Review

Genome-wide alternative polyadenylation in animals: insights from high-throughput technologies

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Alternative polyadenylation (APA) plays an important role in gene expression by affecting mRNA stability, translation, and transloca-tion in cells. However, genome-wide APA events have only recently been subjected to more systematic analysis with newly devel-oped high-throughput methods. In this review, we focus on the recent technological development of APA analyses on a genome-wide scale, as well as the impact of APA switches on a number of critical biological processes in animals, including cell proliferation, dif-ferentiation, and oncogenic transformation. With the highly enlarged scope of genome-wide APA analyses, the APA regulations of various biological processes have increasingly become a new paradigm for the regulation of gene transcription and translation.
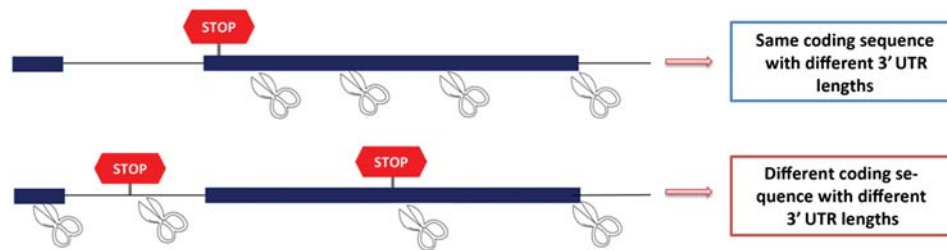
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Introduction

The various mRNA isoforms of each gene, which are produced via alternative splicing (AS), transcription initiation and alterna-tive polyadenylation (APA) are the results of accurate temporal and spatial regulation of gene expression. By recognizing multiple polyadenylation signals, the transcription process can terminate at different APA sites. It has been shown that more than half of the genes in the human genome have APA sites (Tian et al., 2005). APA sites can be classified according to their effects on mRNA: those located in the most 3′ exon, downstream of the stop codon, result in 3′ untranslated regions (3′ UTRs) of different lengths (called tandem 3′ UTRs), while those located upstream of the stop codon may lead to changes in both the protein-coding and 3′ UTR sequences (Figure 1). The differential usage of tandem 3′ UTRs plays an important role in the gene expression network by influencing mRNA stability, transport and translation, generally through the loss and gain of regulatory motifs, especial-ly microRNA-binding sites (Sandberg et al., 2008; Mayr and Bartel, 2009) (Figure 2). A precise map of the expression of differ-ent APA sites across diverse cell types for all genes is urgently needed for better understanding the biology of gene expression.

For decades, tens of genes have been found to use different cleavage sites in their 3′-end formation and regulation by APA (Edwalds-Gilbert et al., 1997), such as *Cyclin D1* (Yarden et al.,

1995; Wiestner et al., 2007), *Amy-1A* (Tosi et al., 1981), and *NF-ATc* (Chuvpilo et al., 1999). Most of these studies used north-ern blotting to detect the transcriptional end of a single gene's transcript. APA was first implicated on a genome-wide scale by an integrative bioinformatics study of expressed sequence tag (EST) data (Gautheret et al., 1998; Beaudoing and Gautheret, 2001). With the accumulation of cDNA/EST data, more than half of the genes in human and over 30% of mouse genes were found to harbour APA sites (Tian et al., 2005). However, due to high cost and limited data yield, the cDNAs or EST libraries fol-lowed by capillary sequencing only provided limited information for the true complexity of APA-directed gene regulation (Carninci et al., 2003). With microarray technology (i.e. exon array), a more cost-effective method, was used to study genes with known APA sites (Sandberg et al., 2008). The limitations for this method were that only genes with known APA sites and only known polyA sites within these genes could be studied. The second-generation sequencing technologies have made it possible to overcome these limitations by sequencing cDNA derived from cellular RNA. Based on the second-generation se-quencing technologies, RNA-seq was developed for the quantifi-cation of genes expression and AS, the discovery of new fusion genes, the improvement of genome assembly, and the identifica-tion of the start and end of a transcript (Cloonan et al., 2008; Mortazavi et al., 2008; Wang et al., 2008; Maher et al., 2009; Hawkins et al., 2010; Ozsolak and Milos, 2011). To study APA sites in a whole genome fashion, RNA-seq lacks the efficiency to profile complex APA events deeply because only a very small

