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REVIEW

RNA: Networks & Imaging

Marc Kenzelmann[1,*], Karsten Rippe[2] and John S Mattick[3]

[1] Department of Cellular and Molecular Pathology, German Cancer Research Center, Heidelberg, Germany,
[2] Kirchhoff-Institut für Physik, Molecular Biophysics Group, Heidelberg, Germany and
[3] Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland, Australia
* Corresponding author. Department of Cellular and Molecular Pathology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Tel.: +49 6221 42 4368; Fax: +49 6221 42 4352; E-mail: m.kenzelmann@dkfz.de

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The past few years have brought about a fundamental change in our understanding and definition of the RNA world and its role in the functional and regulatory architecture of the cell. The discovery of small RNAs that regulate many aspects of differentiation and development have joined the already known non-coding RNAs that are involved in chromosome dosage compensation, imprinting, and other functions to become key players in regulating the flow of genetic information. It is also evident that there are tens or even hundreds of thousands of other non-coding RNAs that are transcribed from the mammalian genome, as well as many other yet-to-be-discovered small regulatory RNAs. In the recent symposium RNA: Networks & Imaging held in Heidelberg, the dual roles of RNA as a messenger and a regulator in the flow of genetic information were discussed and new molecular genetic and imaging methods to study RNA presented.
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Introduction

The diverse informational, structural, and catalytic activities of RNA have been known for many years leading to the coining of the phrase 'RNA world' (Gesteland *et al*, 2006). Recently, an increasing number of non-coding RNAs (ncRNAs) have been discovered, that form a 'RNA underworld' (Mattick, 2006), in which RNA exerts regulatory functions in transcription and translation. These RNA transcripts do not contain any clear open reading frame and some have been known for many years (Mattick, 2003; Costa, 2005) but until recently have largely been regarded as fascinating but exotic exceptions rather than being part of a sophisticated RNA-based regulatory network. However, the discovery of small interfering RNAs (siRNAs) and micro-RNAs (miRNAs), new members of the family of ncRNAs, and the elucidation of their basic mechanism of action, known as RNA interference (RNAi), has now attracted the broad interest of life scientists. Our current knowledge of ncRNAs places them at key positions in various molecular and cellular events in eukaryotic cells. ncRNAs range from about 21–25 nucleotides (for siRNAs and miRNAs) to 100–200 nucleotides (for small RNAs normally found as transcriptional and translational regulators) and up to >10 000 nucleotides for ncRNAs involved in gene silencing (Mattick, 2003; Costa, 2005; Mattick and Makunin, 2005, 2006; Furuno *et al*, 2006). Thus, RNA transmits genetic information but at the same time also appears to form a highly structured network that regulates gene expression and translation in the cell (Mattick, 2003; Costa, 2005; Mattick and Makunin, 2005; Esquela-Kerscher and Slack, 2006). This network needs to exert specific temporal control over functions distributed over various subcellular locations and compartments. To understand its complex spatiotemporal dynamics, sensitive imaging methods are required to visualize its components at the single-cell and sometimes even at the single-molecule level and follow them on their way to action and degradation (Shav-Tal *et al*, 2004a, b).

The fascinating roles of RNA in eukaryotic cell and developmental biology attracted international experts to the symposium 'RNA: Networks & Imaging' organized by the DFG Research Training Group 886 (http://www.ma.uni-heidelberg.de/ag/grk886/index.html) in Heidelberg in April 2006. Here, we review the topics covered at the meeting in relation to regulatory RNA networks, their use in elucidating the function of molecular and cellular mechanisms, and new applications and developments of imaging-based approaches to study RNA.

