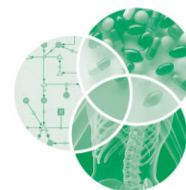
Focus Article



Circuitry of mRNA regulation

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Some of the classical paradigms of gene regulation have been challenged by global-scale analysis of eukaryotic transcriptional and post-transcriptional gene regulation (PTGR), made possible by the development of genomics and proteomics tools. Post-transcriptional events in particular are increasingly being recognized as important sources of gene regulation. The hundreds of regulatory RNA-binding proteins that exist in eukaryotes may regulate dozens to hundreds of functionally related RNA targets. Likewise, the expression of considerable fractions of many eukaryotic genomes is affected by hundreds of non-coding RNAs, e.g., microRNAs. These findings suggest an enormous regulatory potential for PTGR that may affect virtually every message in a cell. All gene regulatory systems are composed of simple network circuits that coordinate the transfer of regulatory signals to a target gene/message.. © 2009 John Wiley & Sons, Inc. *WIREs Syst Biol Med*

INTRODUCTION

On the basis of recent global studies identifying targets of transcription factors (TFs), RNA-binding proteins (RBPs) and microRNAs, we highlight some of the network motifs that have been found in both transcriptional and post-transcriptional control. We then consider the crosstalk between the different systems, including some recent studies proposing novel mechanisms of interaction. Finally, we speculate how the data and future work could add up to the construction of a composite network model of gene expression regulation.

Gene expression in eukaryotes is regulated at multiple levels. However, transcriptional control, mediated by TFs assembling on DNA sequences proximal to their target genes and recruiting RNA polymerases for the synthesis of primary RNA transcripts, has been largely considered to be the primary force regulating gene expression. Although it is well established that RBPs mediate numerous processes that are essential for the processing, quality control, localization, translation and turnover of RNA transcripts,[1–4] the enormous regulatory potential of this class of proteins may have been underestimated. Besides RBPs, mRNAs are also post-transcriptionally regulated via direct physical interactions with small ncRNAs. The best characterized class of such RNAs is constituted by microRNAs (miRNAs), ∼22-nucleotide-long RNA molecules that negatively regulate gene expression.[5,6] The relevance of post-transcriptional gene regulation (PTGR) is evidenced by considering the limited correlation between mRNA abundance and respective protein levels,[7–9] and the fact that the hundreds of RBPs and miRNAs encoded in eukaryotic genomes,[10] may equal or even surpass the number of members in other classes of gene regulators, such as TFs and post-translational modifiers.

Strikingly, many features of RBP- and miRNA-mediated gene regulation closely resemble those of TFs: While TFs generally bind DNA motifs upstream of a given gene,[11–13] RBPs and miRNAs typically bind sequence or structural features of mRNA molecules, primarily located in their untranslated regions.[6,10,14,15] Moreover, much like TFs are organized into transcription initiation complexes or ‘enhanceosomes’, RBPs and miRNAs assemble into highly dynamic transient ribonucleoprotein complexes termed mRNPs and miRNPs, respectively; these complexes assemble on specific targets in a combinatorial fashion, and their composition may eventually define unique fates for each target.[16–22] Finally, TFs, RBPs and miRNAs often bind targets that code for functionally or cytotopically related proteins.[10,12,16,23–26] The development and application of genome-wide analysis tools like DNA microarrays revealed fundamental insights into the logic of gene regulatory programs. Chromatin immunoprecipitation (ChIP-Chip) assays have been implemented to systematically map the binding sites of DNA-associated proteins, leading to the identification of transcriptional network motifs.[27–29] For instance, Young and colleagues systematically analyzed the TF-binding sites for almost

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all known transcriptional regulators (203 proteins) in *Saccharomyces cerevisiae*.[12,26] Likewise, ribonomics approaches have revealed the RNA, in particular, the mRNA targets for dozens of RBPs and thus shed light on the organization of PTGR systems.[16,19,24,30–32] Recently, Brown and colleagues have identified RNA targets for more than 40 tagged yeast RBPs with RBP ImmunoPurification followed by DNA microarray analysis (RIP-Chip) experiments.[10,23,33] Finally, several recent studies globally identified targets of individual human miRNAs, either using quantitative proteomics,[34,35] or a RIP-Chip approach,[36–38] each in the presence or absence of specific miRNAs. For the latter approach, mRNAs were defined based on their association with members of the Argonaute family of RBPs, essential components of miRNPs and mediators of the binding of miRNAs to their cognate targets.[18]