Figure 1 Two types of alternative polyadenylation configurations. Type II polyadenylation occurs in more than one polyA site, all of which are located in the 3′-most exon and generate mRNAs with the same protein-coding sequence with different 3′ UTR lengths; type III polyadenylation occurs when alternative polyA sites are located in different exons or introns and generate different protein-coding sequences with different 3′ UTRs. The type II alternative polyadenylation is usually called tandem 3′ UTR.
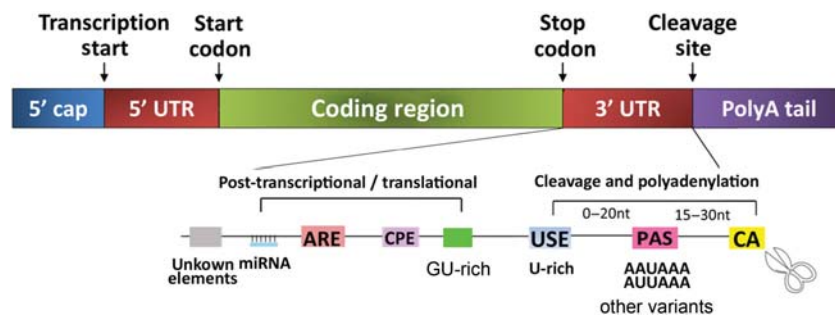
Figure 2 The basic structure of eukaryote mRNA and representative *cis*-elements in the 3′ UTR. The upstream U-rich element (USE), polyA signal (PAS, usually AAUAAA or AUUAAA, but other variants exist) are involved in the regulation of cleavage and polyadenylation. ARE (AU-rich element), microRNA target sites, cytoplasmic polyadenylation [(pA signal and cytoplasmic polyadenylation element (CPE)] and GU-rich elements are involved in post-transcriptional/translational regulation, usually by affecting mRNA stability or translation efficiency. There may be important unknown elements that exist in the 3′ UTR.

proportion of reads are generated from the 3′-ends of mRNAs (Wang et al., 2008; Pickrell et al., 2010). Recently, several new methods based upon RNA-seq have been developed to profile APA sites genome-wide; consequently, various biological effects of APA sites have been investigated, including tumourigenesis and metastasis (Mayr and Bartel, 2009; Fu et al., 2011), embryonic development (Ji et al., 2009; Mangone et al., 2010), immune responses (Sandberg et al., 2008), and neuron activity (Zhang et al., 2005; Flavell et al., 2008).

Although several excellent reviews (Danckwardt et al., 2008; Lutz, 2008; Neilson and Sandberg, 2010; Di Giammartino et al., 2011; Lutz and Moreira, 2011; Proudfoot, 2011; Tian and Graber, 2012) have described APA sites, including their mechanisms and implications for disease, there is still a shortage of reviews on genome-wide studies of APA sites, especially a comparison between different APA profiling strategies. In this review, we focus on genome-wide studies of APA switching and its biological consequences, and we discuss the potential research directions for the APA regulation.

High-throughput techniques for studying APA sites

With the emergence of vast amounts of EST data made available by Sanger sequencing at the end of the last century, APA studies have also entered into the omics era, where they have progressed rapidly, benefiting from the microarray and second-generation sequencing technologies.

EST by sanger sequencing

Gautheret et al. (1998) first analysed human APA sites with 3′ EST library data. These researchers clustered 164000 3′ ESTs, constructed contigs and visualized the alignments to find the putative polyA sites; they subsequently filtered the internal priming by distinguishing the adenine stretches in the contigs flanking the 3′ ESTs. Later, this group further analysed polyA signals and tissue-biased APA sites (Beaudoing et al., 2000; Beaudoing and Gautheret, 2001). These researchers mapped the 3′ ESTs to the 3′ UTR database with BLAST, and they filtered the internal priming. Subsequently, they combined the mapping positions within 30 nt of each other to define the polyA sites for which two or more ESTs were required. Although the results were imperfect because of the limited data size and the lack of a corresponding genome, the findings provided a primitive method to identify the different polyA sites.

