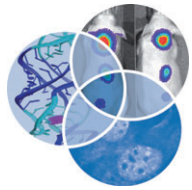
Advanced Review



The organization and regulation of mRNA–protein complexes

Olivia S. Rissland[1,2*]

In a eukaryotic cell, each messenger RNA (mRNA) is bound to a variety of proteins to form an mRNA–protein complex (mRNP). Together, these proteins impact nearly every step in the life cycle of an mRNA and are critical for the proper control of gene expression. In the cytoplasm, for instance, mRNPs affect mRNA translatability and stability and provide regulation of specific transcripts as well as global, transcriptome-wide control. mRNPs are complex, diverse, and dynamic, and so they have been a challenge to understand. But the advent of high-throughput sequencing technology has heralded a new era in the study of mRNPs. Here, I will discuss general principles of cytoplasmic mRNP organization and regulation. Using microRNA-mediated repression as a case study, I will focus on common themes in mRNPs and highlight the interplay between mRNP composition and posttranscriptional regulation. mRNPs are an important control point in regulating gene expression, and while the study of these fascinating complexes presents remaining challenges, recent advances provide a critical lens for deciphering gene regulation.

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INTRODUCTION

In the cell, messenger RNAs (mRNAs) do not exist as naked transcripts, but are instead dressed with protein factors to form mRNA–protein complexes (mRNPs). Eukaryotic mRNP composition is determined by a complicated mix of ingredients: namely, common RNA elements (such as the 5′ cap), specific RNA sequence motifs, RNA modifications, protein modifications, and cellular context. Linked to nearly every RNA regulatory process, mRNPs represent a key node in gene regulation.

mRNPs are also challenging to study. Unlike many other macromolecular complexes, by their very nature mRNPs are diverse, highly dynamic, and often transient. Not only do mRNPs vary between genes, but for a given transcript, the associated proteins will also change throughout their life cycle. This variety can render some scientific questions (e.g., what is the structure of the mRNP?) nonsensical, despite their utility for understanding other RNA–protein complexes like the ribosome.

In an mRNP, the mRNA naturally holds special prominence. From a structural perspective, it can be thought of as the organizing scaffold that recruits a variety of proteins. Each mRNA can be broadly divided into five portions, which bind specific sets of RNA-binding proteins (RBPs) and thus have distinct roles in mRNP organization: the 5′ cap, the 5′ untranslated region (UTR), the open reading frame (ORF), the 3′UTR, and the 3′ poly(A) tail (Figure 1). Of course, an mRNA not only provides the foundation for an mRNP but also encodes the information necessary to make a specific protein, the production of which is influenced by the rest of the mRNP.

*Correspondence to: olivia.rissland@sickkids.ca

[1]Molecular Structure and Function Program, The Hospital for Sick Children Research Institute, Toronto, ON, Canada

[2]Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada

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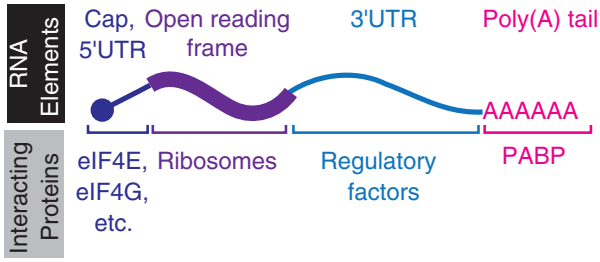

FIGURE 1 | RNA elements and proteins combine to form an mRNP. Translation initiation factors, such as eIF4E and eIF4G, interact with the 5′UTR and cap, while PABP binds the 3′ poly(A) tail. Because the cap and poly(A) are found on nearly all transcripts, these proteins are considered core factors. In contrast, regulatory factors recognize specific motifs, often in the 3′UTR, and so bind and regulate a specific subset of transcripts.

RBPs, in turn, can be classified by an ability to either bind all transcripts (through common RNA elements) or recognize specific transcripts (through specific motifs). In general, those of the first class, which I will refer to as 'core factors,' tend to directly affect gene expression, for example, by stimulating translation or mediating mRNA decay. On the other hand, those of the second class, or 'regulatory factors,' often bind to the UTRs and then alter the binding of core factors. One particularly well-understood class of regulatory factors is microRNAs (miRNAs), which predominantly destabilize their targets by recruiting decay factors.

This review will discuss the components of cytoplasmic mRNPs (the mRNA itself, core factors, and regulatory factors) with an emphasis on understanding how they interact and affect one another. I will focus on miRNA-mediated repression as a case study to reveal common themes in mRNP organization.

CORE FACTORS

The 5′ cap and 3′ poly(A) tail are found on nearly all RNA polymerase II transcripts, and, in many respects, these two elements can be considered the foundation of an mRNP. Replication-dependent histone mRNAs are the major class of mRNAs lacking a poly(A) tail and instead terminate with a specific stem loop, a structure analogous to a poly(A) tail that carries out many of the same functions,[1] but this class will not be discussed further. The cap and poly(A) tail are intimately involved in two processes central to all mRNAs: translation and decay. Thus, much of gene regulation will, as an ultimate endpoint, impact the cap, the poly(A) tail, or the proteins binding to these structures.

