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Review

RNA regulons and the RNA-protein interaction network

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Abstract

The development of genome-wide analysis tools has prompted global investigation of the gene expression program, revealing highly coordinated control mechanisms that ensure proper spatiotemporal activity of a cell's macromolecular components. With respect to the regulation of RNA transcripts, the concept of RNA regulons, which – by analogy with DNA regulons in bacteria – refers to the coordinated control of functionally related RNA molecules, has emerged as a unifying theory that describes the logic of regulatory RNA-protein interactions in eukaryotes. Hundreds of RNA-binding proteins and small non-coding RNAs, such as microRNAs, bind to distinct elements in target RNAs, thereby exerting specific and concerted control over posttranscriptional events. In this review, we discuss recent reports committed to systematically explore the RNA-protein interaction network and outline some of the principles and recurring features of RNA regulons: the coordination of functionally related mRNAs through RNA-binding proteins or non-coding RNAs, the modular structure of its components, and the dynamic rewiring of RNA-protein interactions upon exposure to internal or external stimuli. We also summarize evidence for robust combinatorial control of mRNAs, which could determine the ultimate fate of each mRNA molecule in a cell. Finally, the compilation and integration of global protein-RNA interaction data has yielded first insights into network structures and provided the hypothesis that RNA regulons may, in part, constitute noise 'buffers' to handle stochasticity in cellular transcription.

Keywords: gene regulatory networks; non-coding RNA; post-transcriptional gene regulation; protein-RNA interaction; RNA-binding protein.

Introduction

In 1961, François Jacob and Jacques Monod described the first gene regulatory system in *E. coli* – the bacterial *lac* operon (1). An operon refers to a single, polycistronic transcriptional unit composed of a cluster of functionally and/or structurally related genes that are under the control of a single regulatory unit that can be activated or repressed by a *trans*-acting factor. The operon concept was revolutionary as it implied that transcriptional units might bear information for the coordinate expression of functionally related proteins. Such an integrative architecture enables prokaryotes to quickly coordinate gene expression in response to environmental stimuli. However, DNA operons limit the flexibility to regulate the expression of genes individually and would therefore be detrimental to the regulation of genes that have multiple functions.

DNA operons, which lead to the production of polycistronic transcripts, predominantly occur in prokaryotes (2). In contrast, most eukaryotic genes are monocistronic, with their own promoter and transcription terminators at the 5′- and 3′-ends, respectively. So, how is the coordinated expression of functionally related genes achieved in eukaryotes? The answer is certainly complex because such coordination is seen at all stages of gene expression, from chromatin structure and transcription through to RNA processing, localization and decay to the synthesis of proteins and their post-translational modifications. Regarding the first steps in the control of gene expression, one of the earliest possibilities for coordination is provided by the chromatin structure of the genome. The structure can be separated roughly into heterochromatin, which is transcriptionally silent, and euchromatin, which governs access to transcription factors through the density of packaged structural proteins, such as the histones. Continuing from this, the physical linkage of genes allows for co-expression, exemplified by the homeobox genes, which form a sequential cluster on the chromosome to facilitate a developmental cascade (3). Ultimately, the transcription of genes is coordinated by a host of cell and tissue-specific transcription factors (TFs) (e.g., homeobox proteins) that bind to specific DNA promoter sequences and recruit RNA-polymerases for RNA synthesis. Hence, TFs generally activate sets of genes coding for proteins that are structurally or functionally related.

As cellular mRNA transcript levels are limited indicators of protein abundance (4–7), additional layers of control must exist to ensure coordinated protein synthesis. Moreover, the presence of the nuclear membrane in eukaryotes, which results in a spatial separation of transcription and translation, requires structures to coordinate production and distribution

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of the large number of mRNA molecules present in a cell (~150,000 mRNA molecules in a mammalian cell). Such post-transcriptional coordination is mediated by a plethora of RNA-binding proteins (RBPs) that dynamically associate with specific subpopulations of transcripts. Additionally, RBPs form ribonucleoprotein particles (RNPs) with small non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), that participate in complementary binding to mRNA targets and repress translation or promote decay of their target mRNAs. Hundreds of RBPs and ncRNAs are expressed in eukaryotes – most of them with unknown functions – suggesting an elaborate system for post-transcriptional control that may affect virtually every mRNA in a cell.

With the development of genomic tools, it became feasible to study the extent and logic of post-transcriptional gene regulation on a global scale. These technologies enabled the comprehensive identification of mRNAs specifically associated with RBPs and miRNAs (see below). They also enabled the comprehensive measurement of RNA processing events in the nucleus (e.g., alternative-splicing and polyadenylation), the systematic exploration of mRNA localization, and the global determination of mRNA decay and translation rates. In summary, genomics tools have provided important clues about the architecture of the post-transcriptional regulatory system. Importantly – and by analogy with prokaryotic DNA operons – they showed that groups of mRNAs coding for functionally related proteins are commonly regulated and/or bound to regulatory components, such as RBPs and/or miRNAs. This supports a model in which functionally related messages are dynamically controlled by *trans*-acting factors that interact via *cis*-acting elements in the RNA, forming so-called RNA regulons (8, 9).