On the basis of these systematic investigations on the targets of TFs, RBPs and/or miRNAs and with a focus on PTGR, we summarize here some of the basic circuitries of gene regulation and elaborate on the interplay between transcriptional and post-transcriptional gene regulatory networks (GRNs). For a more detailed discussion of the concepts of GRNs, the reader may also consult some related reviews.[39–41]

BASIC COMPONENTS OF GRNs

In contrast to linked list and hierarchical data structures, networks are multidimensional and thus neither exclusively linear, unidirectional nor 'rooted'. In the case of GRNs, regulators and targets are interconnected by particular regulatory events that either activate or repress the expression of the target. Whereas both TFs and RBPs either increase or decrease the expression of their targets, miRNAs are thought to predominantly repress the target expression, although some exceptions have been reported.[42,43]

Multiple Outputs: One Regulator Binds many Regulatees

As GRNs are nonlinear, within each class of regulatory molecules there are instances where one regulator binds to and controls the expression of two or more targets (Figure 1A). The regulator is usually activated by a signal which could either be an inducer molecule that binds to the regulator or a protein modification of the regulator mediated by a signal-transduction cascade. The frequency of this network motif in GRNs is apparent from elaborate studies analyzing TF-binding sites and RBPs in the yeast *Saccharomyces cerevisiae*.[10,12,26] Lee et al. found that each of 106 yeast TFs under study bound up to 181 promoter regions ($P < 0.001$), with an average of 38 bound promoter regions per regulator.[26] Similarly, Hogan et al. found that 43 of the 46 RBPs—including two 'negative' control proteins—bound more than one RNA target; 11 of them binding less than 10 targets, and 6 of them binding more than a 1000 different RNAs, mainly mRNAs [false discovery rate (FDR) < 1%].[10] Although perhaps attributable to the different confidence levels applied for target definition as well as a bias from the RBP selection, it is nevertheless striking that RBPs are associated with an average of about 300 mRNA targets, a number almost 10 times as high as the average number of targets for TFs. Whether the larger numbers of RBP targets go along with diminished regulatory impact on individual messages or have other functional implications is not known; yet it illustrates that PTGR networks are at least as densely constructed as TF systems.

A similar scenario has been observed for miRNAs, where five recent studies have systematically analyzed potential targets for individual human miRNAs, suggesting that each miRNA may bind to and regulate between dozens and thousands of mRNAs.[34–38] For example, Karginov et al. found that Argonaute 2 (Ago2) proteins associated with 294 unique messages upon overexpression of *miR-124* in HEK293 cells.[37] Applying the same approach, Hendrickson et al. defined 419 *miR-124* target messages, which substantially overlapped with the ones defined by Karginov et al.[36] Applying a quantitative proteomics approach, Selbach et al. found the abundance of 1544 proteins changed in response to *miR-124* overexpression.[35] Although this number will certainly include secondary effects, it clearly demonstrates the far-reaching consequences of PTGR.

Multiple Inputs: Many Regulators Bind One Regulatee

Another hallmark of nonlinear GRNs is cooperative control, which implicates the binding of two or more regulators to a single target (Figure 1B). Lee et al. mapped almost 4000 individual interactions between TFs and promoter regions, providing evidence for the regulation of 2343 of 6270 yeast genes (37%) and an overall connectivity of 1.7 TFs per gene.[26] Likewise, Hogan et al. mapped 12,000 individual interactions between 46 RBPs and 4300 mRNAs, indicating a fairly dense overall connectivity (2.8 RBPs per message).[10] Extrapolating this to the hundreds of regulatory RBPs present in yeast, each mRNA message might interact with a dozen or more different

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RBPs during its lifetime. These data indicate that RBPs possess a considerably higher combinatorial arrangement than TFs, supporting our speculation that PTGR networks are meshed more densely than transcriptional networks.

