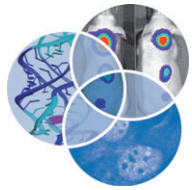
Advanced Review



Computational challenges, tools, and resources for analyzing co- and post-transcriptional events in high throughput

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Co- and post-transcriptional regulation of gene expression is complex and multifaceted, spanning the complete RNA lifecycle from genesis to decay. High-throughput profiling of the constituent events and processes is achieved through a range of technologies that continue to expand and evolve. Fully leveraging the resulting data is nontrivial, and requires the use of computational methods and tools carefully crafted for specific data sources and often intended to probe particular biological processes. Drawing upon databases of information pre-compiled by other researchers can further elevate analyses. Within this review, we describe the major co- and post-transcriptional events in the RNA lifecycle that are amenable to high-throughput profiling. We place specific emphasis on the analysis of the resulting data, in particular the computational tools and resources available, as well as looking toward future challenges that remain to be addressed.

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INTRODUCTION

Co- and post-transcriptional regulation encompasses a multifaceted and interconnected group of events including RNA processing, translation, and decay. Each stage involves multiple regulatory steps and interactions with complexes containing RNA-binding proteins (RBPs) and noncoding RNAs.[1] The list of regulators, which often participate in multiple processes, is long, with a possible >1000 RBPs and thousands of non-coding RNAs in human.[2,3] Dissecting co- and post-transcriptional regulatory events at the genomic level poses numerous challenges in terms of methods and computational analyses.

RNA biology reached genome-wide scale only recently, when RIP-chip (ribonucleoprotein immunoprecipitation followed by microarray analysis), the first approach for *en masse* identification of RBP targets, gained popularity in the early 2000s.[4] Other methods are still under development. For instance, ribosomal profiling (RP), which is now the method of choice for the study of translation regulation, was developed just a few years ago and continues to evolve.[5,6] As a result, computational methods to support these technologies have yet to reach the level of maturity seen, e.g., in the transcriptomic field. Also in contrast to transcriptomics, where some consensus has been reached in terms of methods and analysis pipelines,[7–10] RNA biologists continue to use a range of different experimental and analysis approaches. For

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example, although still used, RIP-chip and RIP-seq have been mostly replaced by a plethora of different cross-linking methods such as cross-linking and analysis of cDNAs (CRAC)[11] and CLIP (Cross-linking and Immuno-Precipitation) approaches, i.e., HITS-CLIP, PAR-CLIP, and iCLIP.[12–15] All methods have their pros and cons and, due to their technical differences and biases, deliver slightly different datasets.[16] When comparing datasets, it is hard to say why one method but not the others captured a particular binding site. We clearly need to conduct more extensive comparative analyses coupled with functional assays to better understand what each method is producing. An understanding of the idiosyncrasies of each technology used in the lab and how they relate to analysis methods is essential. They will give us the means to improve computational tools and include filters that at the end will deliver the highest number of functional RBP sites with a minimum of false positives.

At a higher level, the need for effective integration of disparate data sources in the study of co- and post-transcriptional regulation is particularly pronounced. Assigning function to RBP binding can be a complex task due to the polyvalent nature of these proteins. For example, binding of a given RBP to 3′ UTRs (untranslated regions) could affect mRNA decay, translation, or interfere with poly(A) site selection; multiple angles of analysis are necessary, but data integration is nontrivial. There is need to centralize all co- and post-transcriptional datasets and develop tools to allow cross-platform comparisons.

Figure 1 summarizes the relation between the major experimental high-throughput assays with both the stages and regulators of the RNA lifecycle they inform on. In the next sections, we cover different high-throughput approaches used in RNA biology, tailoring the discussion to the computational methods available and challenges in terms of development and data integration.

PROFILING RNA-BINDING PROTEIN ACTIVITIES

Experimental Methods

RNA binding proteins are, next to non-coding RNAs, the central drivers of co- and post-transcriptional regulation, and can have hundreds to thousands of target mRNAs thanks to flexibility in their binding specificity. *En masse* identification of *in vivo* binding has become possible only within the last decade, first with RIP and then with CLIP. They were developed by the Keene and Darnell labs, respectively.[4,12] They both consist of immuno-precipitation approaches where RNPs containing the RBP of choice are isolated and associated mRNAs are subsequently purified and identified. Quantification of the resultant RNA, was originally carried out using microarrays or Sanger sequencing, but is now more commonly performed using next- and second-generation deep sequencing. When RIP was established, there were some concerns regarding the possibility of re-assortment of RNPs during the IP process. This issue was essentially raised by a study from the Steitz lab,[17] in which a very simplistic analysis was conducted. To the best of our knowledge, similar claims have not been reported by other scientists using RIP. In fact, RIP was used successfully in cell systems and organisms to generate cell type specific gene expression profiles and no problems of cross-contamination between cell types have been reported.[18–20]

We focus on the analysis of data from these high-throughput assays, termed RIP-seq and CLIP-seq. While CLIP-seq is more frequently used, RIP-seq continues to be used, especially if there are limitations in terms of antibodies, or the amount and type of tissue. Recently, 'reversed CLIP' assays have been developed in which mRNAs are extracted; the binding sites and identities of bound proteins are determined by RNA-seq and proteomics, respectively.[21,22] These studies have revealed the enormous extent of the protein–RNA interaction landscape. In a more recent study Tombe et al.[23] developed a high-throughput sequencing-RNA affinity profiling (HiTS-RAP) assay that employs high-throughput sequencing to measure RNA aptamer affinities in large scale by quantifying the binding of fluorescently labeled protein to millions of RNAs anchored to sequenced cDNA templates. This is an extension of high-throughput sequencing–fluorescent ligand interaction profiling (HiTS-FLIP) protocol[24] that was previously developed to image and analyze the binding of fluorescently labeled proteins to DNA clusters for direct quantitative measurement of protein–DNA binding affinity.