This review summarizes concepts and features of RNA regulons and how their effectors are controlled. We mainly focus on cytoplasmic events that govern the coordination of the localization, translation, and decay of mRNA molecules. We highlight recent studies and observations that support and further extend the RNA regulon model, describing features of a highly complex post-transcriptional gene regulatory system.

Post-transcriptional gene regulation through RBPs

Post-transcriptional regulation is mainly controlled by RBPs that specifically bind to RNA molecules to form stable or transient RNP complexes. RBPs often contain characteristic RNA-binding domains that specifically interact with sequences or structural elements in the RNA, and the combination of multiple RNA-binding motifs can further increase the specificity and affinity for a given RNA (10–12). Prominent RNA-binding domains in eukaryotes are the RNA-recognition motif (RRM), the heterogeneous nuclear ribonucleoprotein K-homology domain (KH), zinc finger/knuckles domains (ZnF), and the double-stranded RNA-binding domain (10–13). In yeast, about 600 proteins (~10% of protein coding genes) are known or predicted to function as RBPs (14). In the worm *Caenorhabditis elegans* and in the fruitfly *Drosophila melanogaster* approximately 2% and in humans about 5% of the protein coding genes are annotated as 'RNA binding' (UniProt/ Swissprot release 2012_2). However, it is likely that the number of RBPs is even higher in light of the recent discoveries of many potentially novel RBPs (15, 16). Furthermore, several of the RNA-binding domains, such as the RRM, underwent drastic amplification during animal evolution (12), in parallel with the evolution of highly specific post-transcriptional processes, such as alternative splicing, allowing an increased genetic diversity from a limited repertoire of genes. This may have consequences for human disease: many mutations in RBP genes are linked to muscular and neurodegenerative disorders, which parallels rapid evolution of neurological functions in primates (17); an increasing number of RBPs are being linked to cancer by acting as oncogenes or tumor suppressors (18); some RBPs coordinate the initiation and resolution of inflammation (19).

Messenger RNAs can also be regulated via direct physical interactions with ncRNAs. The best-characterized class of such RNAs is the large family of microRNAs (miRNAs), ~22-nucleotide-long RNA molecules that negatively regulate gene expression in metazoans [reviewed in (20, 21)]. MicroRNAs are processed from larger precursors in a series of steps, and they are finally incorporated into the RNA-induced silencing complex (RISC) – the main component of which is a member of the Argonaute (Ago) protein family. 'Seed' sequences in the miRNA (typically seven to eight nucleotides at the 5′-terminus) guide the miRNA-loaded RISC (miRISC) complexes towards partially complementary sequences in 3′-untranslated regions (3′-UTRs) of target mRNAs and thereby inhibit translation or promote mRNA decay. Almost 2000 miRNAs have been discovered in human cells so far (22), with an increasing number of them linked to important physiological and developmental processes and diseases (23).

Ribonomics – global methods for the identification of RNA-protein interactions

Whereas many classical studies focused on the functional impact of individual RBP-target interactions, the recent development of genome-wide analysis tools, such as DNA microarrays or high-throughput sequencing, now allows researchers to acquire a 'systems' level view of post-transcriptional gene regulation and of the underlying RNA-protein interactions. In 1999, Robert Cedergren coined the term 'ribonomics' for the search for RNA genes, their structures, and functions (24). However, the term now refers to the global approaches aimed at the systematic identification of RNA molecules that are bound and regulated by RBPs. The field has developed enormously in recent years, and new techniques have been established that allow relatively precise mapping of RNA-protein interactions. These typically involve the affinity purification of an endogenous or epitope-tagged RBP, and the subsequent analysis of bound RNAs with DNA microarrays or high-throughput sequencing. In the following paragraphs, we briefly outline some of the experimental procedures that

have been developed in this field, and we refer the reader to some recent reviews on this topic for more details (25, 26).

One approach called RBP-immunopurification-microarray (RIP-Chip) was initially employed by Jack Keene's lab to study RNAs associated with three RBPs (human antigen B [HuB], poly(A)-binding protein [PABP], eukaryotic translation initiation factor 4E [eIF-4E]) in embryonal carcinoma stem cells (27). Thanks to its simplicity and robustness, this method has since been employed to determine the RNA targets of more than one hundred RBPs in mammalian cells, flies, worms, trypanosomes, and in yeast [a comprehensive list of RBP experiments is given in (28)]. It was also the first technique that was applied for the experimental exploration of miRNA targets through immunopurification of Ago proteins and other miRISC proteins from human cells (29–36), flies (37, 38), and worms (39, 40). Of note, RIP allows concomitant identification of the protein components of RNPs and other associated regulatory proteins by mass-spectrometry (MS) (41). Drawbacks of the RIP method include concerns that mRNAs may dissociate during the isolation steps or that other RNAs may associate with RNP complexes in the extract, or that the RBP interaction may be indirect rather than direct with a particular mRNA.