The 'RNA underworld' on a grand scale

The increasing knowledge about the 'underworld of RNA' leads to fundamental questions, in particular how the expression of large numbers of ncRNAs fits into the generally accepted model of gene expression (DNA→RNA→protein, the central dogma of molecular biology, elaborated between 1941 and 1952; Beadle and Tatum, 1941) and what may be the roles of these ncRNAs in biological complexity? John Mattick (University of Queensland, Australia) presented a new perspective on these questions and discussed the structure of genetic programming in higher organisms. Recent studies have reported the discovery of tens of thousands of new ncRNAs (Bertone *et al*, 2004; Carninci *et al*, 2005; Cheng *et al*, 2005) whose transcription spans at least 70% of the mammalian genome, frequently on both strands (Katayama *et al*, 2005). It becomes clear now that there are more conserved non-coding than coding DNA sequences in the mammalian genome (Waterston *et al*, 2002) and that many ncRNAs are evolving quickly (Pang *et al*, 2005). Mattick suggested that most of the genome is devoted to a hidden layer of ncRNAs that direct the

trajectories of differentiation and development via a range of mechanisms including control of epigenetic memory, promoter selection, alternate splicing, RNA editing, mRNA stability, and mRNA translation (Mattick, 2003, 2004). Although it is accepted that DNA encodes information digitally, little consideration has been given to the possibility that RNA may also transmit information digitally, a feature that renders RNA optimal for regulatory purposes (Mattick and Gagen, 2001; Mattick, 2003, 2004, 2006). According to this latter concept, eukaryotic genomes would produce two parallel outputs: proteins, whose biochemical activities represent an analog signal, and ncRNAs, which send sequence-specific, hence digitally encoded, signals to RNA and DNA targets. As is the case for digital systems, ncRNA-based signals and the consequent actions are separated by a decoding process involving specific enzymatic activities (e.g. the RNAi machinery or enzymes involved in DNA methylation). This idea is underpinned by the recent discoveries about the action of small RNAs in fine-tuning expression levels of mRNAs (controlled by siRNAs) or by modulating stability and translational activity of mRNAs (mediated by miRNAs). Accordingly, a new definition of the term 'gene' and a revision of the central dogma of molecular biology describing the flow of genetic information are required (Figure 1; Mattick, 2003, 2004). The concept of RNA as a molecule transmitting information digitally opens fascinating new clues to the generation and maintenance of biological complexity. Both information theoretic analysis and empirical data show that regulatory information rises nonlinearly with complexity and indicates that a system based on regulatory modules operating in an analog manner (i.e. using regulatory proteins) quickly reaches its complexity limits as in prokaryotes (Mattick, 2004). A digitally operating system based on RNA as the regulatory molecule, however, may expand the limits allowing for the generation and maintenance of much more complex systems in eukaryotes (Mattick and Gagen, 2001; Mattick, 2004). This model also provides a fascinating evolutionary explanation for the 'Cambrian explosion' (∼520 My), a period in which complex animal life exploded in parallel to the incidence of intronic and other non-coding sequences (Mattick and Gagen, 2001; Mattick, 2004).

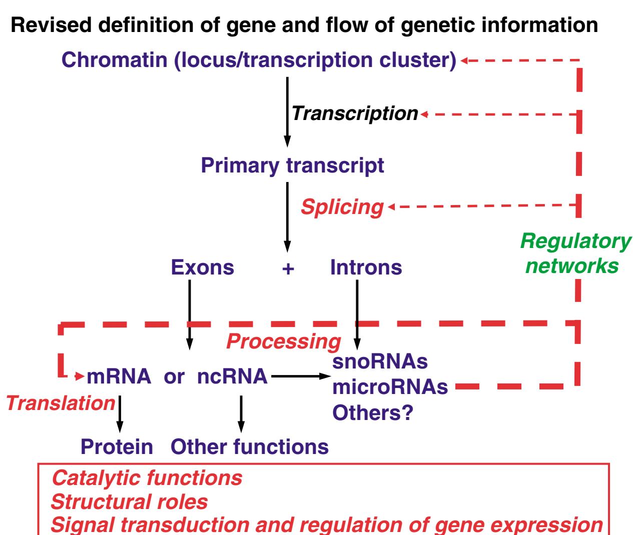

Figure 1 Regulatory 'feedback' and 'feed-forward' networks involving ncRNAs. ncRNAs are involved in the regulation of gene expression at different levels including the control of chromatin structure, RNA transcription and processing, mRNA stability and translational activity, and self-regulation (adapted from Mattick, 2003).