Finding Targets and Binding Sites of RNA-Binding Proteins

RIP and CLIP aim to answer two closely related questions: which transcripts are bound by an RBP, and where. The key distinction lies in resolution. Generally, RIP-seq does not involve digestion of bound RNA fragments, and provides transcript-level resolution, enriching reads in bound RNAs but not necessarily with positional information. In contrast, CLIP-seq allows for much higher resolution. From a technical perspective though, identifying targets at the full transcript level and finding binding sites at the resolution of 10 or 20 nucleotides are essentially the

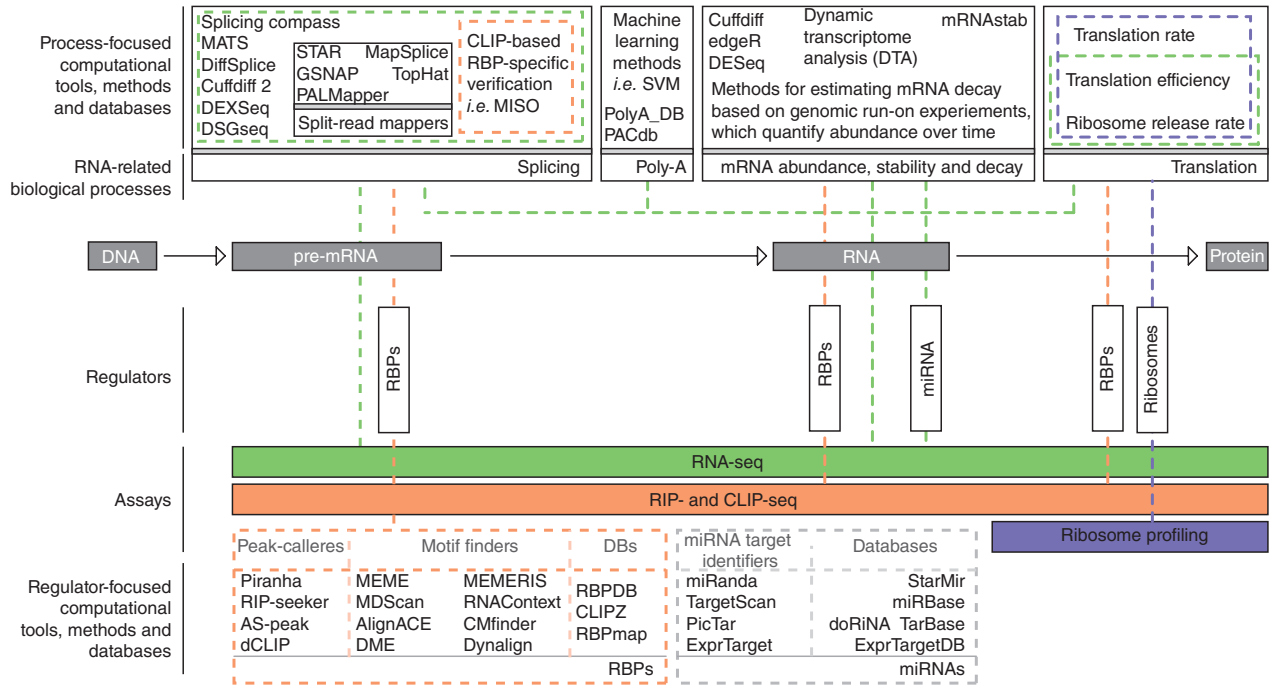

FIGURE 1 | Summary of post-transcriptional regulation processes and corresponding computational methods.

same problem: we search for genomic regions which are enriched for reads. This process is referred to as *peak-calling*, and forms the basis for any downstream analyses. Peak-calling follows read-mapping (alignment of short sequenced reads to the reference genome), which we will not address as it has been covered fully elsewhere.[25] Peak-calling assumes that some loci will receive reads, but not all of these represent true binding sites. There are a number of possible reasons for this, including transient or nonspecific interactions,[26–29] cross-linking biases (modest uridine preference caused by UV cross-linking in HITS-CLIP and iCLIP),[30] re-association after cell lysis[17] (the artifactual RNA–protein complexes formed in cell lysate, depending on lysis conditions, generally only a problem with RIP-seq), and background cross-linking (background caused by random UV cross-linking of RNAs to proteins that are not the RBP of interest).[31] However, it is expected that such false-positive loci will generally accumulate few reads. There is generally no specific way of defining such binding activities and different groups use different measure. For instance Friedersdorf et al.[31] performed an experimental method to define background cross-linking in PAR-CLIP data. Freeberg et al.[32] calculated the cross-link score (CLS) for each T in the genome, where CLS is the ratio of CLIP reads containing one or two T-to-C conversion events to the number of mRNA-seq reads and associate low CLS values to transient binding.[32] Similar methods can be used to define cross-lining biases and background cross-linking. Peak-calling aims to differentiate these loci from those that represent targeted binding of the RBP, i.e., are true-positives. This differentiation is particularly important in RIP, where the lack of cross-linking and RNAse treatment results in much higher background signal. Although CLIP has a high-degree of accuracy that cannot be achieved by RIP, it exhibits both cross-link biases and background cross-linking. In addition, due to inefficiency of UV cross-linking[33] it is not clear what proportion of binding activities is really captured by cross-linking. Nonetheless, even with these problems CLIP has proven to be useful for identifying mRNA targets of RBPs. However, due to the above-mentioned problems rendering careful separation of signal from noise essential.[17,30,31]

The simplest peak-calling scheme considers only the number of reads mapped to a locus. The exact read-count threshold to use must be calibrated for each dataset, since sequencing depth varies. A major challenge is selecting an appropriate resolution. Reads are counted into bins tiled along the genome. If bin size is too small, it is difficult to distinguish the underlying distribution of the read counts in peaks from the background. If the bin size is too large, resolution suffers. Most methods defer the decision to the analyst, although there are some attempts to automize selection of resolution, such as RIP-Seeker.[34]