To circumvent some of these limitations and to detect relatively weak RNA-protein interactions, more elaborate procedures that involve crosslinking of RNA-protein complexes either by UV irradiation or with chemicals prior to immunoprecipitation have been developed. Two of these methods, termed cross-linking-immunopurification (CLIP) (42) and, if high-throughput sequencing is applied, HITS-CLIP, were originally established by Robert Darnell and colleagues (43, 44). Recent improvements in computational analysis of HITS-CLIP data (45, 46), and advances in the preparation of samples for sequencing [e.g., (47)], now allow binding site determination at nucleotide resolution [for a review see (25)]. Recently, a modification of the method was introduced by growing cells in the presence of the photoactivatable ribonucleoside 4-thiouridine (48), which enhances the UV-crosslinking efficiency between RBPs and their targets (49). As crosslinked 4-thiouridines cause the misincorporation of guanosines on the opposite strand of RNA during reverse transcription, this method – called PAR-CLIP [Photo Activatable Ribonucleoside enhanced CrossLinking and ImmunoPurification (48, 50); reviewed in (51)] – enables identification of binding sites in the RNA at single nucleotide resolution. The CLIP-based techniques and their variants have been extensively used during the last few years to map the RNA targets and binding sites of several RBPs in human and murine cell lines and tissues. This includes miRISC components (48, 52, 53), the Pumilio and FBF [Fem-3 binding factor] (PUF) domain family member Pumilio-2 (PUM2), the insulin-like growth factor 2 mRBPs 1, 2, and 3 (IGF2BP1-3), Quaking (QKI) (48), heterogeneous nuclear ribonucleoprotein (hnRNP) particles (47), T-cell intracellular antigen 1 (TIA1) and the related TIA1-like 1 (TIAL1) protein (54), the splicing regulators TAR DNA binding protein 43 (TDP-43) (55), Nova-1 and Nova-2 (43, 56), fragile X mental retardation protein (FMRP) (57), the FET family proteins fused in sarcoma (FUS), Ewing sarcoma breakpoint region 1 (EWSR1) and TATA box binding protein (TBP)-associated factor 15 (TAF15) (58), and human antigen R (HuR) (59, 60). Furthermore, PAR-CLIP has been applied in worms (61) and yeast (62). Of note, databases for the deposition and visualization of CLIP experiments, such as CLIPZ (63) and PARalyzer (64), have been set-up and are available to the community for data deposition or exploration (65). However, one drawback of CLIP procedures is that they are not well-suited for recovering RNP complexes for MS analysis, and the procedures are labor intensive. Finally, the exposure of cells to UV induces a stress response that could alter the RNP structure and add bias to target identification. This may become an important issue if differences in RNA target compositions are to be analyzed under low stress conditions (e.g., exposure of cells to drugs) or during cellular differentiation. Importantly, comparison of data obtained from parallel RIP-Chip and PAR-CLIP experiments to map the RNA targets of HuR, a human RBP that binds to AU-rich elements (AREs) in mRNA transcripts, revealed a substantial overlap in identified targets (60). It appears though that RIP-Chip enriches for stable, more functionally responsive interactions, whereas PAR-CLIP captures both stable and transient interactions (60). In conclusion, both methods are suitable for the comprehensive analysis of the RNA targets for RBPs; ultimately, the choice of method will depend on the resources available and the specific biological question to be addressed.

Features of RNA regulons

Coordination – organization of functionally related messages

Application of RIP or CLIP techniques combined with a systematic analysis of associated/bound RNA molecules via DNA microarrays or sequencing has provided valuable insights into the organization and conservation of post-transcriptional gene regulation (9, 28, 66). These studies also added strong support for the presence of RNA regulons, as most RBPs in eukaryotes appear to coordinate/regulate groups of mRNAs coding for functionally related proteins. Furthermore, this characteristic can be seen in diverse species, suggesting that RNA regulons constitute a universally conserved feature of post-transcriptional gene regulation in eukaryotes (28, 66). For instance, impressive RNA regulons are found among five yeast Puf-family RBPs, for which mRNA targets share striking common functional and cytotopic features (67). Most notably, the Puf3 protein binds almost exclusively to mRNAs coding for mitochondrial proteins, many of them acting in translation. Puf3p-bound messages contain a characteristic binding motif (5′-UGUAnAUA-3′), which is preferentially located in 3′-UTRs. The targets were slightly up-regulated in *puf3* mutant cells, which is consistent with observations that Puf proteins generally destabilize their mRNAs targets (67). Taken together, these results strongly suggested roles for Puf3p in the biogenesis and function of mitochondrial proteins. Indeed, later studies confirmed the roles of Puf3p in mitochondrial function and in the localization of mRNAs

(68, 69), which suggests that Puf3p manages an RNA regulon with an impact on the biogenesis and motility of mitochondria.

However, while many global analysis studies have revealed functionally related mRNA targets for RBPs, only a few of these putative RNA regulons have been validated for their biological/physiological impact. For example, in budding yeast, we have recently shown that the yeast La-related protein Sulfide production 1 (Slf1) associates with and stabilizes messages that are linked to copper homeostasis (70). Over-expression of Slf1p renders cells resistant to elevated copper concentrations in the medium, and this effect is dependent on the interaction of Slf1 with target mRNAs. From this, we deduced that Slf1p regulates a copper regulon, thus allowing cells to adapt to elevated copper concentrations (70). Such a mode of regulation would be analogous to a previously identified yeast RBP network triggering coordinated responses to iron deficiency (71). Under conditions of low iron, the RBP cysteine-three-histidine protein 2 (Cth2) down-regulates mRNAs encoding proteins that participate in iron-dependent processes, ensuring that the availability of limited iron is maximised. Cth2p interacts with AREs of target mRNAs in the nucleus, guides them to the cytoplasm and then promotes their degradation (72). Likewise, post-transcriptional control of iron homeostasis in mammals has important physiological consequences (73): The iron response protein binds to a number of mRNAs which code for proteins that mediate iron uptake or storage and either prevents their translation (binding to 5′-UTRs) or affects their stability (binding to 3′-UTRs). Thus, the post-transcriptional control of cellular programs for dealing with changing concentrations of transition metals appears to be a common theme in physiology.