Insights into the 'RNA underworld' on the small scale

Illustrative examples of how ncRNAs can modulate gene expression at different levels were given by Ingrid Grummt (Heidelberg, Germany), Gunter Meister (Martinsried, Germany), and Witold Filipowicz (Basel, Switzerland). In her lecture, Ingrid Grummt presented a sophisticated mechanism by which a 150- to 300-nucleotide long intergenic transcript determines the epigenetic state of rRNA genes (rDNA). It has been known for almost two decades that transcripts originating from the intergenic spacer that separates rDNAs are expressed but their biological role, if any, was unknown. rDNAs exist in two distinct epigenetic states: about half are active (characterized by unmethylated promoters, euchromatic histone acetylations, and their association with RNA polymerase I and respective transcription factors) and half are inactive. Switching between the two states is mediated by a protein complex called NoRC (nucleolar remodelling complex; Strohner *et al*, 2001) that mediates silencing of rDNA (Zhou and Grummt, 2005). It has now been shown that heterochromatin formation by NoRC requires RNA and that TIP5, a NoRC subunit, binds RNA. Specifically, TIP5 recognizes the secondary structure of a ncRNA of 150–300 nucleotides complementary in sequence to the rDNA promoter and originating from a spacer promoter. Strikingly, binding of TIP5 to this ncRNA changes the structure of both RNA and NoRC in an induced fit mechanism thereby 'switching on' NoRC-mediated heterochromatin formation of rDNA (Mayer *et al*, 2006). Recognition of structural features is a common mechanism used to decode the digital information carried by ncRNA. This also holds true for the mechanism of RNAi, as demonstrated by Gunter Meister. Argonaute (Ago) proteins are key components of RNA silencing complexes guided by small RNAs. They bind single-stranded (ss) siRNAs and miRNAs. Although eight Ago proteins exist in humans, only Ago2 mediates cleavage of RNA targeted by siRNAs and miRNAs (Meister *et al*, 2004). Structural studies revealed that the 5′ phosphate of the guide strand is attached via a basic pocket of Ago2, whereas the mRNA is positioned for site-specific 'slicing' by the RNase H-like activity of Ago2's PIWI domain (Yuan *et al*, 2005). Using proteomic approaches, several Ago2-associated proteins were identified including Dicer (containing RNAseIII-PAZ domains), TRBP, the putative RNA helicase MOV10 (containing DEAD box), the RRM-motif-containing protein KIAA1093 (these components are required for small RNA-guided RNA cleavage), Gemin3/4, and eEF1-α (Meister *et al*, 2005). Interestingly, ss siRNAs are functional without 5′ phosphates, whereas double-stranded (ds) siRNAs require 5′ phosphates. These observations support a role for Ago-containing complexes in specific recognition of characteristic

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features of small RNA duplexes as well as their incorporation into silencing complexes.

Ago as well as MOV10 and KIAA1093 colocalize intracellularly to cytoplasmic processing bodies (P-bodies), sites of degradation of cellular mRNAs that contain decapping enzymes and exonucleases (Newbury *et al*, 2006). Witold Filipowicz reported that miRNA-mediated translationally repressed mRNAs (but not mRNAs targeted for degradation as has been suggested before) can be visualized in P-bodies. This observation led him to propose a two-step model of miRNA action: (i) miRNAs mediate translational inhibition at the initiation level in a 5′-cap-dependent mechanism (Pillai *et al*, 2005), a mode of action Witold Filipowicz favors over the alternative models of miRNA action by mRNA destabilization (Wu *et al*, 2006) and translational inhibition at the elongation level (Petersen *et al*, 2006), and (ii) relocation of repressed mRNAs to P-bodies. An interesting question is whether repressed mRNAs can be relocated from P-bodies and enter translation again. Using a cationic amino-acid transporter 1 (CAT-1) assay with CAT-1 being a target for miR-122, it could be shown that under conditions of cellular stress, miR-122-repressed CAT-1 mRNA can exit P-bodies and re-enter translation again, demonstrating a storage function of the P-body in mammalian cells. Importantly, the CAT-1 mRNA departure from P-bodies was accompanied by its recruitment to polysomes. The mechanism of relief of miRNA-mediated repression seems to involve HuR, an ELAV-like RNA-binding protein that translocates upon stress from the nucleus to the cytoplasm, where it may act as a modulator of miRNA repression (Bhattacharyya *et al*, 2006). Similarly, components of the miRNA machinery were found in the chromatoid body of male germ cells. These perinuclear, cytoplasmic cloud-like structure seems to operate as an intracellular center of the miRNA pathway, thereby underscoring the importance of post-transcriptional gene regulation in the control of post-meiotic cell differentiation (Kotaja *et al*, 2006).