Cooperativity has also been observed for miRNA-mediated PTGR. For example, expression of NOTCH1, a major receptor protein of the Notch signaling pathway, appears to be combinatorially regulated by at least four different miRNAs (*miR-24*, *miR-27b*, *miR-34*, and *miR-236*).[44,45] Likewise, synthesis of the phosphatase PTEN, a tumor suppressor gene and downstream target of NOTCH1 signaling,[46] is controlled by at least three different miRNAs.[47–49]

Autoregulation

The directional regulatory events described above are passed down from one or more regulators to one or more regulatees (e.g., regulons). A true network, however, is multidimensional, such that each regulator is itself subject to regulation. For instance, circular motifs are ubiquitously found within GRNs. The simplest form of such a circuit is constituted by an autoregulatory loop, where one regulatory molecule activates (positive feedback loop) or inhibits (negative feedback loop) its own production or activity (Figure 1C). Importantly, autoregulatory loops are thought to be crucial for the modulation of the response time of gene circuits to a signal and to affect cell–cell variation in protein levels ('noise'). While negative autoregulation generally speeds up the response time of transcriptional gene circuits and reduces cell-cell variation in protein levels, positive autoregulation leads to slowed response times and variation is usually enhanced.[39] These circuits may allow rapid adaptation to new environmental conditions (negative loop) or to differentiated states (positive loop).

Global TF-binding site analysis in yeast revealed 10 high confidence ($p < 0.001$) autoregulatory loops among the interrogated 106 yeast TFs (9%).[26] Likewise, 9 out of the 46 RBPs (20%) surveyed by Hogan et al. were reproducibly associated with their own message.[10] Interestingly, this number is doubled to 18 autoregulation loops (39% of all studied RBPs) when applying a less stringent cutoff (FDR < 5%). In contrast, miRNAs have not been reported to be organized in autoregulatory loops because miRNAs are not translated but rather target mRNAs in the cytoplasm.[5] Whether the high incidence of such loops in RBP-mediated PTGR has general regulatory implications remains to be analyzed.

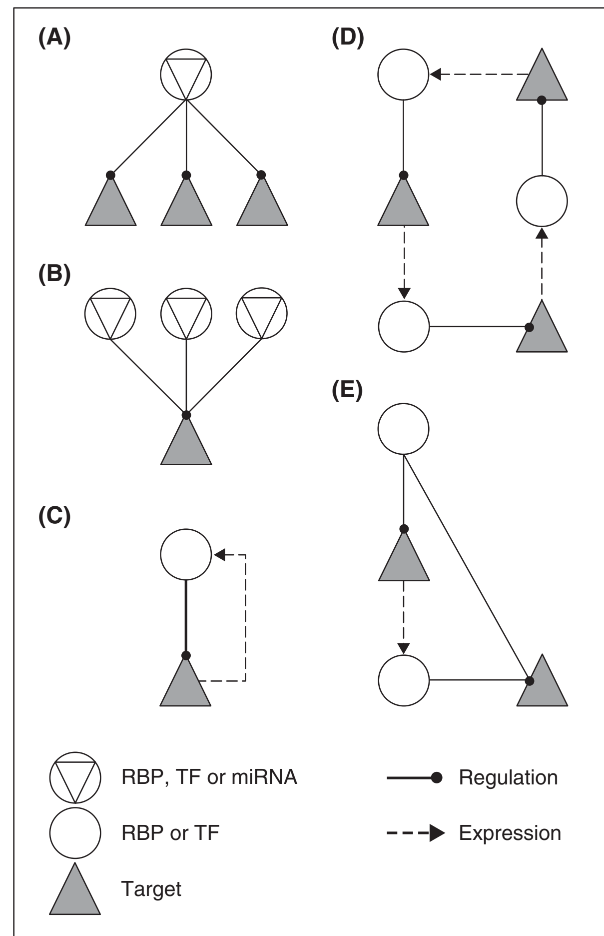

FIGURE 1 | Basic network motifs in transcriptional and post-transcriptional gene regulation. Each motif consists of one class of gene regulators, which may either activate or repress target gene expression. Note that miRNAs generally, but not always, act as repressors.[42,43] (A) Regulation of multiple messages by a single regulator. (B) Regulation of a single message by multiple regulators. (C) Autoregulation of gene regulators. (D) Multi-component regulatory loop. (E) Feed-forward loop.