Further, one must consider the statistical distribution of the read counts. In previous work, we demonstrated that read-counts are Poisson overdispersed in CLIP-seq datasets.[16] An appropriate model to capture their distribution is thus the negative binomial. When only a single sample is analyzed, loci with zero-counts are not considered, and in this case it is better to use a zero-truncated negative binomial, which appropriately adjusts for the missing zero counts. These distributions were used as the basis for the Piranha peak-caller.[16] In addition, other methods proposed Hidden Markov Model (HMM) for modeling and analyzing CLIP-seq data, such as dCLIP[35] and MiCLIP.[36] At the first step, dCLIP normalizes CLIP-seq data across datasets and subsequently employs an HMM to detect common or different RBP-binding regions across conditions.[35] MiCLIP uses two rounds of HMM to first infer enriched versus nonenriched regions and then to distinguish binding sites of RBPs versus nonbinding sites within those enriched regions.[36]

Additional information beyond read counts can be used to improve peak-calling. One example is transcript abundance. The number of reads mapping to a given genomic locus will be proportional to the binding strength of the RBP to that site, but also the abundance of the RNA. Abundant RNAs will take a greater slice of the sequencing pie, leaving less abundant RNAs, even if strongly bound, starving for coverage. Piranha was developed to account for this sequencing inequality, allowing the significance threshold, at which a locus is considered a true interaction, to vary as a function of RNA abundance, measured by RNA-seq.[16] AS-peak[37] is another peak caller, tailored specifically to RIP-seq data, that considers transcript abundance.

Other markers of true RBP–RNA interactions are modifications in nucleotide reads as a result of UV cross-linking, coined cross-link induced mutation sites (CIMS). In HITS-CLIP, CIMS are 'deletions' at the cross-linked nucleotide,[38] while in PAR-CLIP, reads exhibit T-to-C nucleotide conversions due to incorporation of 4SU photoactivatable-ribonucleoside into transcripts.[15] Not only are these changes useful in distinguishing true from false interactions, but they have also been used to improve localization. Without considering CIMS, only iCLIP achieves single-nucleotide resolution. Zhang and Darnell proposed a systematic method based on CIMS for the analysis of HITS-CLIP, elevating HITS-CLIP to single-nucleotide resolution, and allowing exact localization of the cross-link location.[38] They applied their genome-wide analysis to Nova and Ago HITS-CLIP data, identifying CIMS deletions in ~8% of mRNA tags mapped to Nova targets. Corcoran et al.[39] proposed a method for PAR-CLIP data, based on the characteristic conversion. They allow a read to contain up to two mismatches restricted to T-to-C conversions during the mapping. At each genomic locus, they calculate the likelihood of T to C conversion and use this to predict interaction sites.

To date, most analyses employing CLIP- and RIP-seq have been restricted to identifying targets and binding sites under single conditions. Moving forward, comparative analyses will become more important, and a few studies have already taken steps in this direction.[35,40–42] Firstly Tenenbaum et al.[4] used RIP-chip to determine dynamic changes in mRNA targets during neuronal differentiation. Moreover, Mukherjee et al.[43] employed Gaussian Mixture Modeling to RIP-seq data with probabilistic LOD scores and background quantification of each mRNA target to quantify dynamic changes in mRNA targets during T cell activation. However, computational tools to facilitate comparative peak-calling are few. To date, only Piranha and dCLIP provide support for identifying differential binding.[16,35]

Most tools for identifying interaction sites are stand-alone programs intended to run on a local machine. There are some online tools that can be used for CLIP data analysis, e.g., PIPE-CLIP[44] and pyCRAC,[45] both of which run on the web-based Galaxy[46] platform.

Characterizing and Understanding RBP Specificity

Nucleic acid binding proteins interact with their substrate (DNA or RNA) and participate in biochemical reactions that lead to specific cellular functions.[47] In the case of RNA, these interactions happen between a subset of residues in the protein (the RNA binding domains, or RBDs) and a subset of nucleotides within the RNA (the binding sites). Certain nucleotide sequences present high affinity for the protein's RBDs, causing the protein to bind to these locations with high frequency. These patterns are called *motifs*, and observing these patterns in a genomic location is called a *motif occurrence*. Motifs can be characterized by both sequence and structural elements and show tremendous variation amongst RBPs, even between members of the same RBP family.[48]

Until the early 2000s, characterization of binding sites was mostly restricted to individual studies involving a particular RBP and one target gene/binding motif. Such studies include a variety of assays from mutagenesis and binding shifts to more elaborate analyses involving 3D structures of RBP bound

to RNA.[1,49,50] One exception, SELEX experiments, combined a recombinant RBP and large pools of short random RNA sequences. After several rounds of selection, a consensus motif is defined based on the sequence of RNA fragments preferentially bound.[51,52] RNAcompete is another *in vitro* method that is much less expensive than SELEX due to a smaller designed pool of RNA oligonucleotides.[53]

Finding statistically enriched motifs in biological sequences is one of the most well studied problems in computational biology. The inherent variability in the motif sequence for RBPs renders methods based on exact matches of little use.[54] More flexible models have been proposed, the most well established being the position weight matrix, constructed by counting occurrences of each type of nucleotide at each position in the motif.[47,55–57] Methods employing this representation can generally be divided into two groups, (1) exhaustive enumeration methods, which are based on enumerating possible motifs then progressively narrowing the search to the neighborhood of highest scoring motifs and (2) probabilistic models, which construct the motif model and find the occurrences of the motif simultaneously in an iterative manner.[58] Much of the extensive body of work on motif discovery is due to the attention paid to transcription factors and the need to understand transcriptional regulation through protein–DNA interactions. MEME,[59] MDScan,[60] AlignACE,[61] and DME[62] are just a handful of the highly successful methods. The interested reader is encouraged to pursue one or more of the extensive reviews written on the details of these methods.[63–67] In comparison, motif finding in RNA brings its own unique set of challenges that must be considered. Early applications of motif-finding algorithms optimized for transcription factor binding sites to finding regulatory regions in RNA, especially RBP-binding sites, encountered a number of challenges, chief among which are the shorter length of RBP motifs[68,69] and the role of RNA secondary structure in binding site recognition.[70]

An early approach for modeling RNA structure involves covariance models (CMs).[71,72] CMs deliver both a sequence alignment and a consensus structure for a set of RBP-bound RNA sequences. Training a CM constructs a model from a set of sequences, which in turn can be used for aligning new sequences in an integrative approach. Other methods, such as Dynalign, a software for simultaneous sequence and structural alignment of RNA molecules using dynamic programing,[73] evolutionary methods,[74] and text indexing approaches[75] have been used for sequence and structural motif discovery for RNAs. However, evolutionary and text indexing methods are very limited in terms of the range of RNA secondary structures that they can discover, while CM and Dynalign are computationally expensive.