Using the machinery of the 'RNA underworld' to reveal gene functions

Although many genomes, including those of man and mouse, have been fully sequenced, the function of most of the protein-coding genes is still unknown. An elegant way to uncover the functional roles of proteins comes from the 'RNA underworld': By specifically targeting and destructing the mRNA of the respective protein of interest with RNAi, its function within the living cell can be evaluated ('loss-of-function' assay).

Andrew Fraser (Cambridge, UK), Michael Boutros (Heidelberg, Germany), and Jan Ellenberg (Heidelberg, Germany) reported on high-throughput RNAi-based screening methods to examine genetic interactions in *Caenorhabditis elegans* development, in *Drosophila* signalling, and in mitotic regulation of human cells, respectively. Using an *Escherichia coli*-produced dsRNA 'RNAi library' that targets ∼17 000 genes of *C. elegans*, genetic interactions were studied. This work benefits from the ease of dsRNA delivery to *C. elegans*: they just eat it (Kamath *et al*, 2001, 2003)! Using different strategies to map genetic networks in vulval development, Fraser reported on the identification of several highly connected 'hub' genes (Figure 2). When these hubs are disrupted, the phenotypic consequences of mutations in components of the majority of the examined signalling pathways are enhanced. Interestingly, the hub genes encode many chromatin-modifying components and may represent 'genetic buffers'. As overall susceptibility to genetic diseases is a heritable trait, inherited variation in the activity of such 'genetic buffers' may play a significant role in this respect. Extrapolation of *C. elegans* gene maps showed that 'genetic buffers' are conserved in other animals as well, indicating that network connectivities may enable prediction of the probabilities of phenotypes in a 'cross-species' manner. Using similar high-throughput RNAi screening strategies in *Drosophila* cells, Boutros *et al* (2004) found several novel genes involved in different developmentally important signalling pathways such as JAK/STAT (Muller *et al*,

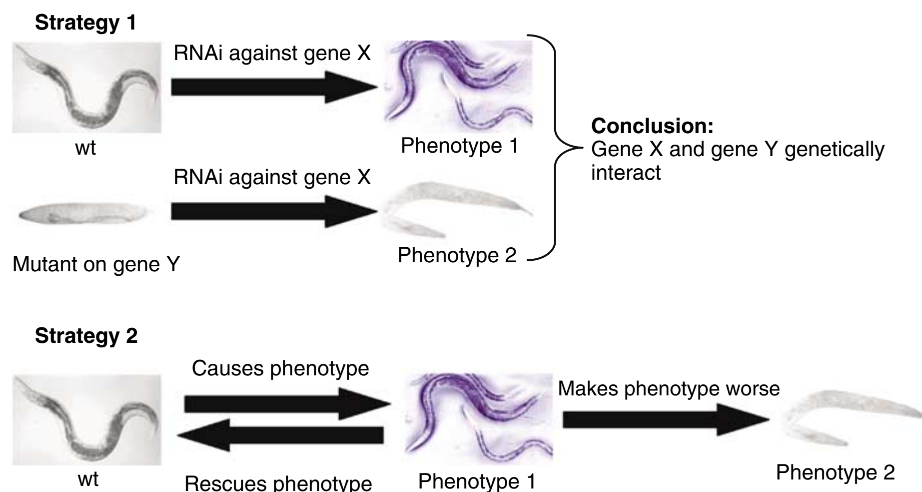

Figure 2 Schematics illustrating two strategies to map genetic interactions based on RNAi. The top panel illustrates the 'guilt-by-association' principle: if the knockdown of gene *x* in a wild-type background causes a specific phenotype that is different from the one caused in an already mutated (e.g. gene *y*) background, then these two genes (e.g. genes *x* and *y*) must genetically interact. Another strategy is based on causing a distinct phenotype by knocking down a specific gene. Additional knockdowns are then performed, which may either rescue or enhance the phenotype seen after the first genetic knockdown. Both results indicate genetic interactions between the genes that have been knocked down (bottom panel).