Microarray

The design of an expression microarray with multiple probes within the last exon has provided one method for analysing tandem APA sites. The 3′-IVT expression array by Affymetrix designs 11 probes within the 600 bp near the 3′-end of each transcript, while the exon array designed ∼4 probes within each exon. Based on this 3′-IVT array analysis, Flavell et al. (2008) have found that neuron activity-dependent genes in the rat switched to proximal APA sites. In addition, an exon array analysis revealed that activated T lymphocytes tended to use shorter 3′

UTRs (Sandberg et al., 2008). Briefly, these researchers first chose genes with two polyA sites based upon their prior knowledge, and later genes with at least two probes on each of the common and extended regions of 3′ UTR were designed to analyse the APA site switching. The 3′-IVT array may be more accurate than an exon array for APA studies because of the more usable probes, but it cannot analyse genes with APA sites that are >600 bp from each other.

RNA-seq

The development of parallel sequencing has changed nearly the entire field of genome biology. The RNA-seq technique can not only measure the gene expression levels digitally, but it can also detect the AS and gene fusion. Recently, two groups have also used these methods to study the APA sites (Wang et al., 2008; Pickrell et al., 2010). Polyadenylated mRNA is fragmented followed by reverse transcription with random primers, and it is subsequently sequenced by the second-generation sequencing. Intuitively, the reads that are tangled by polyA could be used to identify APA sites. From 1.2 billion reads, Pickrell et al. (2010) found 70 million reads containing polyA, from which they identified 7296 putative cleavage sites. However, for the limited putative cleavage sites, this method is obviously inefficient. Another group developed a Bayes model to investigate APA switching (Wang et al., 2008). These researchers also restricted their study to genes with only two tandem APA sites, and they applied all of the reads that were mapped to the 3′ UTR of these genes for the analysis.

3′-end sequencing

RNA-seq provides a powerful tool for profiling the RNA transcripts of the whole transcriptome, including the 3′ UTR region, but it is far from efficient at deeply profiling APA events in a genome-wide fashion. Thus, for the purpose of sequencing polyA sites using the RNA-seq strategy derived, several modified methods have been developed to precisely sequence the 3′-end of the mRNA based using the second- and third-generation sequencing platforms (Figure 3); a comparison between these methods, including the data yield and operability, is summarized in Table 1.

PolyA capture. The polyA capture was developed to profile the 3′ landscape of *Caenorhabditis elegans* (*C. elegans*) using the 454 FLX system (Mangone et al., 2010). For the construction of the 454 sequencing library, the double-stranded cDNA was synthesized by reverse transcriptase and DNA polymerase. To obtain a suitable size, the double-stranded cDNA was digested by *Dpn*II, a restriction enzyme that recognizes a 4-bp sequence of GATC. After adaptor ligation and PCR amplification, the resulting library was sequenced with 454 FLX. Over 2.5 million reads were generated using this method. As the first published high-throughput sequencing method, polyA capture provides much information about the 'UTRome' in *C. elegans*. Because the average length of a sequencing read generated by 454 FLX is >400 nt (and over 800 nt by the latest 454 sequencer), this method was also suitable for addressing the 3′ UTR landscape of non-model animals. One problem related to this method is that the use of *Dpn*II can induce bias for incomplete digestion and enzyme site location (Torres et al., 2008; Zaretzki et al., 2010).

3P-SEQ. Jan et al. (2011) developed a more complicated method, called 3P-Seq, which is intended to eliminate the internal priming sequence during the sequencing library preparation. To remove the internal A-rich regions of transcripts, a series of RNA manipulations were carried out before double-stranded cDNA synthesis. Firstly, polyA+ RNA was annealed to a biotin-tagged primer with a 3′ oligo d(T) cohesive terminus. After partial digestion with RNase T1, the polyadenylated ends were captured by streptavidin-biotin selection. The polyA tail was later reverse transcribed with dTTP only. RNase H digestion was applied to remove mRNA polyA. Following gel-purification, adaptor ligation and amplification, the final library was then subsequently sequenced. The final result produced nearly 32 million 3P tags from *C. elegans*. As described, 3P-SEQ can effectively diminish internal priming sequence during the preliminary treatment and increase the proportion of useful data, despite the extreme intricacy of the preliminary treatment.