In addition to the aforementioned well-characterized iron regulon, a small number of examples of mammalian RNA regulons with physiological implications in disease have been described [reviewed in (9, 19)]. One elegant example is the interferon (IFN)-gamma-activated inhibitor of translation (GAIT) complex, which becomes activated in IFNγ-induced macrophages and coordinates the temporal expression of groups of mRNAs coding for proteins that are pivotal to the acute immune response. The complex consists of four proteins (glutamyl-prolyl-tRNA synthetase, hnRNP Q1, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein L13A) that bind to the GAIT element, a 29-nt hairpin structure situated in the 3′ UTR of a group of mRNAs encoding inflammatory modulators, such as ceruloplasmin (74), a component of the acute phase inflammatory response. IFNγ strongly induces the transcription of ceruloplasmin, whereas the binding of the GAIT complex to its mRNA inhibits translation initiation. The assembly of the GAIT complex on the mRNA takes approximately 16 h, and thus the production of ceruloplasmin is turned off 16 h after IFNγ stimulation, with the effect that protein levels decline to those of uninduced cells. A failure of this mechanism has been linked to an increased risk of cardiovascular disease. Besides the GAIT complex, inflammatory response and cytokine production are also regulated by a group of RBPs that bind to AREs in the 3′-UTRs of mRNAs, such as tristetraprolin (TTP), AU-rich element Binding Factor 1 (AUF1/hnRNP D), KH-type splicing regulatory protein (KSRP), and HuR [reviewed in (75, 76)].

Whereas the direct physiological and biological impact of an RNA regulon is difficult to track, its coordination can often be seen by integration of RNA-protein binding data with other global datasets, particularly from transcriptomics experiments where the impact of altering RBP levels on the steady-state levels of RNA targets can be assessed. However, to date only a few studies have addressed directly and comprehensively how this extrapolates to changed protein levels. For example, we have recently performed a combined transcriptomic and proteomic analysis to measure the effects of the yeast RBP glucose inhibition of gluconeogenic growth suppressor 2 (Gis2) on experimentally defined target mRNAs (77). The protein contains seven retroviral type ZnF RNA-binding domains and binds to G(A/U)(A/U) repeats that are mainly located in the coding regions of hundreds of mRNA targets that can be grouped into functional categories such as rRNA processing, ribosomal function, and cell motility. The matched-sample analysis of the changes of mRNA steady-state levels and protein levels upon Gis2p overexpression revealed distinct outcomes for functionally related subsets of mRNA targets (77). Thus, the various mRNA targets of Gis2p appear to be grouped into different RNA regulons that are differentially regulated. One possible explanation for the differences in the impact of particular RBPs on subsets of mRNA targets could be that combinatorial control by other RBPs defines the regulatory outcome.

Two recent studies measured the impact of mammalian RBPs on their mRNA targets using a proteomics approach. The targets of Musashi-1, a human RBP regulating splicing, were defined by RIP-Chip, and effects of Musashi-1 knockdown on protein levels of respective targets were measured by quantitative proteomics (6). Likewise, transcriptome and proteome data were integrated for the targets of HuR identified by PAR-CLIP (59). In both examples, the proteomic changes correlated with changes seen at the mRNA levels. Likewise, SILAC (stable-isotope labeling by amino acids in cultured cells)-based methods have been employed to monitor the extent of miRNA-mediated translational regulation in cultured cells, showing a general reduction of protein levels of miRNA targets that was consistent with increased mRNA degradation (78, 79). However, a similar study in the worm *C. elegans* revealed different patterns of regulation, which suggests that certain miRNA targets are preferentially regulated at the level of translation rather than mRNA stability (40).

In the future, integrative studies that combine mRNA target analyses with transcriptome, translatome, and proteome analyses will be used to assess comprehensively the impact of RBPs or miRNAs on RNA regulons. This is of particular importance for the study of translational regulators, the functions of which may not be obvious from simple comparisons of mRNA levels. Notably, the cellular abundance of human proteins appears to be predominantly controlled at the level of translation (7), which further emphasizes the

necessity to integrate data from all levels of the gene expression program.

Modularity – is there an RNA code?

One of the most pressing questions is how RBPs recognize their cognate RNA targets. Knowledge of all binding elements of *trans*-acting factors in the transcriptome should lead us to an 'RNA code' that describes the fate of individual mRNAs. This code is comprised of 'letters' (sequence), 'words' (structural RNA recognition elements) and 'readers' (RNA binding domains), which together specify the regulatory impact exerted on RNA. However, unlike DNA-binding proteins that bind to relatively short sequence elements in the DNA, the situation is considerably more complicated with RNA, thanks to their complex structures that are difficult to predict or solve by computational and biochemical methods, respectively. Even if a trans-acting factor specifically binds to a short sequence motif, stable structures in the RNA could prevent its access. In addition to computational prediction, the recent development of new experimental techniques to probe RNA structures, for instance by high-throughput sequencing, will yield insights into the structural organization of the eukaryotic transcriptome – the 'RNA structurome' (80).