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2005) and Wnt. Jan Ellenberg presented a strategy to identify and define the function of genes involved in mitosis using a fascinating combination of high-throughput RNAi screening of human (HeLa) cells and state-of-the-art time-lapse microscopy (Neumann *et al*, 2006). The first level of analysis involves a fully automated method for microscopy-based siRNA screens combining transfected cell arrays, automated time-lapse fluorescence imaging, and computational phenotype analysis of chromosome segregation using histone-GFP reporter cells. This methodology allowed to expand the collection of genes known to be involved in mitosis. To further characterize a large number of novel genes, a second automated level of analysis was developed to allow quantitative 4D imaging of chromosome structure. This strategy led, for example, to a new model of *condensin-1* function in the cell. Condensin-1 was shown to bind dynamically to chromatin after envelope breakdown and to stabilize chromatin in a rigid state allowing mechanically stable spindle attachment, rather than being involved in chromosome compaction (Gerlich *et al*, 2006).

Visualizing biological macromolecules *in vivo* and *in situ*

An average analysis over very large numbers of cells often fails to provide the resolution and sensitivity required to investigate regulatory RNA networks and other molecular processes. However, technical advancements over the past years make it possible to visualize properties of distinct molecular complexes including their composition, proximity, state of association, and covalent modifications. Tom Jovin (Göttingen, Germany) explained how fluorescence microscopy-based approaches can be used to elucidate molecular interactions involved in signal transduction. He developed the concept of a fluorophore as a ‘photophysical enzyme’, and described the Förster (or fluorescence) resonance energy transfer (FRET) method as a unique approach in generating signals sensitive to molecular conformation and separation in the 1–10 nm range (Jares-Erijman and Jovin, 2003). Moreover, the exploitation of fluorescence polarization measurements such as anisotropy fluorescence lifetime imaging microscopy (rFLIM) and energy migration FRET (emFRET) are particularly suitable for probing rotational motion, association, and proximity of cellular components *in vivo* (Lidke *et al*, 2003).

With respect to fluorescent probes, the use of semiconductor nanocrystals known as quantum dots (QD) offers exciting new possibilities for imaging biological macromolecules. QDs are photostable, possess a broad excitation spectrum with a narrow emission band, they are small, bioconjugatable, and their detection is quantitative on a single QD level. This was successfully applied to track ErbB1 receptors on filopodia labelled with epidermal growth factor conjugated to fluorescent QDs with single molecule sensitivity (Lidke *et al*, 2005). The superior spectral properties of QDs point to their use for imaging RNAs as also demonstrated by their recent successful application for labelling mRNA *in situ* (Chan *et al*, 2005). It is probably not too far-fetched to think of an imaging system for small RNAs with one QD for each miRNA thereby allowing quantitative resolution of endogenous miRNAs.

Another elegant approach for conducting analyses at the single-cell level was presented by Ulf Landegren (Uppsala, Sweden). He described a variety of molecular tools based on DNA probes to visualize the distribution and interactions of single molecules in the cell. These tools comprise padlock probes (Hardenbol *et al*, 2003; Larsson *et al*, 2004), selector probes (Dahl *et al*, 2005; Stenberg *et al*, 2005), proximity probes (Fredriksson *et al*, 2002; Gullberg *et al*, 2004), and ‘microfabrication’ devices (Melin *et al*, 2005). These probes can be tethered to nucleic acid or protein molecules, and typically comprise unique identifier DNA sequence elements that serve as a code for the recognized target molecules. Detection of a specific molecular interaction is achieved by formation of specific DNA strands that can be recorded after suitable amplification. Local molecular interactions are thus used to ‘write’ a specific DNA sequence, thus allowing highly specific, sensitive, parallel, and localized measurements of any molecule, being it DNA, RNA, or protein, coupled with background-free signal amplification steps. Amplification is based on ‘rolling circle replication’ (Baner *et al*, 1998), a strategy that is highly specific, sensitive, quantitative, and is suited for multiplexed assays. Using microfluidic devices, the rolling circle products can be counted in real time, allowing for precise quantification and digital molecule detection over an extended dynamic range.

mRNA in focus

Although recent results point to the importance of ncRNAs in regulatory RNA networks, we should not forget that many central aspects of the expression of mRNA are still not understood, particularly the spatial and temporal aspects of transcription in the cell. These topics were addressed in three talks that presented *in vivo* fluorescence microscopy approaches and theoretical descriptions to elucidate the mechanisms of transcription.