SAPAS. Fu et al. (2011) developed another method, sequencing alternative polyA sites (SAPAS), in which a mRNA template-based reverse transcription reaction was used to generate the first strand of the cDNA by an anchored oligo d(T) primer and a 5′ template switching adaptor. The 5′-ends of the primers were tagged with 454 or Illumina adaptors. A modified reaction system with such materials as trehalose and sorbitol was also developed to improve the efficiency of this method. Then PCR was later used to amplify the cDNA and to introduce mutations into the polyA. The number of cycles was determined to ensure that the ds-cDNA remained in the exponential phase of amplification. After size selection of fragments with PAGE gel-excision, the fragments can be sequenced from the 5′-end with 454 or the 3′-end with Illumina GA IIx (Fu et al., 2011).

With the advancement of genome-wide polyA profiling, it is easy to discover thousands of genes with three or more polyA sites that could not be detected by microarray methods. However, the identification of more than two APA sites brings new challenges for testing the statistical significance of APA switching for a gene between two samples because traditional statistical methods, such as the Fisher's exact test or chi-square test can only consider genes with exactly two polyA sites. To overcome this analytical problem in processing data generated from SAPAS, Fu et al. (2011) adopted a linear trend test for APA switching that not only counts reads (the expression level of polyA sites) but also analyses the 3′ UTR length of multiple polyA sites could be analysed simultaneously. Thus, this method provides an unbiased framework for analysing 3′ UTR switching in APA profiling data.