MEMERIS[76] was proposed for RNA binding site characterization, and it takes both sequence and structure into account. MEMERIS calculates the probability of RNA regions to be single-stranded, and uses these values as prior knowledge to guide the search for the motif. RNAcontext[77] is another approach for RNA binding site characterization and motif discovery that takes both sequence and structure of the RNA into account. The model developed in this program has a much simpler representation than MEMERIS: a position weight matrix for describing the motif sequence and an additional vector to describe the structural context of each nucleotide in the motif. RNAcontext performs well, both *in vitro* and *in vivo*, in terms of recovering experimentally validated motifs. However, both MEMERIS and RNAcontext suffer from the assumption that RNA sequence and structure are independent. In addition, MEMERIS takes only single-stranded regions into account, which is a limiting factor for RBPs that bind double-stranded RNA. More recently, a new method called GraphProt was proposed as a machine-learning framework for learning models of RBP-binding preferences from different types of high-throughput experimental data. GraphProt in essence is a supervised learning algorithm that builds a model using positive and negative sets of binding sites and then scans the genome to find instances of binding sites based on sequence and structure profiles.[78] For Identification of miRNA-RISC complex target sites, handful of studies has done CLIP experiment for transcriptome-wide mapping of miRNA targets, which have proven to be quite useful.[15,39,79,80] In addition, the computational methods take advantage of predictive features of the binding regions, most notably sequence characteristics of the seed region, phylogenic conservation of binding sites and secondary structure accessibility of the target.[81–83]

Several databases of RNA–protein interaction sites have been developed. RBPDB[84] contains a collection of experimental motifs of RNA-binding sites from human, mouse, fly and worm. This database includes RBP-binding sites derived from *in vitro* methods, motifs in position weight matrix format, and sets of sequences of binding sites obtained from immuno-precipitation experiments *in vivo*. CLIPZ[85] is a database of binding sites that are constructed from CLIP data for a limited number of proteins. However, users can upload their short-read sequences from CLIP, small RNA sequencing, and mRNA sequencing experiments for analysis.[85] RBPmap is a webserver for prediction of RBP-binding sites. Users can input

their sequences and motif in the form of a consensus sequence or position weight matrix or select from a large database of experimentally validated motifs. The algorithm then searches sequences for the motif, compares matches to the embedded background model, calculates a weighted rank for all the positions, and outputs a summary of all predicted binding sites.[86]

Regulators and Function

Binding of a given RBP to a target transcript can produce a variety of outcomes, both promoting and repressing events—for instance increasing or decreasing translation or mRNA decay, promoting or repressing exon skipping or the usage of a distal poly A site. A variety of genomics methods are necessary to link binding to function. For instance proteomics studies have been combined with RIP-chip and CLIP experiments to identify functional RBP-binding sites, e.g., to characterize the translation regulators such as HuR,[27] Msi1,[87] IGF2BP1-3, QKI, and PUM2,[88] or the splicing regulator RBM20.[89] Other methods combine the analysis of miRNAs with proteome, transcriptome or translatome profiling, e.g. for miR-124,[90] mir-223 and others.[91–93] The analysis of this data (and integration with data from binding assays) brings a new set of computational challenges that we discuss in the remaining sections.

REGULATING TRANSCRIPT ABUNDANCE

Quantifying gene expression is a well-studied problem in computational genomics. Expression profiling is now largely performed by RNA-seq. Read counts are the main source of information to calculate a gene's expression profile, though they must be correctly normalized to obtain meaningful information. There are primarily two concerns during normalization, which arise from transcript length and sequencing depth. The former is the result of RNA fragmentation during library construction in which longer transcripts naturally generate more reads than shorter transcripts even if they have similar abundance. Sequencing depth refers to the variability in the total number of reads sequenced and mapped in each run, which causes variations across samples.[8] To account for these issues, the reads per kilobase of transcript per million mapped reads (RPKM) metric was introduced by Mortazavi et al.[94] to normalize a transcript's read count by both its length and the total number of mapped reads in the sample.[8] With paired-end data, to avoid counting reads that fall into mapped fragments twice, a similar measure called reads per kilobase of transcript per million mapped fragments (FPKM) was developed.[95] However, Wagner et al.[96] showed evidence that RPKM is not suitable for comparison between samples and proposed a new measure called transcript per million (TPM) for this purpose.[96] For a comprehensive review on normalization methods for transcript abundance, refer to Dillies et al.[97]

Often the goal of analyses is to compare expression between conditions and identify transcripts whose concentration changed. Methods such as Cuffdiff,[95] edgeR,[98] and DESeq[99] are frequently used. Cuffdiff[95] is based on beta negative binomial model and estimates the variance of RNA-seq data by t-like statistics from FPKM values. edgeR[98] is based on an over-dispersed Poisson model in order to explain the variation in the read count data. The evaluation of differences across transcripts, are estimated using Empirical Bayes method. DESeq[99] uses a negative binomial for estimation of variability in read count data. Differential expression analysis for RNA-seq is a widely explored area; for a comprehensive survey refer to.[8,100]

RBPs have the capacity to directly regulate mRNA levels. However, many studies observe substantial changes in transcript abundance upon knockdown or knockout of RBPs, but find a surprisingly small overlap with the set of RBP targets identified by binding assays.[101] This discrepancy is most likely due to a large number of indirect effects. As a result, the question of whether data from binding assays can be effectively married with mRNA expression data remains open.