Two- or Multicomponent Loops

More complex regulatory loops are composed of two or more components of a class of regulators (Figure 1D). Lee et al. identified three distinct multicomponent loops that consist of two, and in one case three TFs.[26] Hogan et al. did not explicitly analyze the data from their survey on protein–RNA interactions for the presence of multicomponent loops.[10] We therefore re-analyzed the raw data considering all RNA–protein associations with a FDR of less than 5% (Kanitz and Gerber, unpublished results). We found that 29 RBPs bound to mRNAs coding for at least 1 of the 46 RBPs under study. At least 21 of these 29 RBPs are arranged in multicomponent loops. In particular, we identified 13 two-component and 16

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three-component loops, involving 16 and 17 different RBPs, respectively (Kanitz and Gerber, unpublished data). This high incidence of multicomponent loops among RBP targets is intriguing and naively suggests great regulatory potential. One example of how multicomponent loops may influence gene regulation is represented by feed-forward loops, a special kind of multicomponent loop which involves the regulation of one or more common targets by two regulators, one of which being under the regulation of the other (Figure 1E). Feed-forward loops can trigger delayed responses to signals, which can be useful to filter out spurious pulses of signals.[39]

INTERPLAY BETWEEN GENE REGULATORY SYSTEMS

Since TFs, RBPs, and—to a lesser extent—miRNAs, share basic network motifs, we will now discuss their compatibility and connection, prerequisites for a composite GRN. In the following, we highlight some examples for interactions between RBPs and TFs, and miRNAs respectively.

RBPs Versus TFs

RBPs are selectively regulated by transcriptional activators or repressors. Among 561 known and predicted yeast RBPs,[10] 279 (50%) were targeted by at least one of 106 TFs surveyed by Young and colleagues.[26] This fraction is considerably larger than the total fraction of regulated genes in the genome (2343 regulated genes out of 6270; 37%). Conversely, the messages encoding TFs require the activity of RBPs for their maturation, decay, localization, and translation. Interestingly, 94 out of the 106 TFs surveyed by Lee et al. (87%) were bound by at least one of the 46 RBPs analyzed by Hogan et al. (FDR < 5%).[10,26] Although the study was not exhaustive, this analysis underpins recent observations that RBPs tend to control other gene regulators, such as RBPs and TFs (Figure 2A). Such a 'regulator of regulators' concept has recently been established for particular human RBPs and hence, may possibly be evolutionarily conserved.[50]

RBPs Versus Micrornas

Besides the miRNAs-processing factors required for the biogenesis of all miRNAs, such as the nuclear microprocessor complex,[51] and TRBP/Dicer,[52] recent findings suggest the existence of RBPs that selectively act on specific miRNAs or miRNA families. Lin28, a cytoplasmic mRNA-binding protein, selectively

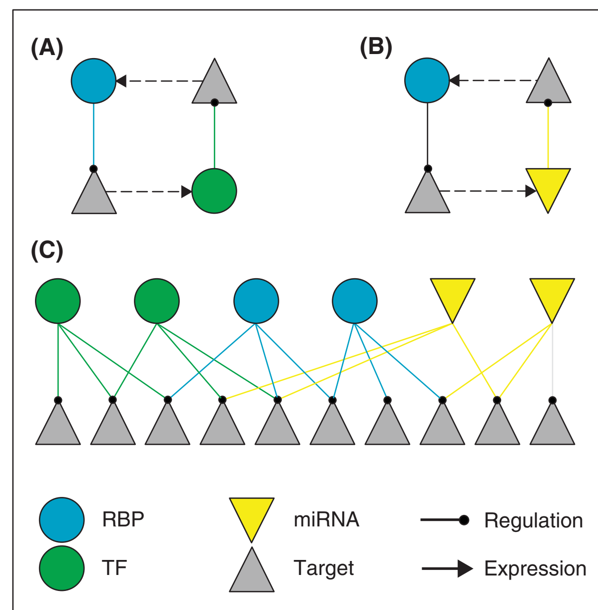

FIGURE 2 | Selected composite gene regulatory network motifs. Proteins are represented by circles [RNA-binding proteins (RBPs) in blue, transcription factors (TFs) in green], while miRNAs and target genes/mRNAs are depicted by orange and gray triangles, respectively. (A) Two-component loop involving one RBP and one TF. (B) Two-component loop involving one RBP and one miRNA. (C) Schematic representation of a hypothetical composite 'regulon'. Each regulator binds and regulates the expression of different subsets of targets. Conversely, each target is bound by a distinct set of regulators in a combinatorial fashion.

