White Paper

Exploring the RNA world:

An introduction to genome-wide analysis of RNA-protein interactions using RNA-binding protein immunoprecipitation (RIP)

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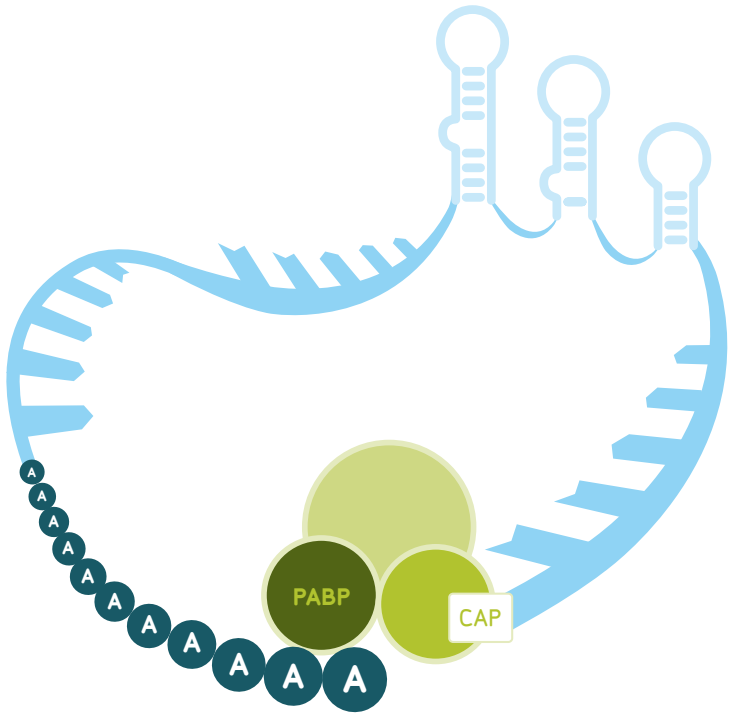
Introduction

Gene expression is regulated at multiple levels to ensure a coordinated synthesis of the cells' macromolecular components[1,2]. For years, research has mainly focused on the first steps of gene expression, namely transcriptional control mediated by transcription factors (TF) that activate genes by binding to DNA promoter sequences and recruit RNA-polymerases for RNA synthesis.

It is now also becoming increasingly recognized that control of the post-transcriptional steps of gene expression has a substantial impact on gene regulation that include pivotal roles in development, metabolism, neuronal function, and aging.

Whereas the roles of TFs, chromatin structure and modifications are undisputed, it is now also becoming increasingly recognized that control of the post-transcriptional steps of gene expression has a substantial impact on gene regulation that include pivotal roles in development, metabolism, neuronal function, and aging[3-5]. The alteration of post-transcriptional gene regulation, such as alternative splicing of mRNAs in cancer[6], can mark or even directly cause diseases, as exemplified by nucleotide repeat expansions in neurodegenerative disorders[7].