The 5′ Cap and 3′ Poly(A) Tail: The mRNA Perspective

One of the most fundamental roles of the 5′ cap and 3′ poly(A) tail is to protect mRNAs from the general action of exonucleases. The cytoplasmic and nuclear 5′→3′ exonucleases (Xrn1 and Xrn2, respectively) are processive, efficient enzymes that recognize 5′ monophosphate RNAs.[2,3] These enzymes are blocked by the 5′ cap, and so removal of the cap by the decapping enzyme is a tightly controlled process.[4–6] The cytoplasmic exosome, which degrades RNA 3′→5′, plays a more minor role in cytoplasmic mRNA decay.[7] Recently, other 3′→5′ exonucleases (Dis3L1 and DisL2) have been found in higher eukaryotes and implicated in degrading uridylated miRNA precursor hairpins and some specific transcripts; their importance in general mRNA decay remains an open and important question.[8–10]

However, the 5′ cap and 3′ poly(A) tail serve much broader functions than working as mere roadblocks to exonucleases. These two RNA elements are fundamental for regulating gene expression, especially at the control points of translation initiation and mRNA decay.[6,11–13] Maximal translational efficiency in a variety of systems depends on the presence of both the cap and poly(A) tail, and these elements stimulate initiation in a nonadditive manner.[12,14–16] Similarly, the roles of the cap and the poly(A) tail in mRNA decay are closely entwined; mRNAs lacking either structure are very short lived in cells,[17–22] and deadenylation stimulates removal of the cap.[6,11,13]

The 5′ Cap and 3′ Poly(A) Tail: The Protein Perspective

Many of the protein players are the same in translation initiation and mRNA degradation,[17,23–25] and so these two pathways are inextricably linked at a mechanistic level.[26,27] In the cytoplasm, there are two main protein factors: the translation initiation factor eIF4E, which binds the 5′ cap, and the cytoplasmic poly(A)-binding protein (PABP; *PABPC1* in humans and Pab1 in yeast), which binds the poly(A) tail. Underscoring the paramount importance of these structures, these two elements and associated proteins appear to be absolutely conserved in eukaryotes.[28,29] eIF4E and PABP, together with the translation initiation factor eIF4G, mediate the so-called mRNA closed-loop structure (Figure 2), which is thought to be important for both the translation and the stability of a transcript.

eIF4E is most known for its role as a translation initiation factor, where it acts as part of eIF4F

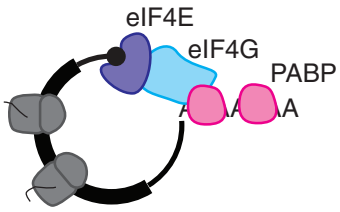

FIGURE 2 | The closed loop model. By binding simultaneously to eIF4E and PABP, which in turn bind the 5′ cap and 3′ poly(A) tail, eIF4G forms a protein bridge that brings the two transcript ends together and allows regulatory information (such as deadenylation) to be transmitted from the 3′ to 5′ end of an mRNA.

(also containing the RNA helicase eIF4A and eIF4G).[30] In addition, because eIF4E blocks access of the decapping enzyme to the 5′ cap, its dissociation is a necessary, although poorly understood, step in mRNA decay.[25] Interestingly, the loss of eIF4E does not appear to be sufficient to stimulate decapping in all scenarios,[31] perhaps indicating that other steps are necessary for mRNA decay or that other proteins can bind the cap and shield it from the decapping enzyme.

PABP is a protein fundamental for both translation and mRNA stability.[17,23] Analogous to the role of eIF4E in protecting the cap, PABP inhibits deadenylation and uridylation, processes that trigger decapping[32–34] (Figure 3). In addition, PABP can inhibit decapping,[24] although the mechanisms underlying this observation are still unknown. Containing four RNA recognition motifs (RRMs), PABP requires ~14 adenosines to bind and has an overall footprint of 26 nucleotides.[35,36] With the average mammalian poly(A) tail being 80–90 nucleotides in length,[37,38] human transcripts can, in theory, accommodate three to four PABP proteins, although the precise stoichiometry of bound PABP remains unknown.

eIF4E and PABP are primary proteins thought to bind the cap and poly(A) tail, but these are not only proteins to do so. For instance, the nuclear cap-binding complex recognizes the cap of newly transcribed transcripts and promotes pre-mRNA processing.[39–41] After export to the cytoplasm, this complex is replaced by eIF4E in a key mRNP remodeling step.[42,43] Similarly, eIF4E2 (also known as 4EHP) can bind the 5′ cap in the cytoplasm, but it

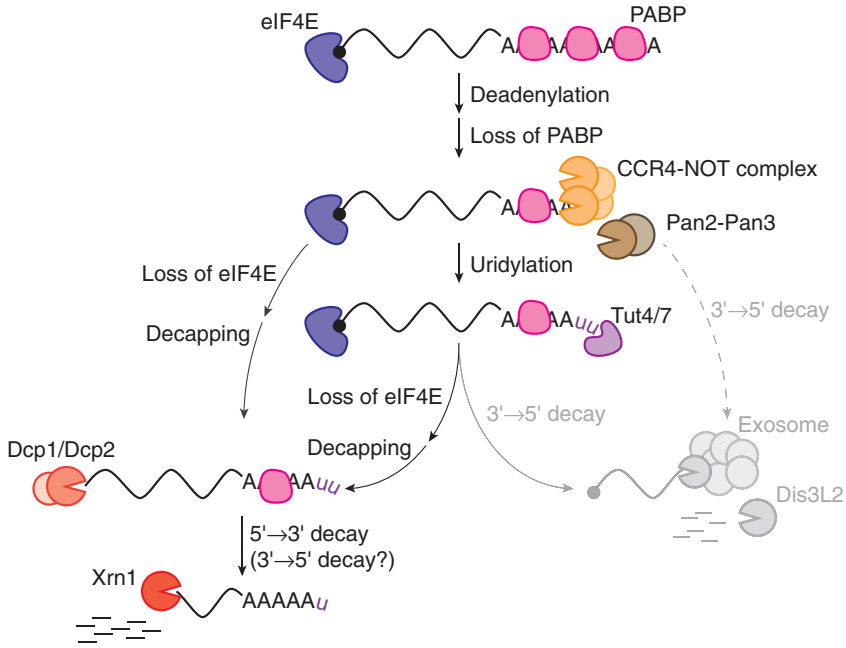

FIGURE 3 | Cytoplasmic mRNA decay. During mRNA decay, the action of the two major cytoplasmic deadenylase complexes (the Pan2–Pan3 complex and the CCR4–NOT complex) leads to shortening of the poly(A) tail and PABP dissociation, although it is currently unclear whether shortening of poly(A) tail leads to the loss of PABP or vice versa. In some cases, deadenylation (or the deadenylase complexes with associated factors) stimulates the dissociation of eIF4E and recruitment of the decapping enzymes. In other cases, deadenylation is followed by uridylation, which serves as a landing pad to stimulate the recruitment of the decapping enzymes. (Note, however, that Saccharomyces cerevisiae lacks TUTases.) Once the message is decapped, the cytoplasmic 5′→3′ exonuclease, Xrn1, degrades the transcript body. In the cytoplasm, 5′→3′ decay represents the major decay pathway, but there are 3′→5′ exonuclease (such as the exosome and Dis3L2) that can degrade deadenylated transcripts and may also act at the same time as 5′→3′ decay.