ALTERNATIVE SPLICING

The 'one gene, one enzyme' hypothesis postulated by Beadle and Tatum[102] is no longer valid; we know that the number of human genes is much smaller than the number of expressed proteins.[103] This discrepancy can be explained by several levels of gene regulation, co- and post-transcriptional modifications, especially alternative splicing.[104]

More than 90% of human genes are alternatively spliced, with a role in many physiological functions.[105,106] Alternative splicing, coupled to nonsense-mediated decay (NMD), can also directly regulate gene expression by producing unstable transcripts that contain premature stop codons.[107,108] Splicing-related changes in gene expression can be triggered in response to stress and other environmental signals,[109] and are increasingly recognized as a participant in many diseases.[110–113] Cancer-related studies have revealed specific changes in alternative

splicing patterns that can be used for diagnosis[65] and therapy.[114]

Many mathematical models, algorithms and statistical methods have been developed and employed to explore alternative splicing. The goal of these methods is generally to identify and quantify the abundance of individual transcripts,[115,116] or more commonly, to profile changes in splicing either at the full transcript level or at the level of individual splice sites and exons.[117–121] The latter task is called differential splicing analysis. An example of such an analysis would be to calculate exon inclusion from exon-junction arrays, microarrays or RNA-seq data, and then compare the values between samples or conditions to infer occurrences of different alternative splicing events. Although some approaches to either problem may employ a reference dataset of exons or splice junctions and only considers splicing events with known splice junctions, a frequent goal is to identify novel splicing events with previously unknown donor and acceptor sites. Addressing this challenge relies heavily on *split-read* mappers, which are able to map reads containing previously unknown splice junctions—a task that regular short-read mappers generally fail with, as the read is not derived from a single contiguous region of genomic sequence, nor one that can easily be constructed *in silico*.[122–132]

Several excellent reviews of computational methods for splicing and alternative splicing analysis already exist; for a detailed review of methods and databases refer to Hooper et al.[133] and the EURASNET website,[134] respectively. Despite much work in this field, it remains challenging to link the observed changes in splicing regulation with their regulators, such as RBPs. In the case of RBPs, one approach is to profile cells with a regulator of interest either silenced or deleted, and compare against the wild-type. RIP and CLIP have been used to match observed changes in splicing to the putative binding sites identified, as has been done, e.g., for Nova,[13] hnRNP proteins (namely, hnRNP C,[14] H1,[116] L,[135] A1, A2, A2B1, F, M, U[136]), TDP43,[137] Fox,[138,139] PTB,[140,141] Mbnl1,[142] TIA1, and TIAL1.[143] However, the analysis is generally ad hoc; no effective computational tools yet exist for linking functional assays such as RNA-seq with binding assays such as RIP- or CLIP-seq. One main reason for this problem is that observing binding activities of an RBPs according to RIP or CLIP experiments is not an evidence of direct binding.

ALTERNATIVE POLYADENYLATION

Polyadenylation is the addition of a stretch of adenosine nucleotides to the end of RNA molecules. This polyA tail aids nuclear export and translation, and protects the transcript from degradation. The point at which the RNA is cleaved and the tail is added can vary—a mechanism known as alternative polyadenylation (APA). APA can result in mRNAs with differences in coding sequence and 3′ UTR, contributing to altered regulation, function, stability, localization, and translational efficiency.[144] Although alternative polyA sites, that are situated between coding exons, can lead to isoforms encoding different proteins,[145] more often APA events result in shorter 3′ UTRs which lack sequences that are targets of microRNAs and RBPs.[146] The earliest examples of APA were described in the mRNAs of IgM and DHFR.[147,148] Subsequently, EST databases and microarray analyses allowed the identification of several other APA sites.[149,150] Recent RNAseq methods have enormously improved our understanding of APA.[151]

Genomic studies have shown that APA is a widespread phenomenon in metazoan genomes. For example, about 70% of mammalian genes and about 50% of the genes in flies and worms are subjected to APA.[146,152,153] This mechanism is known to regulate a range of biological processes, often associated with development, cellular differentiation and proliferation. Shortened 3′ UTRs due to alternative polyadenylation are associated with increased pluripotency and cell proliferation,[154,155] and relaxation of microRNA repression of oncogenes.[156]

Computational methods for the prediction of alternative polyadenylation are mainly based on the Direct RNA Sequencing (DRS) technology,[157] in which RNA molecules are sequenced without prior conversion to cDNA or the need for biasing ligation or amplification steps.[157] This method was employed to develop a map of over 1 million polyA sites in major cancers and tumor cell lines.[158,159] An alternative method, PolyA-seq, allows for the high-throughput sequencing of the 3′ ends of polyadenylated transcripts, and has been used to obtain a global map of polyadenylation sites in human, rhesus, dog, mouse, and rat.[153] Purely computational methods for predicting the locations of polyA signals also exist, such as the classification-based method polyA-predict, which was used to construct a database of predicted sites.[160] Other databases of polyA sites include PACdb[161] and PolyA_DB.[162]

STABILITY AND DECAY

Regulation of mRNA Stability and Decay

Another major contributor to expression regulation is mRNA degradation which has also been linked to several diseases.[163] Two major regulatory routes

control mRNA decay: quality control mechanisms eliminate the production of aberrant protein products while another group of mechanisms influence mRNA life time with the main purpose of controlling protein abundance.