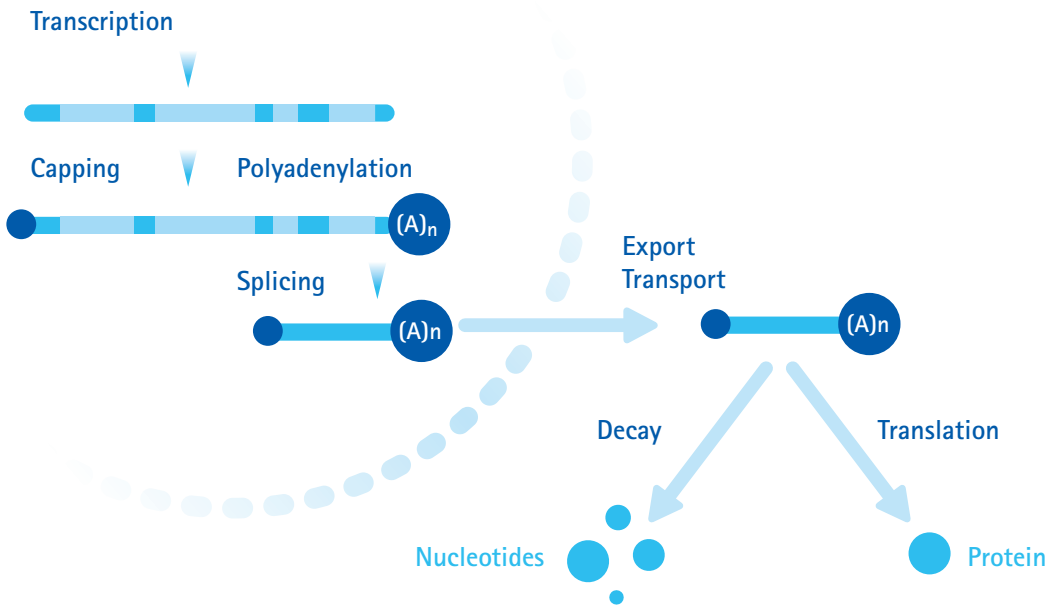


Post-transcriptional gene regulation through RNA-binding proteins and miRNAs

Post-transcriptional regulation is mediated, on the one hand, by RNA-binding proteins (RBPs) that control almost every aspect of an RNA's life in a dynamic manner from maturation, quality control and localization, to translation and degradation[8] (Figure 1). RNA-binding proteins comprise 8 to 15 % of the protein coding genes in eukaryotic genomes[9]. This large number of RBPs may merely reflect the ancient origin of RNA regulation, which is possibly the most evolutionarily conserved part of cell physiology. RBPs often contain characteristic RNA-binding domains that specifically interact with sequences or structural elements in the RNA. Bioinformatics and experimental analysis propose almost one hundred distinct RNA-binding motifs, half of which are believed to have originated at early stages in evolution. Other motifs, such as the classical RNA recognition motif (RRM) found in many proteins required for splicing, are exclusively present in eukaryotes[9]. Notably, some of the RNA-binding domains such as the RRM underwent drastic amplification during animal evolution. This amplification is concurrent with the origin of alternative splicing, allowing the generation of more genetic diversity with a limited repertoire of genes.

On the other hand, the fate of mRNAs is also controlled via interactions with small non-coding RNAs such as microRNAs (miRNAs), which are only around 22 nucleotides long[10]. miRNA precursors are exported from the nucleus to the cytoplasm and converted to miRNAs by an RBP called Dicer. The miRNA is then incorporated into the RNA-induced silencing complex (RISC) – whose main component is a member of the Argonaute (Ago) protein family. The miRNA-RISC micro-ribonucleoprotein complex (miRNP) partially assembles with sequences located in 3'-untranslated regions (3'-UTRs) of mRNA messages, inducing changes to the subcellular localization, the translation, and/or the stability of the mRNA target[10]. Notably, the more than 500 miRNAs that exist in humans are expected to regulate almost one third of all genes.

The hundreds of RNA-binding proteins (RBPs) and miRNAs encoded in eukaryotic genomes rival in number to other classes of regulatory molecules such as transcription factors and kinases and thus, suggests an elaborate system for post-transcriptional control that may affect virtually every message in a cell[11,12]. The relevance of this post-transcriptional gene regulatory system is further evident by the limited correlation between mRNA abundance and their respective protein levels[13,14]. The recent development of genome-wide analysis tools now enables the study of this post-transcriptional system on a global scale[12]. Thereby, the systematic identification of the RNA targets for RBPs/miRNAs leads to the discovery of novel



RNA regulatory networks and to the study their implications for cell physiology and disease. Furthermore, this emerging field provides basic insights into the architecture of post-transcriptional regulatory systems and how the large population of RNAs present in cells are temporally and spatially coordinated[11,12].

RNA binding protein immunoprecipitation (RIP) to identify components of ribonucleoprotein complexes

One of the numerous methods to characterize ribonucleoproteins (RNPs) involves immunoprecipitation or affinity-purification using an antibody to a constituent protein. The RNPs can then be separated into proteins and RNAs that can be further analyzed. This approach (developed 30 years ago) was first employed to identify and characterize the components of small RNPs in mammalian cells[15]. Small RNPs, such as components of the splicing machinery (spliceosomes), are highly expressed in cells (10^3 to 10^8 molecules/ cell) and hence, these complexes are well-suited for biochemical characterization. In fact, initially the RIP approach was not believed to be suitable for the identification of much lower-expressed mRNAs bound to regulatory RBPs. However, the development of reverse transcription-PCR (RT-PCR) methods for amplification of associated RNAs, followed by tedious sub-cloning of cDNAs and sequencing allowed the discovery of at least a fraction of the mRNAs that were bound by specific RNA-binding proteins[16]. In this regard, RIP combined with RT-PCR amplification with specific primers is still widely used to test for RBP binding to a particular RNA.