RBPs often recognize one or several sequence motifs in the primary target RNA via an array of RNA-binding motifs that add up to increase the specificity and affinity towards the RNA (11, 81). For example, in a large-scale study defining the mRNA targets for more than 40 yeast RBPs, the potential cognate consensus recognition sequences could be deduced by bioinformatic methods for 14 RBPs (14, 82). Similar global RIP-Chip approaches led to the identification of many binding sites for RBPs in different species (28, 77, 83). A well-characterized example of an evolutionarily conserved sequence recognition mode is provided by the PUF family of RBPs, whose members bind to single-stranded UGUAAAUA or closely related octamer motifs via eight repeats of the PUF-homology domain (67, 84–86).

An elegant approach to extending the binding repertoire of an RBP is the incorporation of small ncRNAs that are (partly) complementary to sequence stretches within targeted mRNAs. For example, members of the Ago protein family form miRISC complexes with miRNAs, the 5′-end seed regions of which form Watson-Crick base pairs with mRNAs. This and other base-pairing rules for miRNA target recognition, such as the presence of bulges or mismatches in the center region [nts 13–16 of the miRNA; reviewed in (87)], as well as the consideration of evolutionary conservation of putative target sites in the mRNAs, led to the development of various bioinformatics tools for prediction of putative miRNA targets [reviewed in (88)].

The combination of primary sequence and structural elements in the RNA could define functional RBP binding to many RNAs. However, these are relatively difficult to compute. For instance, the AREs recognized by HuR (e.g., the UAUUUAU constitutes the core recognition element of AREs and a high-affinity HuR motif) are generally of low information content (89). In this case, the introduction of CLIP-based assays greatly improved the identification of potentially biologically relevant mRNA targets by revealing the exact binding sites of the protein on the RNA (59, 60). Likewise, the application of PAR-CLIP to identify the mRNA targets for FUS and EWSR, which are highly conserved member of the FET family of RBPs with important roles in oncogenesis and neuronal diseases, revealed a combination of structural and sequence elements in the RNA target that directs the binding of these proteins to it (58). The implementation and refinement of transcriptome-wide analysis and bioinformatics tools [e.g., (90)] to reveal the recognition motifs of post-transcriptional regulators and to decipher the underlying RNA code, remains a major challenge for the future [e.g., (77, 83, 91)].

Dynamics – rewiring the RNA-protein network

Most reported studies on RNA-protein interactions have focused on single physiological conditions, and only a few studies have described the dynamic rewiring of RNA-protein networks or RNA regulons in response to perturbations or during development (27, 84, 92). Dynamics in RNA-protein interactions could be inferred by transcriptional responses that change the levels of RNA targets. However, it can also involve the direct modulation of RBP activity or of co-factors of RNP complexes, e.g., through posttranslational modifications or altered localization in the cell (Figure 1).

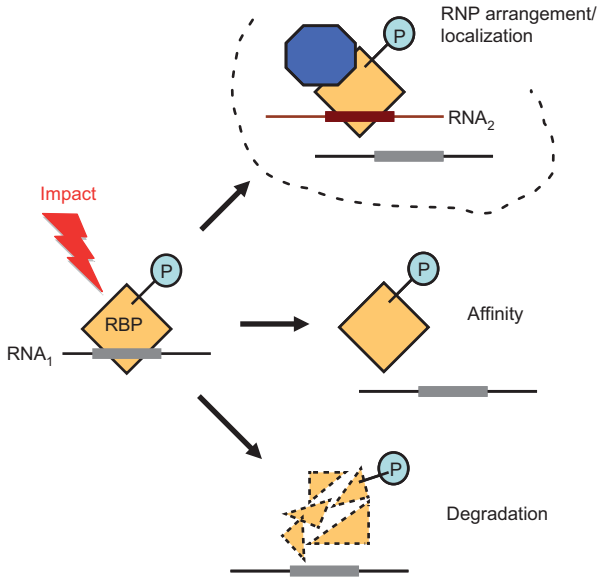

Figure 1 Dynamics of RNP complexes.
Changing internal or external conditions can lead to substantial modulation of RBP activity, which is often accompanied by post-translational modifications (depicted as a phosphorylation; P). In one instance (upper right), phosphorylation of an RBP can lead to altered RNP composition or re-localization, which often goes along with the differential association of RNAs. In another instance (middle right), post-translational modifications change the affinity of an RBP towards RNA. In a third instance (lower right), RBPs can get tagged for degradation, thus causing release of RNA targets. Conversely, RBPs can get stabilized by post-translational modifications (not shown).