A prevalent example of degradation for quality control is NMD, which eliminates mRNAs that prematurely terminate translation.[107] It can be regulated in multiple ways, such as relative concentration and phosphorylation of NMD factors and miRNAs—a detailed review is provided by Kervestin et al.[164]

Another important mechanism is the ARE-mediated mRNA decay. It is predicted that 9% of the human transcriptome contains ARE elements in the 3′ UTR; these are characteristic short AU rich or U-rich sequences.[165] ARE-containing mRNAs have been implicated in important physiological functions as well as diseases and tumorigenesis.[166] Several RBPs like TTP, BRF1, KSRP, and AUF1 interact with ARE-sequences and help recruit degradative enzymes. Another group of RBPs, which include the highly studied HuR, binds ARE elements and increase their stability.[167] These ARE binding proteins have their activities modulated by cell signaling, phosphorylation, and cellular localization.[168,169] For a comprehensive review on mRNA decay see.[170]

Transcriptome-Wide Profiling and Computational Tools

Transcriptome-wide analysis of mRNA decay generally relies on time-series data in which mRNA levels are measured at different time points.[171] For example, data from genomic run-on experiments is used by the computational tool mRNAStab to determine mRNA stability by calculating mRNA half-lives.[172] Dölken et al.[173] developed a pioneering approach to separate total cellular RNA into newly transcribed and preexisting RNA upon metabolic labeling. Other methods are based on Dynamic Transcriptome Analysis (DTA)[174] to calculate mRNA half-lives.[175] From a functional perspective, the influence of RNA sequence and structural elements on mRNA stability and other post-transcriptional regulatory mechanisms has been the subject of recent studies.[176] For instance, TEISER[177] is a computational framework to calculate the correlation between the presence or absence of sequence and structural motifs with experimentally determined mRNA stability. MIST-Seq (Measurement of Isoform-Specific Turnover using Sequencing) is another recently introduced method designed to estimate the decay rate of a population of RNAs accurately.[178] Its application revealed that even minor differences in sequence composition could lead to large changes in decay rates between isoforms, highlighting the functional effect of particular 3′ UTR elements on mRNA stability. Similar studies have been carried out in yeast, comparing mRNA isoform half-lives across different isoforms of particular genes and inferring biological functions for particular sequence elements.[179]

Micro-RNA Biogenesis and Function in mRNA Decay

Over the last decade though, probably the most heavily studied mechanism for regulating mRNA levels has been through microRNAs (miRNAs). MicroRNAs regulate gene expression by base-pairing with complementary sequences in mRNAs.[180] To accomplish this, miRNAs rely on an Argonaute protein to form a complex, called the RNA-induced silencing complex (RISC) that facilitates the binding of miRNAs to mRNAs, and their gene silencing function. However, the actual mediators of gene silencing are members of the GW182 protein family, which regulate all downstream steps in gene silencing.[181–186] Watson-Crick base-pairing between the miRNA and target mRNA determines the specificity of the complex, while the Argonaute protein exerts the gene regulatory function.[187] A given miRNA can have hundreds of targets and a given gene can be regulated by multiple miRNAs. A more comprehensive review on the mechanisms of miRNA gene regulation is presented elsewhere.[188] The end result of miRNA-mediated gene regulation is reduced protein output from the cognate mRNA.[92]

The most successful methods to date for computational identification of miRNA-binding sites have been miRanda,[189] TargetScan,[190] and PicTar.[191] miRanda uses a dynamic programming algorithm to search for complementarity matches between miRNAs and 3′ UTRs. For each match, it estimates the stability of interaction using thermodynamic calculation of the complex free energy and calculates a conservation score with closely related species.[189] Validations have shown this approach to be highly successful. TargetScan[190] takes a similar approach based on the thermodynamics of RNA–RNA interactions and comparative sequence analysis to predict miRNA targets conserved between species. The algorithm in PicTar is based on Ahab,[192,193] which is a probabilistic algorithm for the identification of combinations of transcription factor binding sites[190] and identifies common targets of microRNAs in eight vertebrate genomes.

Several research groups have developed databases of miRNA target sites. ExprTargetDB[194] is a database obtained using an integrative approach

combining the results form TargetScan, miRanda, and PicTar. Other databases include miRBase,[195] the repository for miRNA gene set annotations and TarBase,[196] which is a collection of miRNA gene interactions coupled with experimental observations for any listed interaction. STarMir[197] is a web-server that predicts miRNA binding sites and computes several other features of the targets such as consensus sequence, thermodynamic, and target structure to calculate a measure of confidence for each predicted site.

MicroRNAs act in concert with RBPs. Some databases leverage this for greater accuracy. For instance, Starbase,[198] which uses CLIP experiments to compile a set of computationally predicted miRNA target sites for several species. They also filter false-positive miRNA target sites, which can be used for the detection of false negative binding sites absent from current prediction sets. Another database employing CLIP-seq data is doRiNA,[199] which uses PicTar,[191] and offers the advantage of easy visualization via the UCSC genome browser. Target prediction algorithms for miRNAs that rely on a trusted set of miRNA target sites can greatly benefit from such a feature.[176]

TRANSLATION

Translation and Its Role in Biological Processes

Translation regulation plays an important role in many biological processes.[200–202] It accounts for up to 30% of variation in protein expression in both yeast[203] and mammalian cells.[204] Certain cell types are even more reliant on post-transcriptional regulation than others. Examples include blood platelets, which lack nuclei, so their cellular responses must be modulated post-transcriptionally, and the final stages of sperm development, where transcription is silenced.[205,206] Translation regulation is also essential in development. During early embryogenesis it controls embryonic axis, body patterning, and cell fate, as transcription is largely quiescent at this stage.[207] Since translation reacts faster than transcription, it often forms the basis for rapid responses to environmental changes.[202]

Owing to its important role in cellular biology, translation is also recognized as a nexus susceptible to disruption in diseases. For example, abnormal translation is now a recognized characteristic of tumor cells and a potential target for therapy.[208] Elevated levels of the translation initiation factor elF4E have been found in many cancer cell lines and tumors, and over-expression in rodent cells results in malignancies.[209] Close to 60% of the mRNAs classified as proto-oncogenes have atypical 5′ UTRs with complex structure and high GC content, hindering ribosome binding.[210] There are implications for understanding cancer treatment as well. Radiotherapy is the preferred approach for many tumor types. Genome-wide analyses of irradiated cells revealed that the number of genes with translation affected by radiation is close to 10-fold greater than those with altered transcription.[203]