RIP approaches have also been applied to more 'general' RNA-binding proteins to identify the messages expressed in particular tissues or cell-types. For instance, tagged poly(A) binding protein (PABP or PABPC1) (Figure 2), which binds to the poly(A) tail of cytoplasmic mRNAs, has been expressed within cells or tissues of interest using specific promoters to identify muscle- or ciliated sensory neuron-specific transcripts in the worm Caenohabditis elegans[29,30], or in photoreceptor cells of the fly Drosophila melanogaster[31]. Likewise, tagged ribosomal proteins have been pulled-down to isolate actively translating ribosomes to define gene expression profiles of specific neuronal cells types in the central nervous system of mice[32], and to study reactions of the translatome upon diverse stress conditions in yeast[33]. Finally, the RIP approach has also been used to identify targets that potentially undergo miRNA dependent regulation by profiling RNA and proteins that are bound by Ago proteins[34-37]. The comparison of Ago-associated mRNAs in wild-type and miRNA mutants or over-expressing cells further provides a tool to decipher miRNA-specific targets.

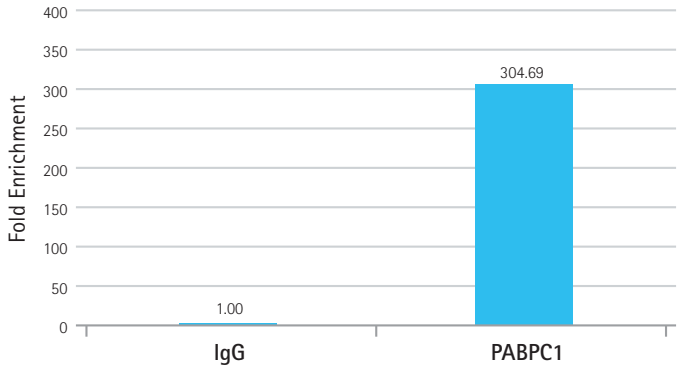


Figure 2.

PABPC1 fold enrichment data for a RIP experiment using Merck Millipore's Magna RIP™ Kit

RIP Lysate prepared from HeLa cells (2 X 10^7 cell equivalents per IP) were subjected to immunoprecipitation using 5 µg of either a normal mouse IgG or the anti-PABPC1 antibody and the Magna RIP Kit (Cat. No. 17-700). Successful immunoprecipitation of PABPC1-associated RNA was verified by qRT-PCR using RIP Primers, ACTB.

Figure 1.

Steps involving post-transcriptional regulation of gene expression

As soon as mRNA-precursors are synthesized by RNA polymerases allocated by TFs, RBPs (not shown) bind to nascent RNA transcripts and mediate the nuclear RNA processing reactions such as 5'-end capping, splicing, editing, 3'-end cleavage, and polyadenylation. The transcripts are then exported from the nucleus to the cytoplasm through nuclear pores by export factors, and some mRNAs undergo further localization to subcellular compartments by complexes that consist of motor proteins and RBPs or by the signal recognition particle. mRNAs are recruited by translation factors and conducted to ribosomes for protein synthesis, which can be controlled by global or transcript-specific mechanisms. Ultimately, most messages are decayed by exonuclease-mediated degradation. RNA transcripts are shown in dark blue; introns in pre-mRNAs are depicted in light blue.

A major advantage of RIP is that it allows concomitant identification of the protein components of RNPs and other associated regulatory proteins by mass-spectrometry (MS). For instance, RIP has recently been used to map RNAs and proteins associated with human Ago proteins[36]. The combination of both RNA mapping and MS allows for the monitoring of RNP complex remodeling upon changing environmental conditions or during cell differentiation. The major drawback of RIP procedures however, is the general concern that certain components may fall-off and others may re-associate with RNP complexes during the procedure. However, whether and how extensive this occurs is not conclusively answered to date and may critically depend on the protein under investigation. To circumvent this concern and to detect also relatively weak RNA-protein interactions, a few laboratories have therefore established protocols to crosslink RNA-protein complexes either by UV or with chemicals prior to immunoprecipitation. One of these methods, termed CLIP[38] or if high-throughput sequencing is applied HITS-CLIP[39], allows relatively precise mapping of the binding sites for RPBs on the RNA. The drawback is that CLIP is ill-suited for recovering entire RNP complexes for mass-spectrometry analysis, the procedure is labor intensive, and there is some concern that cross-linking may alter the RNP structure. The question whether to employ either RIP or CLIP will lately depend on the biological question as both approaches have it advantages and drawbacks.