Xavier Darzacq (Paris, France) reported that tracking the life and motion of mRNA molecules and mRNPs by fluorescence microscopy in living cells using RNA-binding fluorescent protein fusions such as the bacteriophage MS2 coat protein can be a versatile tool in studies of transcription (Bertrand *et al*, 1998; Shav-Tal *et al*, 2004a, b). In combination with the imaging of DNA using the capability of the bacterial lactose repressor to bind to DNA repeats of the lactose operator, it now becomes possible to visualize the different stages of gene expression, including changes in chromatin structure and in transcriptional rates, at a specific gene locus *in vivo* providing unique insight into how these processes are coordinated (Janicki *et al*, 2004). In addition, Darzacq described the kinetic analysis of the RNA polymerase II transcription reaction in living cells and revealed an unexpectedly long engagement of the enzyme with DNA. This suggests the presence of additional rate-limiting steps in the elongation reaction, as for example pausing of the RNA polymerase.

Ralf-Peter Jansen (Munich, Germany) focused on the imaging-based elucidation of the cytoplasmic localization mechanism of mRNAs. Specific cytoplasmic RNA localization is essential for generating high local protein concentrations via localized translation (e.g. β-actin, ‘pre-translational protein sorting’), cell lineage specification (e.g. germ plasm), gradients of morphogens (e.g. bicoid), and associations with specific substructures (e.g. cyclin B at centromeres). The

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localization signals mostly lie in the 3′UTR of the respective mRNAs and involve stem–loop structures, repetitive short sequence elements, and dsRNA parts containing bulges. These structures are recognized by a variety of RNA-binding protein complexes containing KL/RRM/RBD domains. Mechanisms for specific localization range from directional transport including molecular motors such as myosin/kinesin (Lopez de Heredia and Jansen, 2004), diffusion and trapping (e.g. *Drosophila* Nanos), local RNA protection (e.g. *Drosophila* Hsp83) to vectorial nuclear transport (e.g. *Chlamydomonas* β-tubulin). Insights into the respective mechanisms can be gained either by indirect labelling using GFP fusions of mRNA-binding partner proteins or by a variety of *in vivo* labelling approaches such as fluorescently labelled injected mRNA, MS2 tagging, or the use of injected binary 2′-OH-methyl molecular beacons in combination with FRET to enhance the signal-to-noise ratio. In the budding yeast, many localized mRNAs code for membrane and/or secreted proteins, a fact that raises the question about a coordination of mRNA localization and endoplasmic reticulum (ER) trafficking. Using live imaging, a synchronization of mRNP movement (visualized by the MS2-tagging system) and ER tubule movement could be observed (the localized mRNP moves with the ER in a 'piggyback' fashion), corroborating a coordination of mRNAs and ER (Juschke *et al*, 2004).

'Imaging' of RNA molecules or complexes, however, not only refers to the use of sophisticated techniques but may also include 'intuitive visualization'. Peter Cook (Oxford, UK) gave a fascinating example of how a 'simple' mechanical model may direct our view to important molecular details of the basal cellular transcription mechanism (Figure 3; Iborra *et al*, 1996). He demonstrated that active RNA polymerases cluster in 'transcription factories' to loop the intervening DNA. In HeLa cells, for example, the concentration of RNA polymerase II in such a factory is 1000-fold higher than that in the soluble pool so that essentially all transcription is likely to take place in factories. Factory density and diameter are constant in different cells of different organisms under different conditions despite an ∼11-fold range in C-value (haploid nuclear DNA content) and/or an ∼13-fold variation in nucleoplasmic volume. This model elegantly explains many transcriptional events: a promoter near a factory is more likely to initiate than a distant one. Therefore, productive collisions of the proximal promoter attract factors increasing initiation, whereas the longer a distant one remains inactive, the deeper it becomes embedded in heterochromatin (Cook, 1999, 2002, 2003). A prerequisite for this model is looping of DNA. In a theoretical analysis, entropic forces between engaged polymerases were identified to drive the self-organization of genomes into loops (Marenduzzo *et al*, 2006). The model is also consistent with results showing that transcription in a cell is a rare event; this may be beneficial for the cell, as a low transcription rate saves energy and is less mutagenic, and it simultaneously allows regulation over a greater dynamic range (Bon *et al*, 2006).