has distinct binding partners and may have roles in repressing translation or in responding to stress.[44–46] In addition, the decapping enzyme recognizes the cap structure, although, with its enzymatic action representing the committed step of mRNA decay, the decapping enzyme is often not classified as a cap-binding protein. Nonetheless, competition with other proteins binding the cap (such as eIF4E) is an important way of regulating decapping.[25]

As with eIF4E, PABP also has a nuclear counterpart (PABPN1 in humans), which is important for controlling the length of the newly added poly(A) tail.[47,48] Interestingly, while many organisms, such as *Saccharomyces cerevisiae* and *Drosophila melanogaster*, exist with just this cohort of poly(A) tail-binding proteins, most vertebrate genomes have an expanded repertoire.[49] In humans, for instance, there are four PABP genes in addition to the canonical *PABPC1* locus. Although they contain domain architecture similar to canonical PABP, they have distinct expression patterns. Deletions of some can lead to severe mouse phenotypes—for instance, female mice lacking ePABP are sterile[50]—but a careful dissection of the roles of these PABPs in relation to PABPC1 represents a major gap in our understanding.

The poly(A) tail also has a dedicated set of decay enzymes (the PARN deadenylase, the Pan2–Pan3 deadenylase complex, and the CCR4–NOT deadenylase complex) that recognize and remove this element specifically.[13] Additionally, although the preferences of cytoplasmic TUTases (such as Tut4 and Tut7) and poly(A) polymerases (such as Gld-2) are less restricted to polyadenylated RNA, mRNAs represent an important target of these enzymes.[33,38,51] Not all of these enzymes are found throughout eukaryotes (e.g., PARN is absent from *Drosophila* and *S. cerevisiae* lacks TUTases), but the general principle of modifying the poly(A) to affect gene expression is deeply conserved.

THE CLOSED-LOOP MODEL: eIF4E-eIF4G-PABP

Because elements at the 3′ end of the mRNA (namely, the poly(A) tail and PABP) are so intimately linked with events that occur at the 5′ end (namely, translation initiation and decapping), there must be some form of 'communication' between the two ends of the RNA molecule, which can be separated by thousands of nucleotides. Direct evidence of the spatial proximity of the two RNA ends *in vivo* came from early electron micrographs of membrane-bound polysomes.[52] These observations are now explained by the so-called closed-loop model. Here, the translation initiation factor eIF4G simultaneously binds eIF4E and PABP, which, through their interactions with the cap and poly(A) tail, bring the two mRNA ends into proximity[17,19,21,22] (Figure 2). eIF4G interacts with additional initiation factors, such as eIF3 and eIF4A,[30] but its central position in the closed-loop structure is most relevant for the organization and regulation of mRNPs.

At a molecular level, the PABP–poly(A) interaction strengthens the binding of PABP to eIF4G, and PABP enhances the affinity of eIF4F for the cap.[53,54] Similarly, eIF4G also increases the binding of eIF4E to the cap,[55] suggesting that these three proteins may bind cooperatively to circularize mRNAs. By linking the poly(A) tail with the cap, the closed-loop model provides a mechanism by which the poly(A) tail (and PABP) can stimulate translation initiation. The closed loop has also been hypothesized to aid with ribosome recycling and to inhibit decapping.[17,56]

Further support for the closed-loop model comes from a variety of biochemical, structural, and evolutionary angles. Each of the pairwise interactions has been observed in many organisms and is deeply conserved from yeast to humans. In addition, when incubated with eIF4E, eIF4G, and PABP, *in vitro* preparations of capped and polyadenylated mRNAs formed circles.[21] Its conceptual underpinnings are echoed with histone stem-loop-binding protein (SLBP) and with rotavirus NSP3, both of which bind 3′ RNA ends and can form analogous interactions with the 5′ end.[57–60]

But the complete series of interactions [i.e., cap-eIF4E-eIF4G-PABP-poly(A) tail] on a single mRNA has not been observed *in vivo* owing to its technical difficulty, leaving open the question of what fraction of mRNAs exist as circles in a cell, and recent work has begun to call the role of the closed loop into question. For instance, yeast strains lacking the N-terminus of eIF4G1 (the region containing the PABP-binding site) are viable, and the eIF4G1–PABP interaction has been suggested to function by stabilizing eIF4G binding to mRNA rather than by bringing the two RNA ends into proximity.[61] In mammals, eIF4G1 has alternative N-terminal isoforms, depending on start codon usage, and the smallest of these lacks the PABP-binding site.[62] Similarly, in yeast, PABP point mutants that are unable to interact with eIF4G were able to stabilize a reporter when tethered to a 3′UTR.[24] Finally, PABP binds A-tracts within the body of an mRNA,[63,64] and the extent to which these interactions differ functionally (and are distinguished) from interactions on the poly(A) tail is an unexplored issue.