Perhaps the best-investigated RBP in the context of RNP dynamics is HuR. Under normal conditions, HuR is predominantly located in the nucleus. Upon stress or proliferation signals it shuttles to the cytoplasm, where it stabilizes AU-rich mRNAs for translation. Applying RIP-Chip to identify mRNAs associated with HuR in activated Jurkat T cells – a commonly used model for T-cell activation – revealed substantial differences in the HuR RNA target spectrum in stimulated compared to resting cells (92). HuR was associated with different functionally related sub-populations of mRNAs: sets of transcripts acting in pathways in which HuR has known roles, such as 'wingless and Int-1 signal pathway (Wnt) signaling', 'metabotropic glutamate receptor signaling', or 'aging' were generally less associated with HuR, whereas HuR binding to messages coding for 'TFs' or 'RBPs' was significantly increased during T-cell activation. The dynamic profile of these associations was then used to make correlations with gene expression profiles contained in the Connectivity Map (93) (a database of dynamic gene expression data of cultured cells responding to different small molecules). This analysis identified resveratrol as a likely candidate that could oppose HuR activity (92). Thus, the dynamic profile of RBP-RNA associations can be used to select drugs/small molecules that selectively impinge on post-transcriptional gene regulation, offering potential for the discovery of new drugs that target post-transcriptional processes. Another recent study characterized HuR-associated mRNAs upon ionizing irradiation (94). Instead of a re-sampling of mRNA targets, a substantial release of mRNA targets was observed and correlated with phosphorylation of HuR by checkpoint kinase 2 (Chk2). Notably, this was associated with improved cell survival (94).

As exemplified for HuR, there are numerous examples on how the activity of an RBP is modulated by posttranslational modifications. For instance, ubiquitination influences the stability of HuR, which is degraded by the ubiquitin-proteasome pathway following a moderate heat shock (95). Certain classes of RBPs – primarily hnRNPs and serine/arginine-rich (SR) proteins – are site-specifically methylated by arginine methyltransferases (PRMTs) [reviewed in (96)]. Arginine methylation affects the assembly of small-nuclear RNPs, essential components of the spliceosome, and further guides the subcellular localization of both SR and hnRNP proteins. Absence of arginine methylation also induces changes in co-transcriptional recruitment during mRNP biogenesis (96). Other modifications of RBPs may be less prominent but nevertheless have strong functional consequences, such as acetylation (97, 98) or sumoylation (99, 100). Noteworthy, it was recently shown that hydroxylation by a type I collagen prolyl-4-hydroxylase increases the stability of Ago2 protein, which leads to its accumulation (101), particularly under hypoxic conditions; this increases the level of many miRNAs (among them several oncomirs) and enhances the endonuclease activity of Ago2 (102) and could therefore have direct implications for cancer progression. In conclusion, these studies emphasize that an analysis of post-translational modifications to RBPs upon changing internal or external conditions may be crucial to understanding the dynamics of post-transcriptional gene regulation.

Combinatorial control – synergistic and antagonistic interactions between RNAs and RBPs

The presence of multiple distinct binding elements for *trans*-acting factors on the same transcript suggests strong combinatorial regulatory potential and flexibility in terms of redeploying mRNAs to differentially regulated sub-networks or RNA regulons (Figure 2). There is now ample evidence for robust combinatorial control from large-scale genomic studies. A large-scale survey analyzing the RNA targets for 46 RBPs from the yeast *Saccharomyces cerevisiae* showed that RBPs can bind from as little as a few to up to several hundred mRNAs (14). Occasionally, strong overlap of the targets of RBPs, particularly between those that act in similar processes or that physically interact, have been observed. On average, each mRNA interacted with three different RBPs in this study. Extrapolation to the roughly 600 RBPs in yeast indicates that each mRNA molecule may interact with dozens of different RBPs during its lifetime (14). This putative combinatorial arrangement of RBPs on RNA might define particular regulatory effects on subsets of mRNA targets – as exemplified by the yeast KH domain protein 1 (Khd1p) or Gis2p, where differential association of RBPs to subsets of targets leads to different regulatory outcomes [e.g., translational control vs. mRNA decay (77, 103); see also above]. A strong overlap of mRNA targets was also demonstrated for the mammalian RBPs HuR and AUF1, which share 57% of their mRNA targets (104). It was suggested that the composition and fate of

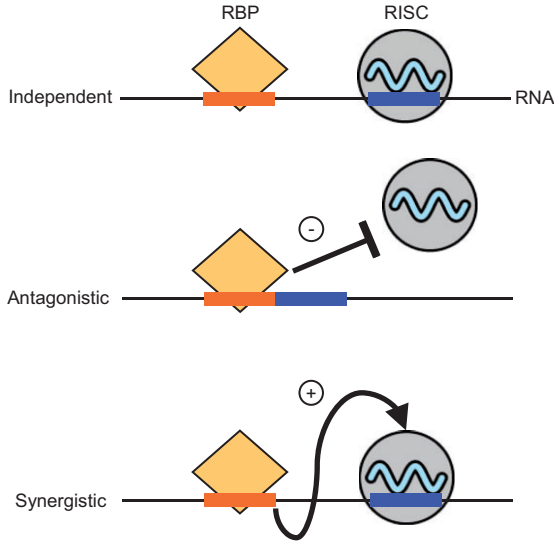

Figure 2 Modes of combinatorial control of mRNAs by RBPs. *Trans*-acting factors – here exemplified as an RBP (yellow) and a miRISC complex (blue) – may act independently of each other on the same RNA molecule (*cis*-acting elements in the RNA are highlighted). The resulting additive effects can, for instance, be seen with miRNAs (119). On the other hand, *trans*-acting factors may act cooperatively, either by competing for the binding to the same RNA molecule (e.g., binding of HuR prevents action of miR-122 or by synergistic interactions (106), or synergistically (e.g., PUM binding changes the local structure of the p27 mRNA required for miR-222 RISC binding (107).