Methods and Challenges

Genome-scale knowledge of translation regulation has lagged behind that of transcription, despite its central role. Integrative analysis of RNA-seq and shotgun proteomics and comparison of protein to mRNA concentrations is one approach to estimate translation efficiency.[211] However, this approach is limited, e.g., by the number of genes covered by proteomics analysis and ignorance of protein degradation. More direct approaches use ribosome binding to mRNAs as a proxy of translation efficiency. For decades polysome profiling has been used to study translation regulation. This method is based on separation of mRNAs that are heavily loaded with ribosome from free mRNAs using ultracentrifugation on sucrose gradients. Coupling polysomal profiling and microarrays or RNA-seq enable translation studies to enter the world of genomics.[212,213] In recent years, the field has experienced a dramatic boost with the advent of ribosome profiling (RP).[6]

Ribosome Profiling

RP is a relatively new method that promises to provide researchers with quantitative information about the relative number and locations of ribosomes bound to RNA.[6] In the RP method, ribosome-protected mRNA fragments are sequenced deeply. Figure 2 demonstrates the detailed steps in this protocol. RP can be used for examining translational control in a range of settings, from basic mechanistic investigations to studies of disease and drug treatments.[216,217] It provides an excellent tool to investigate, discover, and catalog translational products present in a cell type at single-nucleotide resolution. Despite its challenging protocol, the RP technology is now more and more used, and computational analysis tools are under development. Currently, the number and position of reads are used to estimate ribosome binding.

A fundamental contribution of RP has been the identification of open reading frames (ORFs). An ORF is a segment of an mRNA, bounded by a translation initiation site (TIS) and translation termination site

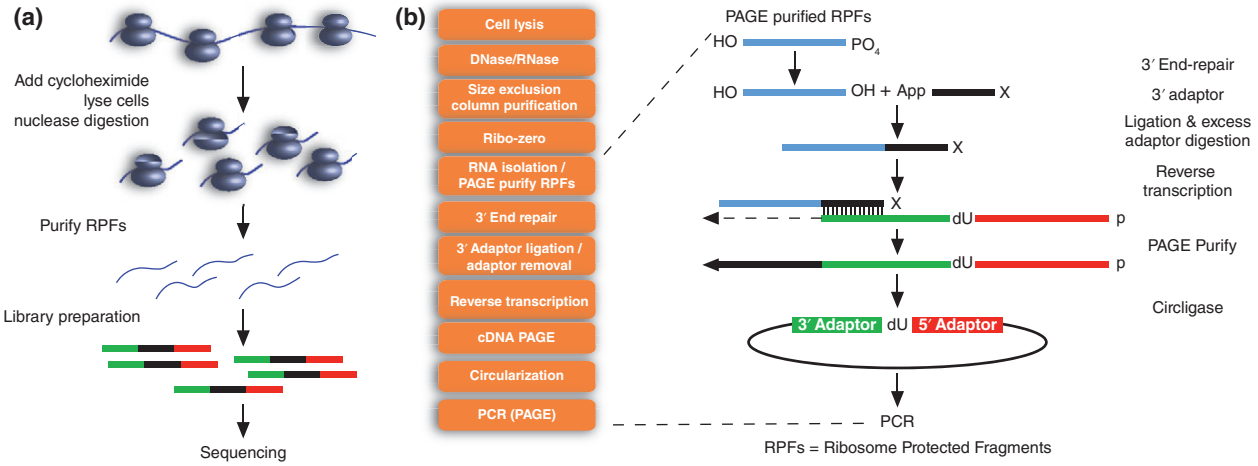

FIGURE 2 | (a) Overview of ribosomal profiling (RP) experiments. (b) Detailed steps in the ArtSeq protocol for ribosomal profiling. The protocol starts with cell fragmentation; the resulting cell extract is submitted to nuclease digestion, which will generate ribosome-protected RNA fragments. Ribosome-RNA complexes are purified using gel filtration columns (SV400 samples) or sucrose cushion (sucrose samples), followed by RNA extraction and elimination of ribosomal RNAs (rRNA). rRNA-depleted samples are submitted to electrophoresis, and ribosome-protected fragments (about 35 nt long) are eluted from gel. These RNAs are used as templates for library preparation and sequencing.[214,215]

(TTS), which causes formation of the elongation-competent 80S ribosome complex.[218] Identifying ORFs is one of the classical analysis problems of computational genomics.[219] HMMs have been used to identify ORFs for more than 20 years,[220] and have done exceptionally well due to their flexibility and the natural sequential dependence within ORFs. The most sophisticated ORF-predicting HMMs were developed in the context of determining the complete gene structure (promoter, exon, intron, etc.).[221]

However, factors such as transcripts with multiple ORFs, internal ribosome entry sites, leaky translation, ribosome shunting, and near-cognate start codons make the purely computational identification of ORFs problematic, as evidenced by the discovery of many novel ORFs by RP studies.[222–225] Despite the success of RP, no public tools are available to date. Many studies simply assume known ORFs.[226] Those that predict them rely on read patterns in RP data from samples treated with elongation inhibitors, which cause ribosome arrest at the TIS (Figure 3). Ingolia et al.[224] employed a classification approach to provide genome-wide maps of protein synthesis. Lee et al.[225] defined a measure based on the number of reads at each position and the total number of reads on the same transcript in their data to identify peaks of ribosome activities and therefore obtain a global map of TISs in mammalian cells. Fritsch et al.[223] employed a neural network method for genome-wide identification of novel upstream ORFs in human. Stern-Ginossar et al.[227] used a method similar to Ingolia et al.[224] to discover diverse short reading frames in human Cytomegalovirus. Clear read patterns denote both TIS and stop codons in untreated samples too, but have not so far been leveraged to improve our definition of ORFs.

Another complication is that elongation-inhibited samples only approximately identify the TIS, since the start of the reads marking the protected fragment is offset from the A-site—by about 12 nucleotides generally.[225,228,229] The TIS is then determined by searching for a sequence (codon) nearby, which requires an existing model and precludes unbiased TIS characterization. Existing methods for detecting the read-pattern indicative of TIS in RP data have been trained on known exemplars,[223,224] which may not always be available and biases toward sites similar to those already known.