THE IMPORTANCE OF POLY(A) TAIL LENGTH

Although the importance of the poly(A) tail for translation and mRNA stability has long been recognized, a key issue has been disentangling the effects of its presence from the effects of its length. This issue has risen, in part, from the difficulties in quantitatively measuring poly(A) tail lengths on endogenous transcripts (especially for many genes simultaneously), from the inefficiencies in introducing exogenous RNAs into classic model systems, such as yeast and human tissue culture cells, and from the inability to fully recapitulate deadenylation-stimulated decapping *in vitro*.

Because of these challenges, the importance of poly(A) tail length on translational efficiency has historically been investigated in *Xenopus* oocytes and *Drosophila* embryos,[65,66] where radiolabeled RNA can be easily injected. Elegant classical studies have demonstrated that, in this biological context, the length of the poly(A) tail is important for controlling translational efficiency, a result that has been recently confirmed with the advent of a protocol measuring poly(A) tail length transcriptome-wide.[37]

Surprisingly, however, this relationship is not universally true: following the maternal-zygotic transition in embryogenesis, it is the presence of a poly(A) tail, rather than its length, that modulates translational efficiency.[37] In the majority of cell types and model systems, in fact, there is no relationship between the length of a poly(A) tail on a transcript and how well it is translated.[37,38] The molecular mechanisms underlying this striking developmental switch are unclear, but likely this change involves global mRNP reorganization. It is notable that oocytes and early embryos appear to be the outlier in this respect, especially because these are the same systems where transcripts lacking a substantial poly(A) tail are stable.[67,68] Although direct evidence is lacking, it is tempting to speculate that these two sets of observations may be causally linked.

Historically, longer tailed messages were also thought to be more stable; however, just as with translation, the mechanistic relationship between poly(A) tail length and mRNA decay requires reexamination. For instance, some of ribosomal protein mRNAs, despite having some of the shortest poly(A) tails in the transcriptome, are also the most stable, with half-lives on the order of the cell cycle.[37] Although deadenylation is clearly important for decapping,[6,11,13] how much of this effect results from the actual shortening of the poly(A) tail? Or does deadenylation cause decapping because it alters the mRNP through removal of PABP and/or recruitment of additional decay factors?

Two lines of evidence support the importance of mRNP reorganization during mRNA decay. First, deadenylation and decapping factors interact through a dense network. Tethering experiments with CNOT1, a scaffold protein in the CCR4–NOT deadenylase complex, have show that it is able to repress gene expression even on reporters lacking a poly(A) tail.[69] Both biochemical and structural data have also highlighted DDX6, a decapping activator and translational repressor that is capable of interacting simultaneously with CNOT1 and the decapping enzyme.[70–72]

Second, removal of PABP is critical for decapping. In *S. cerevisiae* lacking Pab1, mRNAs with long tails are decapped, unlike the scenario in wild-type cells where deadenylation precedes decapping; this observation suggests that Pab1 itself, rather than the poly(A) tail *per se*, inhibits decapping and that deadenylation, in part, serves to remove this block.[23] That PABP, though eIF4G, can strengthen the association of eIF4E with the cap, thus blocking its accessibility to the decapping enzyme, provides a tempting model for how PABP might inhibit decapping. However, the role of PABP as an inhibitor of decapping may extend beyond that in closed loop. As noted above, tethering fragments of PABP that are unable to bind eIF4G are able to stabilize transcripts in yeast.[24] Similarly, *pab1Δ* strains have only been isolated in the presence of the *sbp2Δ* suppressor mutation,[23,73] while yeast strains carrying mutations that inhibit the eIF4G–PABP interaction have no growth defects.[61]

THE 3′UTR CODE

Much of posttranscriptional gene regulation is determined by sequences in the two untranslated regions. In some cases, these regions are sufficient to specify nearly the entire total gene expression program. For instance, in the *Caenorhabditis elegans* germline, adding just the 3′UTR to a GFP reporter was able to recapitulate endogenous expression for many genes.[74] Thus, one major long-term goal is to be able to determine, based on a 3′UTR sequence, how the corresponding gene will be controlled at the posttranscriptional level. This task is not an easy one and requires a detailed knowledge of the 3′UTR code: knowing which protein binds to which mRNA and how those combinatorial interactions integrate to control gene expression.

What is necessary for completing this challenge? This effort, analogous to those dedicated to

understanding splicing or transcription regulation, most likely requires at least four main components, which I will outline here and describe in more detail below. First, there needs to be a compendium of regulatory factors as well as their tissue and developmental expression pattern. Second, the transcripts bound by each factor must be characterized, and the recognized RNA motif identified. Third, how each regulatory factor in turn affects gene expression (e.g., by changing mRNA stability or translational efficiency) must be determined. Currently, the best-characterized examples repress gene expression,[75,76] but it is likely that, as our catalogue increases, factors that increase gene expression will also be identified. Fourth, an individual 3′UTR can be bound by several RBPs simultaneously. Many of these likely regulate gene expression in an independent manner, but some factors are already known to bind or act nonadditively,[77–79] and there are likely additional interactions of this class. Understanding such combinatorial effects is critical for truly being able to dissect the 3′UTR code.

THE CATALOGUE OF RNA-BINDING PROTEINS

Many RBPs can be identified by the presence of domains, such as an RRM or a KH domain,[80] but recent evidence demonstrates that the full catalogue extends beyond this list.[81,82] With the goal of identifying a complete mRNA 'interactome,' two groups used UV-crosslinking to covalently link RNA to bound proteins and purified the entire mRNP collection using oligo(dT); bound RBPs were then determined with mass spectrometry. Although low abundance factors may have fallen below the detection limit, this approach nearly doubled the number of RBPs from ~500 to ~800. At least 15% of these failed to be predicted by computational methods and indicate a substantial contribution of noncanonical RBPs to mRNPs.[81,82] These studies were performed in mammalian cell lines, and it is likely that additional factors will be continue to be identified as this approach is applied to other cell lines, tissues, developmental states, and organisms.