RNA—what's next?

In 1957, when Francis Crick summarized ideas on the flow of genetic information as 'DNA makes RNA, RNA makes protein, and proteins make us' at a symposium at the University College London (Crick, 1958), the function of RNA as the intermediate between gene and function seemed to be clear. In 2000, the central dogma in molecular biology was 'updated' by Gregory Petsko to 'DNA makes RNA makes protein, but sometimes RNA can make DNA and other times RNA makes

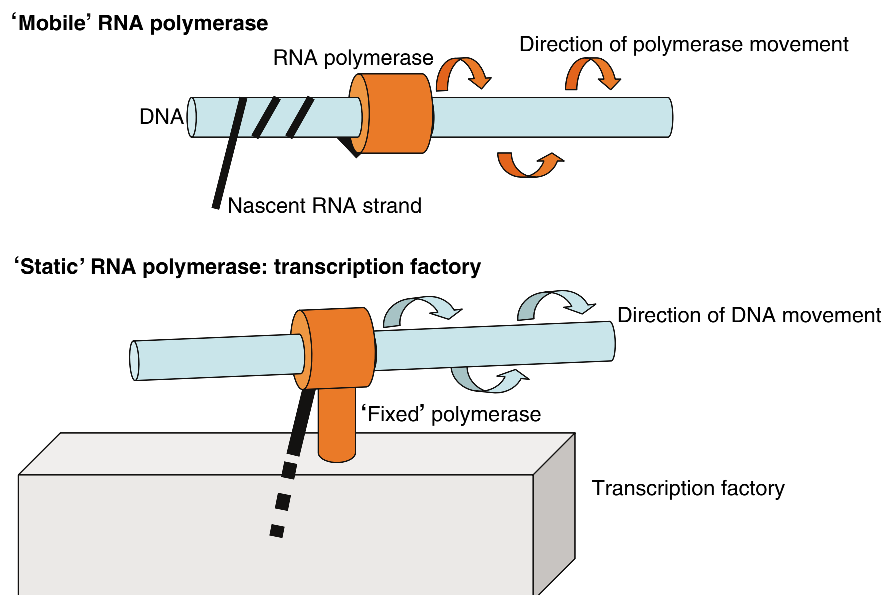

Figure 3 Schematics illustrating two models on RNA transcription with either a mobile or static RNA polymerase. Considering a fully mobile RNA polymerase that translocates and rotates (top panel), the nascent transcript would entwine about the template, and some mechanism would have to be found to untwine the tangle to allow the transcript to escape to the cytoplasm. The untwining problem can be eliminated if the DNA both translocates and rotates while the polymerase is essentially immobile because of its clustering in transcription factories (bottom panel).

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RNA, which makes proteins different from what they would be if only DNA made the RNA, and once upon a time RNA made protein, probably, but no-one knows for certain' (Petsko, 2000). Things obviously have become even more complicated since then. Not only fundamental questions concerning the 'known' RNA functions remain unresolved but RNA is now emerging as a key regulator of transcription and translation. In fact, it may well turn out that regulatory RNAs represent the major output of the genomes of humans and other complex organisms. The results and approaches presented in this symposium have illustrated the ever-increasing diversity of RNA functions in gene expression and translation. It also clearly indicated that we are only just beginning to explore the tip of what is undoubtedly a very big iceberg. Discoveries are however succeeding at a rapid pace and, as in the exploration of uncharted territories, it was apparent that a particular sense of excitement animates those who decided to delve deeper into the RNA (under)world.

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