Identification of ORFs also opens up the possibility of finding and characterizing regulatory reading frames. Many mRNAs contain ORFs upstream of the genic ORF, called uORFs, which also engage ribosomes.[218,230] Whether uORFs produce viable proteins with any function remains open, though the fact that they regulate translation of their downstream genic counterparts is now well established through several recent studies.[218,230–233]

RP analysis provides several measures of translation regulation. It reports the number of mRNAs bound by ribosomes compared to unbound mRNAs (occupancy), it reports the total number of ribosomes per mRNA (density), and the ribosome position at nucleotide resolution. While these data are insufficient to calculate actual rates of translation, they serve as a detailed proxy of translation efficiency per gene. Mass-spectrometry based approaches have

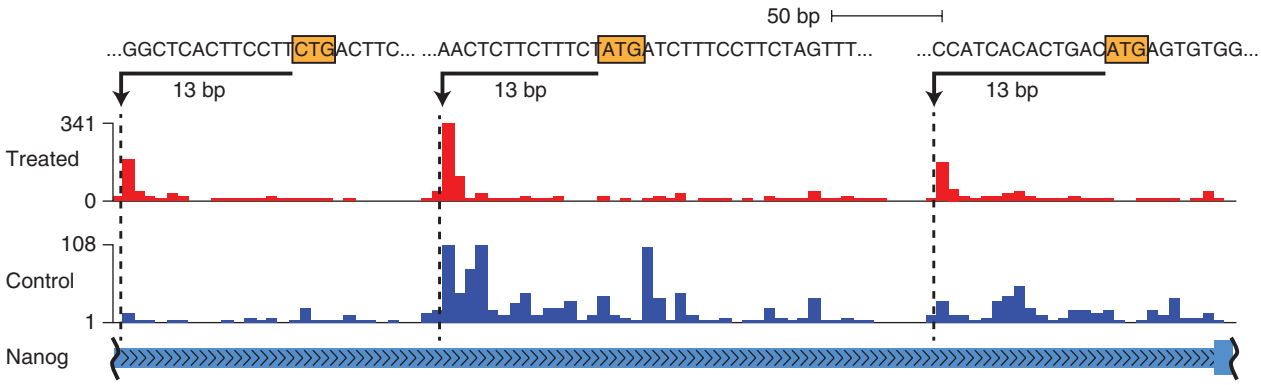

FIGURE 3 | Read profiles of untreated and harringtonine-treated RP data. The genic ORF and two uORFs in the Nanog transcript are shown. Start codons are highlighted, and the offset of the 5′ end of reads is indicated.

recently provided methods to measure actual translation rates,[211,234] but in contrast to RP, these methods only cover a fraction of the human genome. To the best of our knowledge no comparison of RP and actual protein expression levels exists to date.

Via the clever use of time-series data and drug treatments that inhibit translation initiation, RP can also provide insights into translation elongation speed using so-called 'run-off' experiments.[224] Following treatment, ribosomes inside active ORFs will move away from the TIS leaving a 'depleted' region, where RP reads are only observed at the noise level. In addition, we can also define the unaffected region, where ribosomes still exist, and the 'depleting' region, where some intermediate fraction of messages have been depleted of ribosomes (i.e., stochastic variation in speed between molecules with the same ORF). Analysis of the position and lengths of these regions after specific treatment times provides estimates of elongation speed.

Despite the successes of RP, there are a number of outstanding computational challenges. One major challenge is correctly adjusting for ribosome pausing. Protein synthesis by ribosomes takes place at nonuniform speeds between ORFs, and also with varying speeds within an ORF; one extreme is pausing.[229,235,236] Metrics aimed at measuring translation levels must therefore be adjusted to remove the influence of stalled ribosomes. These might be stalled pre-initiation complexes, ribosomes paused during elongation or awaiting release upon termination. Because these ribosomes are not actively translating, they do not contribute to protein levels. Previous studies either ignore the pausing phenomenon, or assume important pausing happens near TIS and stop sites, discarding all reads falling within a fixed distance to these. This discards information, alters the effective size of the region when normalizing, and cannot be done for short coding sequences.

CONCLUSION

Controlled and coordinated binding of one or more RNA binding proteins or miRNAs is the key mechanism that drives co- and post-transcriptional regulation of gene expression. These processes are often complex, inter-related, and dynamic in terms of their timing. Efforts to understand them at global scale therefore require multiple lines of investigation, and necessitate a range of computational methods to interpret the resultant data. Transcriptome-wide profiling of co- and post-transcriptional regulation is still a young field, and the development of computational tools to complement the emerging biological assays is pending. Some fundamental problems still exist. For example it remains unclear what proportion of sites identified in CLIP or RIP are actual binding sites. Moreover, our understanding of what makes a functional RBP-binding site, as opposed to one that has little or no functional impact is still thin. As a result, there are no effective computational tools for determining whether a given RIP- or CLIP-seq site represents functional binding or not. Nevertheless, substantial progress has been made and a range of methods aimed both at fundamental processing of data, and the more high-level goal of understanding specific biological processes are now available. These are supplemented by a growing collection of databases and online resources.

Moving forward, new biological questions will be asked. Questions aimed at expanding our understanding of the interactions between regulators, regulatory networks, the timing of events, and how perturbation of the cellular state affects them. These

questions will drive the next generation of computational methods. One key issue will be the development of tools that effectively handle multifactorial experimental designs, with multiple replicates, and are able to leverage the additional statistical information they bring. First studies exist which combine several of these large-scale approaches. For example, to distinguish functional from nonfunctional RBP-binding sites, proteomics studies have been combined with RIP-chip and CLIP experiments to characterize the translation regulators. Other efforts combine the analysis of miRNAs with proteome, transcriptome, or translatome profiling. As more and more studies on multi-dimensional approaches arise, we need computational methods to integrate and analyze these data. In recent years there has been some studies to gain insight into functions of RBPs by studying mRNA targets of particular RBPs obtained by RIP or CLIP together with changes in mRNA stability or splicing and before and after knockdown of that specific RBP.[237] These approaches will help to drive the consolidation of information about co- and post-transcriptional gene regulation into more holistic and comprehensive models.

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