HuR- and/or AUF1-containing RNP complexes on a particular target mRNA likely depends on the RBP abundance, stress conditions, and the subcellular compartment (104).

There are several examples describing the interplay between RPBs and miRNAs (105). One early example showed that HuR, upon translocating from the nucleus to the cytoplasm following exposure to stress, relieves cationic amino acid transporter 1 (*CAT1*) mRNA from miR-122 mediated repression in the cytoplasm of liver cells (106). Likewise, Dead End 1 (DND1) counteracts the function of several miRNAs in human cells, and in zebrafish primordial germ cells, by antagonizing miRNA association with mRNA targets (107). A global analysis of the targets of the human PUF family proteins PUM1 and PUM2 in human cancer cells revealed that PUM binding motifs were enriched in the vicinity of predicted miRNA binding sites. Likewise, high-confidence miRNA binding sites were generally enriched in the 3′-UTRs of the experimentally determined PUM targets, predicting strong crosstalk between human PUF proteins and miRNA targeting (85). Indeed, PUM1 induces a structural change within the 3′-UTR of the tumor suppressor cyclin-dependent kinase inhibitor 1B (CDKN1B/p27) transcript, thus permitting access to miR-221/222 with consequences for cell cycle progression (108). Likewise, human PUM proteins cooperate with miRNAs for regulation of the transcription factor 3 (E2F3) oncogene (109). Thus, in contrast to HuR and Dnd1 outlined above, PUM proteins and miRNA appear to interact synergistically to dampen gene expression.

To our knowledge, it is not known whether miRNAs directly interact with each other. Nevertheless, mRNAs, but also transcripts from pseudogenes or long ncRNAs (lncRNAs) (>200 nts), could serve as endogenous 'miRNA-sponges' in order to buffer levels of available miRNAs, as demonstrated by Poliseno et al. (110): Overexpression of the pseudogene phosphatase and tensin homolog tumor suppressor (PTEN) P1 led to a Dicer-dependent increase of the tumor suppressor protein PTEN. The proposed explanation for this effect is that the pseudogene transcripts sequester miRNAs that may otherwise target PTEN for negative regulation. Interestingly, the PTENP1 locus is selectively lost in several human cancers, and thus its absence could contribute to the reduced expression of PTEN in cancer cells. Therefore, pseudogenes and other lncRNAs could serve as 'competing endogenous' RNAs (ceRNA), suggesting that, in addition to the functional miRNA → RNA interaction, a reverse RNA ← miRNA sequestration may occur where mRNAs compete for a limited 'reservoir' of miRNAs (111). In this regard, it is likely that RBPs may become critically involved in this model as well, as they may bind to and ultimately define the access and activity of ceRNAs (Figure 2).

Towards RNA-protein interaction networks

It has been recognized for some time that RBPs tend to bind to messages coding for regulatory proteins such as RBPs and TFs. In line with this 'regulator of regulator' concept, a recent comprehensive analysis of the yeast RNA-protein interaction network indicated that a significant fraction of RBPs potentially regulate their own expression at the post-transcriptional level, either directly or indirectly via other RBPs (112). Remarkably, there is a significantly higher incidence of potential autoregulatory loops among RBPs (~40%) than among TFs (~10%) (112–114). Autoregulation provides a means for independent control of expression, which could be crucial in processes that require rapid responses to intracellular changes or external perturbations, such as during development or cellular differentiation, where RBPs dictate the fate of transcripts in a just-in-time fashion [e.g., (115)]. Autoregulatory RBPs further exhibit low protein expression noise, and they are usually highly connected with other RBPs, either by direct interactions or by binding to mRNAs coding for other RBPs (112). In addition to autoregulatory circuits, other motifs were also found to be commonly overrepresented among RBPs, such as the feed-forward loops, wherein a top-level RBP controls two target transcripts and one of these further regulates the other target (112). This apparently highly dense post-transcriptional network among RBPs is supported by direct experimental evidence obtained with human cells showing post-transcriptional gene regulatory interactions among several ARE-binding proteins (HuR, AUF-1, TIA1, KSRP) (116): This group of RBPs is controlled, at least in part, at the post-transcriptional level through a complex circuitry of self- and cross-regulatory RNP interactions.

A recent system-wide study integrated transcriptional and translational data in yeast (117) with large-scale regulatory networks of TFs and RBP interactions (114). This work confirmed the presence of feedback and other network motifs among post-transcriptional regulatory networks. Interestingly, from analysis of cell cultures subjected to six different conditions of stress, clusters of functionally related genes were strongly co-expressed and functionally more coherently connected to the translatome data (i.e., all mRNAs associated with ribosomes engaged in translation) rather than to the transcriptome data (which reflects the steady-state levels of mRNAs). This study also suggested that RBPs often partner with specific TFs to filter noise and equalize expression profiles of translationally co-regulated, but only weakly coexpressed genes (114). Therefore, post-transcriptional regulation may fine-tune the transcriptional output signal by filtering noise from transcriptionally co-regulated genes.