With the advent of the DNA microarray technology, it became feasible to identify the messages that are selectively associated with RBPs on a global level **(Figure 3)**. The so called RNA binding protein immunoprecipitation-microarray (RIP-Chip) approach was first employed by Jack Keene's lab to study RNAs associated with three RBPs in a cancer cell line[17]. They found that each RBP was associated with a distinct subset of mRNAs present in total cell lysate, and the groups of associated mRNAs changed upon induction of cell differentiation. These early results lead to the proposal that groups of mRNAs encoding functionally related proteins are organized into so-called 'post-transcriptional operons'[18]. In analogy to prokaryotic operons, this model predicts that specific RBPs may coordinate groups of mRNAs coding for functionally related proteins in eukaryotes. Cis-acting elements in the mRNA may provide the means to mimic the coordinated regulatory advantages of clustering genes into polycistronic operons[11,18].

Diverse laboratories have now established RIP-Chip protocols in many species including yeast, flies, plants, and mammals[19-24]; and the RNA targets for more than 20 human RBPs have been mapped under various conditions[21,25-28]. Next to specific insights into the role of particular RBPs for the control of its targets, these studies have substantiated and further extended the 'post-transcriptional operon model' proposed by Keene and colleagues, and lead to the discovery of several common aspects of RBP mediated gene regulation. First, RBPs bind to unique sets of RNA comprised of 20 to 1,000 distinct transcripts. Second, the bound mRNAs often encode functionally and/or cytotopically related proteins. This is perhaps best seen among the targets for Puf proteins, a conserved family of RBPs that generally represses gene expression of its targets with widespread physiological implications. Each of the five yeast Puf proteins binds to a distinct set of messages with strong common themes - most striking, Puf3p binds almost exclusively to messages encoding mitochondrial proteins[20]. Third, the spectra of targets may substantially overlap with other RBPs, suggesting strong combinatorial binding of RBPs. Fourth, sequence or structural elements could be identified among mRNA targets using bioinformatics tools, and have been further verified in various instances. Fifth, the RNA-protein network appears to be extremely dynamic and responds to environmental signals altering the RNA and protein content of RNPs.