In contrast, identifying RBPs bound to specific transcripts has proven more challenging. All currently available strategies rest upon a final step of mass spectrometry to identify proteins enriched on an RNA of interest, and thus are sensitive to various technical concerns, such as protein and RNA abundance, the fraction of protein bound to the RNA of interest (and vice versa), and the purity of the selected mRNP. The long-noncoding RNA (lncRNA) field is particularly concerned with protein factors bound to a given transcript, and therefore much of the technical innovation has come from this area.[83] The most successful has focused on highly abundant lncRNAs, such as Xist.[84] In one strategy, crosslinking stabilizes mRNPs to allow for stringent washes during purification; here, unlike in mRNA interactome studies, a specific transcript is isolated through the use of biotinylated complementary oligonucleotides (see, e.g., ChIRP-MS).[84] However, given the inefficiency of UV-crosslinking, it is likely that alternative approaches will need to be used for less abundant transcripts and for mRNAs that exist in many different complexes. Alternatively, a specific mRNA can be purified through an aptamer or using highly specific, exogenous RBPs, such as the MS2 hairpin/coat protein system.[85] Another approach is to make use of lysate systems. Here, an *in vitro* transcribed and biotinylated RNA of interest is incubated with lysate, and then streptavidin is used to purify interacting proteins.[86] UV-crosslinking can be used to increase the stringency of the purification. However, in all approaches, applying these techniques to many different transcripts is challenging, and capturing finer resolution of mRNP dynamics (e.g., defining how protein components change throughout the mRNA life cycle) seems to be well beyond current technical capabilities.

microRNAS: A CASE STUDY

Knowing the targets of a specific regulatory factor provides a complementary, and critical, view of mRNPs. However, the path from an RBP with unknown mRNA targets and unclear regulatory effects to a well-characterized regulatory pathway is riddled with challenges. The dissection of the mammalian miRNA pathway represents a stereotypical example of such scientific development, and the hurdles encountered here provide insights for studies into other RBPs.

As we now know, miRNAs are a class of small RNAs that posttranscriptionally repress gene expression. Loaded into the effector protein Argonaute (Ago), these ~22-nucleotide small RNAs direct the silencing complex via base-pairing with the target transcript and predominantly stimulate mRNA decay.[75,87,88] Upon their initial identification in *C. elegans*,[89,90] however, the widespread impact of these small RNAs was not clear. But once the extensive conservation of *let-7* was discovered and other miRNAs identified,[91–94] the hunt was on for their targets and regulatory effects.

The widespread adoption of transcriptome-wide techniques (initially microarrays and then next-generation sequencing) greatly accelerated the pace of this research, and these large datasets gave researchers substantial statistical power. Because miRNAs base-pair with their targets and mismatches are poorly tolerated,[75] there is little variation in the binding site; in this respect, miRNAs differ from typical RBPs, which often bind degenerate sequences.[95] The signature of this interaction was identifiable through transcriptome-wide datasets, where analyses revealed the importance of the so-called miRNA 'seed' region (nucleotides 2–7), which base-pairs with target transcripts and directs the specificity of the complex.[96] In addition, many miRNAs, as well as their target sites, are deeply conserved, which opened the door to evolutionary-based analysis, further confirming the importance of the seed region and identifying other features of effective sites.[97,98] Although hurdles still remain in predicting effective sites, these types of experimental and computational approaches led to substantial improvements in miRNA target prediction algorithms. For many RBPs, on the other hand, correctly identifying targets and/or the recognized motif remains a significant challenge, and the extent to which conservation can be used to shed light on this issue will most likely differ for each factor. Recent work characterizing dozens of RBPs has created a comprehensive resource that will be invaluable for these investigations.[99]

Another aspect important for the development of the miRNA field was the ability to transfect exogenous miRNAs into cell lines.[100–102] The ability to perform clean overexpression studies greatly increased the quality of the data, and these approaches, together with transcriptome-wide quantification, gave enough statistical power that the rules of miRNA targeting could be determined bioinformatically. These rules were then confirmed for endogenous miRNAs, often using mouse knock-outs.[87,101] In contrast, for constitutively expressed protein factors, this type of overexpression/deletion approach has been more challenging and, until recently, researchers have relied upon RNAi knockdown in cell lines, which, if incomplete, often leads to uninterpretable data. Additionally, some regulatory factors have paralogues, and possible redundancy can further confuse the interpretation of depletion studies.

The combination of the ease of transfecting miRNAs and the precise definition of predicted sites made reporter-based experiments straightforward.[102] Here, a 3′UTR of interest is placed behind a luciferase reporter. Through a series of controls (transfecting a noncognate miRNA and mutating the target site), the direct repression mediated by a miRNA can be determined. These approaches have been invaluable in the miRNA field and are an important component of any investigation into mRNA regulation by regulatory factors. In contrast, many of the current gold-standard approaches (e.g., CLIP- and RIP-based techniques) for determining targets of regulatory factors, despite being motivated by the problem of understanding miRNA targeting principles,[103,104] played a relatively small role in delineating these rules.

There were notable challenges in characterizing miRNAs and their impact on gene regulation. Until recently, the effect that miRNAs have on gene expression—whether this regulation primarily results from transcript destabilization or translational repression—was controversial.[88,92,105,106] It is now clear that in the vast majority of cell types the bulk of repression results from a decrease in mRNA levels, which then results in a decrease in protein levels.[88,107] Nonetheless, disentangling these two effects proved to be surprisingly difficult and highlights potential pitfalls for studies of other regulatory factors. Part of this difficulty was due to the relatively modest effects that miRNAs have on their targets, and so it was unclear how generalizable results from mechanistic investigations of specific genes or individual reporters were. Similarly, the amount of repression mediated by a miRNA for a specific target is affected by numerous factors (such as the extent of base-pairing with the miRNA, the local 3′UTR environment surrounding the site, and the overall stability of the transcript).[102,108] Finally, although overexpression of specific miRNAs is straightforward, isolation of specific miRNA–RISC complexes is not. Many different miRNAs are all expressed simultaneously, and each is bound by Ago: thus the easiest purification handle (i.e., Ago) is shared between many different miRNAs, while the unique portion (i.e., the miRNA) base-pairs with targets and thus can be unavailable for purification. New strategies have recently been developed to circumvent this difficulty,[109] which have already shown to be important not just for purification but also for biochemical studies.[110,111]