DRS. As the third-generation high-throughput sequencing technology, the direct RNA sequencing (DRS) developed by Helicos Bioscience Corporation can be used to profile global ployA sites directly. This sequencing platform used polyd(T)-coated flow cell surfaces with which the polyA+ RNA species could hybridize directly. The sequencing-by-synthesis process was performed by a modified DNA-dependent DNA polymerase that also had reverse transcriptase activity; virtual terminator nucleotides, a type of nucleotides analogues containing removable fluorescent dye, were used in this process. The library preparation for DRS does not require reverse transcription, ligation or amplification steps, and it can avoid artificial cDNA. Ozsolak et al. (2010) reported comprehensive maps of global polyadenylation sites in
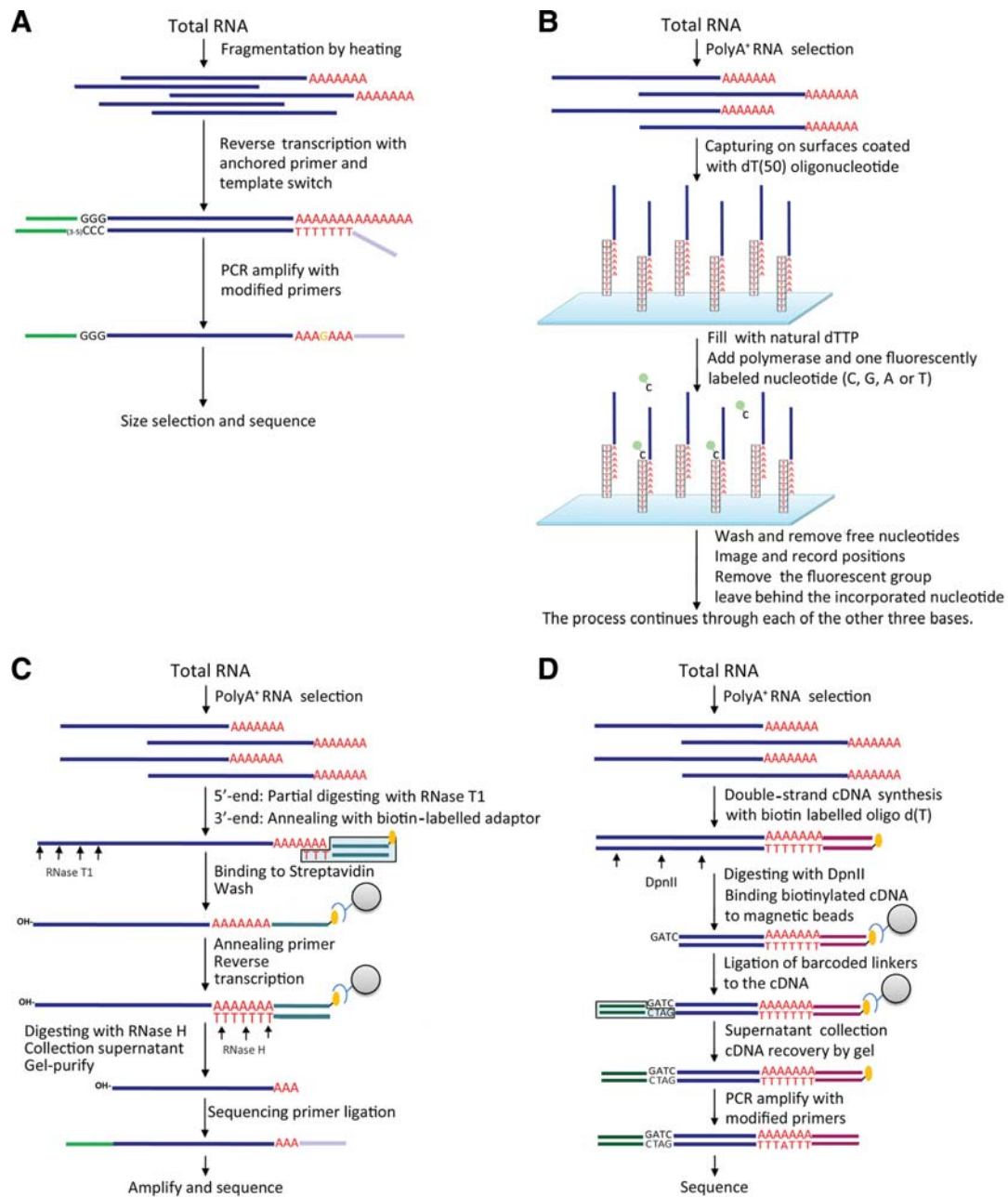
Figure 3 Experimental outlines of four high-throughput 3′-end sequencing methods: (**A**) SAPAS, (**B**) DRS, (**C**) 3P-SEQ and (**D**) polyA capture. See text for details.

Table 1 Summary of methods for genome-wide polyA site profiling by deep sequencing.

	PolyA tail capture	Fragmentation	Fragmentation bias	Platforms	Length (bp)	Error rate	IP*	Manipulation complexity	Species profiled	Reference
SAPAS	Olig(dT)	Heat or fragmentation buffer	No	454/illumina	400/100	0.05%/1.5%	Yes	Easy	Human	Fu et al. (2011)
PolyA capture	Olig(dT)	DpnII	Yes	454	400	0.05%	Yes	Easy	*C. elegans*	Mangone et al. (2010)
3P-SEQ	Biotinylated primer with splint ligation	Partial digestion with RNase T1	Little	Illumina	100	1.5%	No	Difficult	*C. elegans*	Jan et al. (2011)
DRS	Polyd(T)- coated flow cell surface	–	–	Helicos	35	~4%	Rare	Easy	Yeast & Human	Ozsolak and Milos (2011)

* IP, internal priming.

human liver and yeast with DRS technology. The advantage of DRS is obvious, i.e. no PCR amplification is needed, and results were in more accurate quantification of expression levels. However, the current drawbacks of this method are that the sequenced reads are too short (∼35 nt), there is a high sequencing error rate (∼4%) and the cost of the Helicos machine itself is prohibitively high.

Biological functions of APA events

APA has been reported to be associated with biological functions since the 1980s (Alt et al., 1980; Early et al., 1980; Rogers et al., 1980), and we have summarized APA genes that have been reported biological functions in Table 2. Recently, with the application of the genome-wide methods of polyA profiling mentioned above, global 3′ UTR switching have been revealed in different biological processes and various cell statuses including tumourigenesis and metastasis, animal development, immune response, and neuronal activity (Table 3). The widespread APA switching phenomenon stresses a potentially new layer of gene regulatory networks in physiological and pathological regulation.