blocks the processing of pre-miRNAs of the *let-7* family in human cells.[53] Interestingly, the message encoding the Lin28 protein is itself regulated by its target miRNA *let-7b*,[54,55] thus providing an example of an RBP/miRNA two-component feedback loop (Figure 2B).

Several studies indicate that RBPs may selectively modulate miRNA function, both synergistically and competitively, to alter translational repression.[56–58] For example HuR, which has been found to be a component of stress granules, relieves the miRNA-mediated repression of CAT-1 mRNA upon stress.[56] Likewise, the dead-end 1 (DND1) protein prevents binding of *miR-221* to the 3′-UTR of the tumor suppressor p27 mRNA and relieves translational repression.[57,59] Finally, translational repression of *hbl-1*, the *C. elegans* ortholog of the developmental timing regulator *hunchback*, is dependent on the presence of Puf-8, a member of the conserved PUMILIO family RBPs.[58] As the 3′-UTR of *hbl-1* contains both putative Puf-8 and *let-7* miRNA binding sites, combinatorial binding of both the RBP and miRNA may act synergistically to maintain proper

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levels of the *hbl-1* gene product. With regard to the Puf family of proteins, recent ribonomics analyses determined hundreds of targets of the two human Puf family members PUM1 and PUM2 in human cancer cells.[60,61] Interestingly, bioinformatic analysis revealed that conserved PUM- and miRNA-binding sites ('seed' sequences) were preferentially located in close vicinity among the experimentally identified targets, suggesting extensive crosstalk between the two regulatory systems.[60] Furthermore, it was demonstrated that PUM1 has a role in mRNA degradation and, similar to HuR, localizes to stress granules, providing further support for the putative crosstalk between Puf proteins and the miRNA system.[61]

Composite GRNs

The high degree of interplay between transcriptional and post-transcriptional gene regulation (PTGR) should eventually lead to the characterization of composite GRNs.[39] For instance, the combination of the multiple-input and multiple-output motifs for functional related gene classes leads to dense and overlapping composite 'regulons' (Figure 2C). Such regulons are widespread phenomena in the control of gene expression at different levels, and can be thought of as gate arrays, processing multiple inputs from regulators to multiple targets.[39,41] However, in order to understand the functional implications of these regulons, the input function of each regulator (either positive or negative) has to be known, requiring quantitative measurements of the abundance and activity of diverse components of the network. In the future, such analyses will allow a systems-level understanding of the multilayered gene expression programs.

CONCLUSION

The advent of global and quantitative analysis tools for the study of gene expression allowed the detection and quantification of network motifs in gene regulatory systems. Generally, it appears that principles and structures of transcriptional regulatory networks are also preserved at the post-transcriptional level. However, systems analysis of PTGR is still in its infancy. The development of novel techniques for PTGR network analysis will hence be crucial to obtain sufficient data for understanding the 'RNP code'. The combination of ribonomics approaches with crosslinking techniques and high-throughput sequencing will help to systematically map RBP-binding sites.[62] The use of next-generation sequencing methods will allow a more robust and quantitative detection of RNAs, including rare and unknown RNA molecules/species.[63] Furthermore, the application of quantitative proteomics should permit the quantitative description of mRNA translation and degradation.[64] The data obtained from such studies can then be used for the development/refinement of mathematical models of gene regulation that will improve in accuracy and predictive power. First attempts to integrate different layers of gene regulation have already been undertaken in fission yeast, uncovering widespread and substantial coordination between the different regulatory layers of gene expression.[65] Such analyses will certainly lead to new systems-level insights into the logic of cellular and physiological functions and disease-causing perturbations and, hence, may lead to new and unexpected approaches for their cure.

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