INTERPLAY BETWEEN CORE AND REGULATORY FACTORS: miRNA-MEDIATED REPRESSION

Ultimately, by changing the localization, stability, or translatability of a transcript, regulatory factors alter the cytoplasmic fate of the target RNA. Initially, though, each alteration will involve a change in the mRNP. What are the molecular mechanisms

underlying such changes in mRNP organization? Most are unknown and represent an exciting avenue of investigation, but some examples, such as miRNA-mediated repression, have revealed some mechanistic themes. Note that the best examples involve repression of gene expression, and it is unclear how generalizable these are for factors that activate gene expression.

First, with the exception of endonucleolytic regulatory factors, RBPs typically recruit additional factors to assemble a repressive complex. For instance, regulatory factors that stimulate mRNA decay typically recruit decay enzymes, such as the deadenylase complexes.[69,112,113] Second, the effects of regulatory factors eventually funnel into changes in core factors, typically the closed-loop components eIF4G and PABP, which may be mediated directly or indirectly by loss of the poly(A) tail.[114,115] Third, mechanisms may initially involve proteins at the mRNA 3′ end, but these effects are then transmitted to the 5′ end to affect translation or decapping.[116] Fourth, because proteins can rebind and dissociate, changes purely at the level of the mRNP can be reversible, but, especially in the case of those affecting mRNA stability, these will lead to irreversible changes in the mRNA itself (e.g., loss of the 5′ cap).

miRNA-mediated repression is a stereotypical example of how a larger repressive complex is built once the initial binding of Ago has taken place (Figure 4). As discussed above, Ago itself is recruited through complementarity between the seed region of the miRNA and the cognate site in the target transcript.[75] It is important to note that targets containing complementarity to the entire miRNA will be cleaved by Ago2 without requiring the assembly of this larger complex; this 'slicing' activity is utilized in siRNA knockdowns, although very few endogenous mammalian miRNA targets take advantage of this mechanism.[117] In contrast, for the vast majority of animal miRNA targets, the interaction with Ago

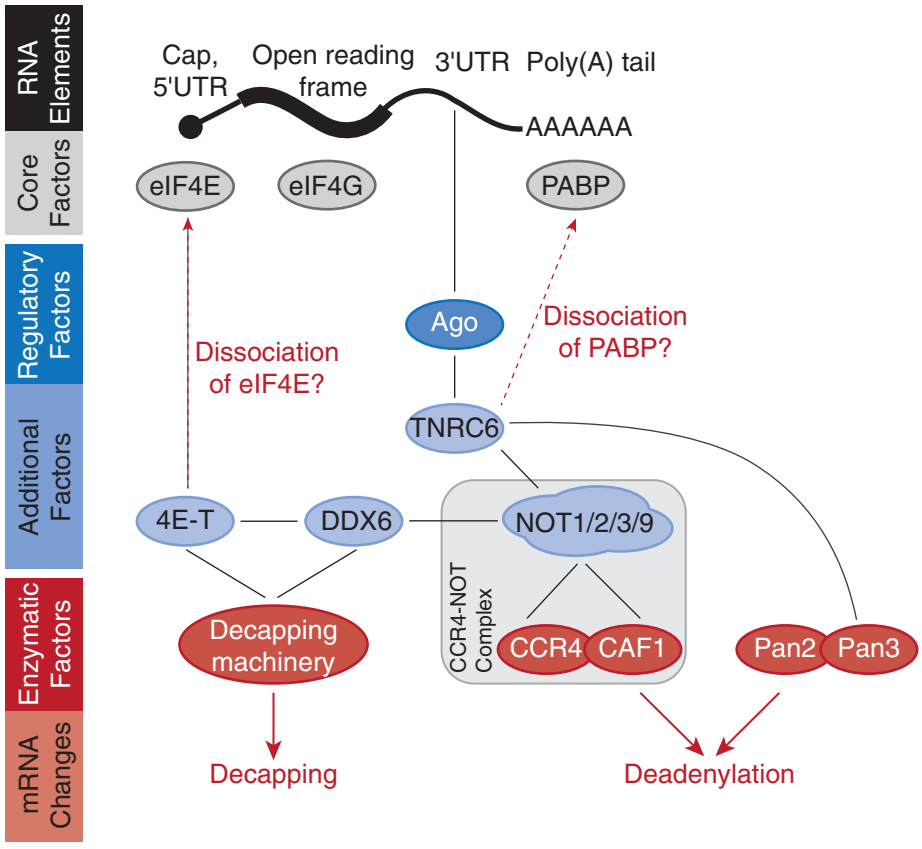

FIGURE 4 | mRNP reorganization during miRNA-mediated decay. Argonaute (Ago) is directed to specific transcripts via base-pairing between the target RNA and the loaded miRNA. TNRC6, which interacts with Ago, then stimulates mRNA decay via the recruitment of additional factors. TNRC6 interacts with PABP, which may stimulate its dissociation. It also interacts with the CCR4–NOT deadenylase complex and the Pan2–Pan3 deadenylase complex, which triggers deadenylation. In addition, CNOT1, a large scaffold protein in the CCR4–NOT complex, interacts with the decapping activator, DDX6, which in turn interacts with 4E-T. In addition to interacting with the decapping enzyme, 4E-T also interacts with eIF4E itself and may stimulate its dissociation from the target.

itself does very little to affect gene expression, and repression relies on an additional factor, a scaffold protein called TNRC6 (also known as GW182 in *Drosophila*).[118,119] TNRC6/GW182 in turn recruits decay factors, especially the CCR4–NOT and Pan2–Pan3 deadenylase complexes.[69,120,121] One component of the deadenylase complex, CNOT1, is itself another large scaffold protein, upon which additional decay factors assemble (for a more in-depth discussion, see Ref 122). These factors, such as DDX6 and 4E-T, then mediate effects at the 5′ end, primarily through the recruitment and stimulation of the decapping enzyme.[70–72,116] Other decay mechanisms, such as those mediated by Pumilio or TTP, also recruit the CCR4–NOT complex,[113,123] and it may be that the subsequent recruitment of DDX6 and 4E-T is a general mechanistic step shared among transcripts targeted for decay.