Expert opinion

Although the study of individual RBP-RNA interactions remains indispensable for the discovery of regulatory mechanisms and for biological validation, a number of global analysis tools have now been developed that allow us to obtain a comprehensive picture of the RNA-protein interaction network of an organism. Integration of global RNA-protein interaction data with measurements of steady-state mRNA levels, ribosome/polysome profiling data, and large-scale quantitative proteomics data will become popular approaches to determine the impact of particular post-transcriptional regulators on splicing, translation and/or mRNA degradation

(31, 40, 77–79, 118). Nevertheless, the meaningful integration of data obtained from different levels of the gene expression program will remain a major challenge and will promote the development of intuitive computational analysis tools that are readily available to support biologists in the generation of new hypotheses.

Whereas the development of new techniques to define the targets and binding sites of RBPs has greatly advanced over recent years, we still have very limited information about how RNA regulons are connected to biology and physiology. This is a demanding task as the regulatory impact of RBPs and miRNAs on mRNA targets is often relatively weak (frequently less than two-fold). Currently, there are two major approaches for experimental validation of RNA-protein interactions. The first approach is mutating a *cis*-acting element in a particular RNA target to abolish the binding of a *trans*-acting factor (RBP/miRNA). The impact is then measured with 'artificial' reporter constructs. However, this type of analysis cannot be currently expanded to monitor the impact of dozens of RNA targets of an RBP. Hence, the development of new tools is needed to specifically target the *cis*-acting elements of entire RNA regulons. This could be achieved by blocking the binding sites for a *trans*-acting factor with antisense oligonucleotides or small molecules. In the second approach, the potential involvement of a particular *trans*-acting factor on RNA regulation is analyzed by over-expression or silencing of the *trans*-acting factor in a cellular system or *in vivo*, followed by a specific or global read-out of changes in the fate of gene products (mRNA or protein). However, the high level of cross-talk between several key players of the post-transcriptional system could lead to substantial secondary effects, which may mask the direct effects on subsets of targets or RNA regulons. As such, it is difficult to fully understand functions of miRNAs without consideration of the RBPs because of the high degree of cross-talk between them. We tentatively suggest that one function of RBPs may be to protect mRNAs from the action of miRNAs and other recently discovered ncRNAs.

Outlook

Diverse tools for the transcriptome-wide analysis of RBP targets have been developed (e.g., RIP/CLIP). Their implementation by different laboratories allows rapid expansion of our knowledge of binding elements and RNA targets for dozens or hundreds of RBPs from various species. Systematic analyses have hitherto been mainly performed with cultured cells grown under one set of conditions. Future studies are likely to focus on system-wide investigations of the dynamics of mRNP and RNA regulon composition in response to environmental perturbations during development and in diseased states. These efforts will generate huge datasets requiring new databases and tools for the integration of data obtained from the study of different levels of gene regulation. Sophisticated and intuitive computational methods will need to be devised or refined to understand the logic and architecture of post-transcriptional gene regulatory circuits. In the long term, we will make advances in the development of predictive models based on 'RNA codes'.

Wide-scale data integration to gain a holistic view of post-transcriptional gene regulation in combination with specific, streamlined and standardized follow-up procedures will enhance our currently rather fragmented picture of how RNA regulons perform in biological/physiological processes. Importantly, we will hopefully acquire more information on how perturbation of RNA regulons and the underlying RNA-protein interactions are linked to human disease. This knowledge is likely to lead to the development of novel drugs targeting RNA regulons, either through specific interactions with *cis*-acting elements or through direct targeting of regulatory RBPs.

Highlights

- The development of robust high-throughput sequencing techniques enables the comprehensive discovery of the RNA targets for RBPs. In combination with crosslinking procedures, it allows high resolution mapping of *cis*-acting elements within RNAs (e.g., CLIP procedures).
- Integrative analysis of global protein-RNA binding, transcriptomics and proteomics data provides evidence for coherent responses by RNA regulons.
- Evidence for combinatorial regulation of mRNAs by RBPs suggests that RBPs and/or miRNAs may interact independently, antagonistically, or synergistically. Combinatorial control may direct different outcomes for subsets of functionally related mRNA targets.
- Computational analysis of the architecture of the RNA-protein interaction network suggests that RBPs are highly connected and are subject to autoregulation.
- By organizing mRNAs into RNA regulons, RBPs may potentially reduce transcriptional noise in eukaryotes.

Glossary

DNA operon: A cluster of functionally related genes under the control of a single regulatory region. Genes are generally transcribed as one polycistronic mRNA.

Proteome: The entire set of proteins present in a cell or tissue at a given time, including all isoforms and other variations.

Ribonomics: The systematic study of ribonucleoprotein complexes through systems-wide analysis tools, such as DNA microarrays or high-throughput sequencing.

RNA regulon: A ribonucleoprotein structure that coordinately regulates sets of functionally related mRNAs.

RNA code: A postulated 'cipher' or code within RNA molecules. *Cis*-acting elements, often present in the untranslated regions of mRNAs, serve as 'letters' of the code and direct *trans*-acting factors, such as RBPs or ncRNAs, to the RNA, thereby influencing their expression.

RNA structurome: The entire set of the structural ensembles of the transcriptome.

Transcriptome: The entire set of transcribed RNA species in a cell or tissue at a given time.

Translatome: The entire set of transcripts associated with ribosomes in a cell or tissue at a given time.

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