al*, 2006; Gromak *et al*, 2008).

As splicing occurs on chromatin, global connections between chromatin states and RNA processing outcomes will probably be made in the future. Genome-wide views of chromatin were presented, with O. Rando (Worchester, MA, USA) explaining how 70% of the yeast genome is contained within well-positioned nucleosomes that bear the marks of transcriptional activity (Rando, 2007). Interestingly, although methylation of certain histones is more prevalent in upstream (for example, H3K4) than downstream (for example, H3K36me3) regions, these modifications might only have a direct role in the expression of some genes. For others, these marks might reveal the transcriptional history of the gene in question. Misteli collaborated with E. Meshorer (Jerusalem, Israel) to show that the chromatin of embryonic stem cells is devoid of heterochromatin, is generally decondensed and is rich in active histone marks. Transcription of the embryonic stem-cell genome is hyperactive, but is progressively silenced on differentiation. Although this type of behaviour might help to explain the plasticity of embryonic stem cells, one also wonders to what extent this plasticity is manifested in the RNA that is synthesized.

The flip side to this is the question of how RNA itself influences transcription and chromatin states. One of the highlights of the meeting was a talk by A. Gamarnik (Buenos Aires, Argentina) on the amplification of the Dengue virus, which has a plus-stranded RNA genome. Her group has detected cyclization of the viral genome by long-range RNA–RNA interactions, and showed that this circular conformation of the RNA brings the 5' promoter near the 3' end initiation site for minus-strand RNA synthesis (Alvarez *et al*, 2008). This work reminds us that the structural role that RNAs can have on the relatively small scale of an RNP, such as TLC1, might echo larger roles on the scale of whole genes and genomes. In this regard, X.-D. Fu (San Diego, CA, USA) reported that transcriptional activation of target genes by oestrogen leads to chromosomal movements that are dependent on actin and dynein, and subsequent interchromosomal interactions within the subnuclear compartment—'speckles' or interchromatin granules—usually associated with pre-mRNA splicing (Nunez *et al*, 2008). We now anxiously await further details on the lines of communication that link chromosomes, their genes and their RNAs across nucleoplasmic space.

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Javier Martinez

Karla M. Neugebauer