Interestingly, TNRC6/GW182 interacts with PABP, and it has been hypothesized that this interaction may be involved in dissociating PABP independently of deadenylation.[114,121,124] It has also been reported that eIF4G and/or eIF4A binding is reduced during miRNA-mediated repression,[114,125,126] but it is unclear how these events relate causally to the loss of PABP, the recruitment of the CCR4–NOT complex, and deadenylation. Indeed, some of the effects mediated by CNOT1 seem to not require the presence of a poly(A) tail,[69] which raises the perennial question about the role of deadenylation: how much of the effect of deadenylation results from recruitment of these additional factors and how much results from the shortening of the poly(A) tail? Careful mechanistic studies will be needed to disentangle these two possibilities.

OTHER CONSIDERATIONS FOR AN mRNP: CELLULAR CONTEXT

Armed with an extensive catalogue of RBPs, their targets, and their effect on posttranscriptional regulation, researchers should be theoretically well positioned to predict gene regulation. Of course, though, with its huge diversity of cell states, developmental and pathological contexts, biology is more complex than simple plug-and-play. One important aspect is the expression level of the RBPs themselves, especially in relation to their affinity for binding sites, as well as their posttranslational modifications, which can significantly impact RBP function.[127,128] Moreover, substantial overexpression of RBPs can lead to unexpected phenotypes, perhaps due to the sequestering or inappropriate localization of protein partners.

A further layer of complexity to the expression of factors comes from nonadditive interactions. Here, regulation mediated by an RBP is affected by the binding of others. For instance, as is the case for transcription factors,[129,130] some pairs of RBPs may act synergistically (with one extreme being those that bind mRNA as a complex), while others may compete for binding to the same site.[77,131] At the other end of the spectrum are those RBPs whose binding is relatively unaffected by the expression of other factors. For instance, miRNAs are generally robust to the interactions with other RBPs,[101] which may be partially due to biochemical properties of the Ago–miRNA complex itself.[110] Nonadditive interactions, which are in turn affected by the expression of both factors, are an important consideration in predicting posttranscriptional regulation *in vivo*.

Cellular context can also affect the 3′UTR itself through differences in alternative polyadenylation.[132,133] In any given cell type, the majority of expressed genes express more than one 3′UTR isoform.[101] Because shortening or lengthening a 3′UTR leads to inclusion or exclusion of regulatory sites,[134,135] differences in poly(A) site usage can have a dramatic effect on the posttranscriptional regulation of an mRNA. A recent report indicates that alternative 3′UTR usage can also affect posttranslational regulation by impacting subcellular protein localization,[136] highlighting the importance of 3′ end formation for gene expression. Poly(A) site usage is modulated by diverse processes, such as cellular transformation, differentiation, and tissue type,[101,132,133,137,138] but the molecular mechanisms underlying shifts in poly(A) site usage are unknown.

A final consideration is that some biological contexts, such as in the oocyte or early embryo, appear to operate with a different posttranscriptional logic than most other cell types. In the *Xenopus* oocytes, for instance, a common mechanism for translationally repressing a transcript is via deadenylation.[68,139] Notably, unlike in more differentiated cell types, such deadenylation does not trigger decapping and degradation of the transcript. Similarly, in the early zebrafish embryo, there is strong coupling between poly(A) tail length and translational efficiency, which is lost after the maternal-zygotic transition (MZT); in the early embryo, again, deadenylation fails to stimulate decapping.[37] Crucially, although the binding of miRNAs (and likely other factors) is unaffected by the context of the pre-MZT embryo, this difference in regulatory logic changes the effect of miRNAs.[37,105] There may be other biological contexts where posttranscriptional logic undergoes a similar switch, and so cellular

context represents an important consideration for gene expression.

mRNA SECONDARY STRUCTURE

Curiously, very little of discussions on mRNP organization focuses on mRNA secondary structure. This omission is partly due to challenges in computationally determining RNA secondary structures for molecules that are several thousands of nucleotides in length, portions of which are being dynamically unwound by the ribosome. However, another important reason for this omission is a more fundamental one: how much resolution of mRNA secondary structure is needed to understand a particular mRNP? In some cases, very little is necessary. For instance, in the ORF, unfolding by the ribosome most likely renders many stem-loops as nonfunctional. Yet, in other cases, local structure is absolutely fundamental to the organization of an mRNP. For instance, for factors that recognize double-stranded RNA or motifs in loops, such as Staufen or Smaug, secondary structure is a necessity.[140–142] Similarly, for factors that recognize single-stranded RNA, structured regions can occlude binding sites.[102,143] In fact, miRNA prediction algorithms incorporate a local secondary structure measurement, penalizing those regions with high structure.[108]

There are other wrinkles in discussing *in vivo* secondary structure. For instance, the binding of an RBP can alter mRNA secondary structure, which can, in turn, affect the binding of a second factor.[78] Similarly, RNA modifications, such as N6-methyladenosine (m6A), can affect the propensity of a region to fold, which then alters RBP binding.[144] Finally, the actions of helicases can disrupt secondary structure, and, generally, mRNAs are thought to be more unstructured *in vivo* than predicted by folding algorithms.[145] Recent protocol developments, such as DMS-seq, have generated *in vitro* and *in vivo* mRNA folding data on a transcriptome-wide scale,[145,146] and these approaches may spur additional insights into the relationship between local mRNA secondary structure and the organization of an mRNP as a whole.