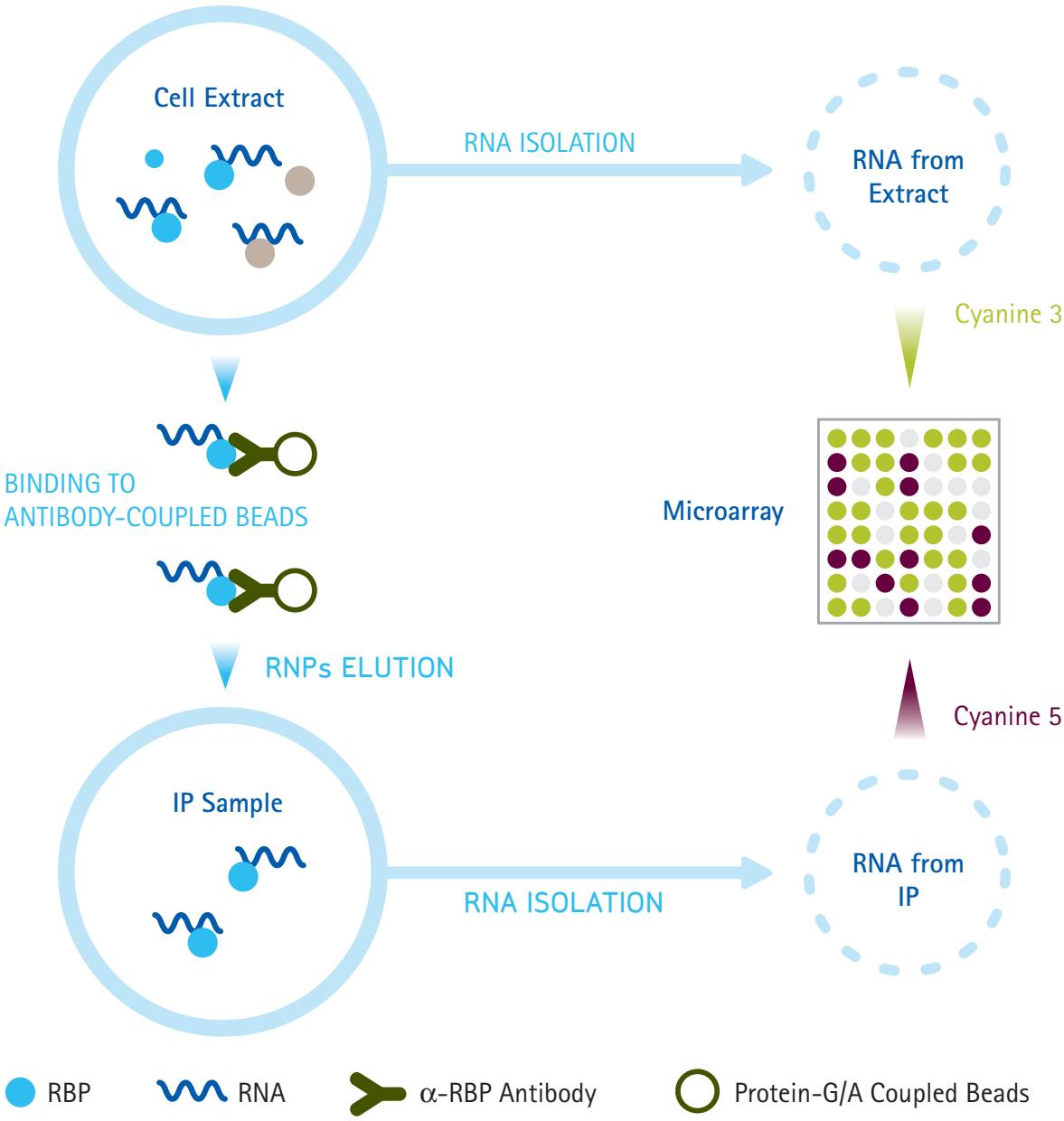


Figure 3.

RIP-Chip to systematically identify RNA targets of RBPs

RBPs are immunoprecipitated or affinity-purified via a tag from cellular extracts. Total RNA isolated from cell extracts, and the RNAs associated with the particular RBP under study are labeled with different fluorescent dyes and competitively hybridized to DNA microarrays. The fluorescence ratio for each locus reflects its enrichment by affinity for the cognate protein.

Perspectives

The systematic analysis of RBPs and the RNA to which they bind enables the discovery of RNA-protein networks and the study their implications for post-transcriptional gene regulation. RIP has become an important tool used to study the structure and composition of RNP complexes, to map targets and binding sites of RBPs and miRNAs, and to discover how these sites may be altered in various disease states. The integration of data obtained from RIP-Chip or RIP-Seq experiments (next-generation or high-throughput genomic sequencing technologies instead of microarrays) with data from other levels of gene expression research will certainly elucidate new and exciting perspectives and interconnections. This growing interest in RIP has now lead to commercially available RIP kits and reagents (Figure 4). The development of robust and easy to handle RIP assays and antibodies will certainly help provide the tools to explore the remarkably complex and highly interlinked post-transcriptionally regulatory networks that comprise this emerging field of research.

Figure 4. Merck Millipore's Magna RIP Kit for RNA-binding protein immunoprecipitation

The Millipore universal RIP immunoprecipitation kit is fully compatible with a wide range of RIP-validated antibodies, and contains all reagents needed for robust, specific enrichment of RBP-associated RNAs.

Description	Reactions	Catalogue No.
Magna RIP Kit	12 reactions	17-700
EZ-Magna RIP Kit, with positive control antibody and primers	12 reactions	17-701
Magna RIP Quad Kit	48 reactions	17-704

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Lit No. AN4663ENEU XLS-SBU-12-06611 6/2012 Printed in the USA.