THE ROLES OF THE ORF AND 5′UTR IN AN mRNP

With the translating ribosome capable of disrupting nearly all RBP–mRNA interactions, the 3′UTR takes on an outsized role in recruiting regulatory factors and controlling gene expression. Many types of regulatory motifs, such as miRNA sites, fail to mediate robust repression when they occur within 5′UTRs or ORFs.[102,147] Correspondingly, translational inhibition increases the repression mediated by ORF sites,[107] and it may well be that, in cellular contexts with limited translation, recruitment of regulatory factors to the 5′UTR and ORF may be an important component of controlling gene expression.

Nonetheless, the ORF can affect mRNP organization, primarily through the recruitment of decay factors. The best-understood examples are various co-translational surveillance pathways that recognize aberrant coding regions.[148] However, evidence is mounting that the ORF plays a larger role in mRNA turnover than just in surveillance pathways. A pioneering report from the Coller lab demonstrated that in yeast, codon optimality is an important determinant for RNA stability, above and beyond specialized quality-control pathways.[5] These results have been echoed in *Escherichia coli* and zebrafish,[149,150] suggesting that this pathway may be an ancient mRNA decay mechanism.

5′UTRs, despite their small size and the disruptions by the ribosome, have long been recognized as playing an important role in the mRNP. Interestingly, unlike elements in the 3′UTR that can affect both translation and mRNA stability, most examples in the 5′UTR primarily modulate translational efficiency. For instance, internal ribosome entry sites (IRES) bypass cap-dependent translation initiation by direct recruitment of initiation factors.[151,152] Although the most notable examples come from viral transcripts, these highly structured RNAs are also found in cellular transcripts and can promote protein production during events like mitosis where translation is globally repressed.[153] Intriguingly, a recent study indicated that m6A within a 5′UTR can stimulate translation through the recruitment of the initiation factor eIF3 in a cap-independent initiation pathway, and this modification is particularly important for the translation of Hsp70 during heat shock.[154] Although RNA modifications remain relatively poorly understood, this result underscores the importance of RNA modifications for some, and perhaps many, mRNPs.

Interactions between 5′UTR elements and RBPs can also inhibit gene expression. For instance, the 5′UTR of *PABPC1* contains a long A-rich stretch very close to the start codon.[155] This region, which is capable of binding PABP,[63] forms the basis for an autoregulatory feedback loop where translation of the transcript is repressed when PABP is present at high levels.[64,156] Interestingly, the presence of this A-rich tract in the PABP 5′UTR is highly conserved and can be found even in *S. cerevisiae* (O.S. Rissland,

unpublished results). Another example of a 5′UTR element that inhibits gene expression is the terminal oligopyramidine (TOP) motif. Much as their name suggests, TOP motifs are directly adjacent to the 5′cap and are a string of pyrimidine nucleotides. Found in important growth and cell-cycle genes, such as ribosomal protein genes, these motifs enable regulation by the mTOR pathway.[31,157] When mTOR is inhibited, translation of TOP-containing mRNAs is repressed through the displacement of eIF4G by 4E-BP1/2 and eventual dissociation of eIF4E.[31]

Other aspects of a 5′UTR, such as its length and structure, can also impact gene expression. eIF4A, a component of eIF4F, acts as the core RNA helicase in scanning 5′UTRs, and is increasingly required with increased length and secondary structure.[158] Some helicases, such as eIF4B and eIF4H, augment the activity of eIF4A,[159] while others, such as DDX3, are important in the translation of a subset of mRNAs.[160] Although these auxiliary helicases are clearly important for the expression of some genes, delineating their substrates has proven difficult thus far.

PERSPECTIVES

The advent of high-throughput sequencing technology combined with the concerted efforts of many labs has greatly increased our understanding of mRNP organization. Nonetheless, these experiments are just the start: mRNPs still have many mysteries that we are only now glimpsing. With the catalogue of regulatory factors, their targets, and their effect on gene expression within grasp,[99] a key question now is how, for an mRNA with many different motifs, all of its regulatory factors are integrated into its mRNP as a whole. Answering this question will likely require three additional components: first, understanding each of the underlying regulatory molecular mechanisms; second, characterizing the affinities for target sites of relevant regulatory factors and their concentrations; and, finally, analyzing the kinetics and dynamics of a variety of interactions, such as the regulatory factor binding to the RNA and the regulatory factor binding to additional factors like deadenylases.

Of these three components, the third is the most unknown. Recent studies of mouse Ago2, though, underscore the potential of biochemical analyses.[110,111] A more detailed understanding of kinetics, especially for core factors such as eIF4E, eIF4G, and PABP, will also touch upon larger questions of mRNPs dynamics, an issue that so far has remained intractable. Indeed, acquiring finer resolution of how an mRNP changes through the life cycle of a specific mRNA has been very challenging with current technologies. Understanding such dynamics on a transcriptome-wide scale seems out of reach, despite representing a fundamental question for the field, and may require technical innovation.

Another critical issue is how subcellular localization affects mRNP organization. Subcellular considerations for mRNA regulation are clearly important for some classes of mRNAs (such as those translated on the endoplasmic reticulum or the mitochondria) and for some systems with localized translation (such as neurons). Similarly, localization to membraneless structures, such as P bodies and nuage, has important repercussions. Nonetheless, in all of these situations, there are serious technical limitations in isolating large enough quantities of mRNPs for current molecular biology approaches. More broadly, although some important attempts have been made,[161] even characterizing the spatial organization of mRNAs on a transcriptome-wide scale represents a major undertaking.

In summary, mRNPs are, in many ways, the relevant unit for posttranscriptional gene regulation in the cell. Diverse and dynamic mRNPs have classically been hard to study, but recent innovations have begun to illuminate these complexes and, in doing so, have revealed how much remains unknown about the fundamental nature of these structures.

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