Immune response

It has been over 20 years since the discovery that the immunoglobulin (Ig) M heavy-chain gene switched its APA sites from the distal site (full-length transcript) to the proximal site (truncated mRNA) during mouse B cell activation (Alt et al., 1980; Early et al., 1980; Rogers et al., 1980). This splicing-dependent switching transforms the IgM protein from the membrane-bound form to the secreted form (Peterson and Perry, 1989), which is a process that is accompanied by a high concentration of the polyadenylation and cleavage factor CstF-64 (Takagaki et al., 1996). Recently, it was found that hundreds of genes switched their polyA sites with different 3′ terminal exons during T-cell activation (Sandberg et al., 2008). While the number of switching events from distal-to-proximal and proximal-to-distal are not significantly different between splicing-dependent events, 86% of the genes in activated T lymphocytes increased their relative expression of short 3′ UTR isoforms, leading to global 3′ UTR shortening during T-cell activation. This phenomenon was confirmed in other immune responses, including the stimulation of human B cell by anti-CD40 and interleukin-4 and the stimulation of human monocytes by lipopolysaccharides and interferon-γ, suggesting a strong association between tandem 3′ UTR shortening and cell proliferation. The differential usage of 3′ UTR isoforms could impact protein output, with the shorter isoforms typically producing more proteins potentially through escaping the microRNA-mediated repression (Sandberg et al., 2008), and degradation by other regulatory elements such as AU rich element (ARE) (Barreau et al., 2005) and GU-rich elements (Vlasova et al., 2008). As both the rates of RNA polyadenylation and protein synthesis increased during T-cell activation (Coleman et al., 1974; Hauser et al., 1978), APA confers another regulatory layer, which in this case accelerates the procession of the central dogma from DNA to protein synthesis in this case.

Neuron activity

The most interesting example of APA regulation in neurons is found in the brain-derived neurotrophic factor gene (*BDNF*), which has two mRNA isoforms with exactly the same CDS sequence but different 3′ UTR lengths (Timmusk et al., 1993; Ghosh et al., 1994; An et al., 2008). It was found that the short and long 3′ UTR mRNAs have different subcellular localizations: the short 3′ UTR mRNAs are restricted to the somata, whereas only the long 3′ UTR mRNAs are found in dendrites. In neurons lacking the long 3′ UTR mRNA, the average spine head diameter is smaller, and there are more spines in the apical dendrites (An et al., 2008). Even more interestingly, a recent study suggests that APA is a widespread regulatory mechanism in neuron activity (Flavell et al., 2008). In the central nervous system, environmental stimulations activate a series of transcription factors (such as MEF2) that modulate the expression of hundreds of genes (called activity-dependent genes) (Flavell and Greenberg, 2008). Recently, it is found that extracellular stimulation also induces APA that produced truncated transcripts in many of these neuron activity-dependent genes (Flavell et al., 2008). Interestingly, a recent study showed that the regulation of transcription activity by APA seemed to be a widespread mechanism (Ji et al., 2011). With an arsenal of transcription factors that are activated during neuron response, it is not surprising that the APA regulation of target genes in the downstream cascade has been tightly modulated by the transcription machinery.

It is also worth noting that transcripts with relatively long 3′ UTRs are preferentially expressed in neuronal tissues, such as the brain and spinal cord (Zhang et al., 2005; Hilgers et al., 2011). The underlying mechanism, however, and the functional consequence of this preference are still unknown. Following the current model once exposed to environmental stimulations, the normal equilibrium in the neuron tissues breaks, resulting in with transcript initiation and APA activation by activity-regulated transcription factors, which leads to short 3′ UTRs or, in certain cases, truncated proteins. The production of transcripts with short 3′ UTRs could accelerate and increase protein synthesis (Sandberg et al., 2008; Mayr and Bartel, 2009; Ji et al., 2011) while truncated proteins could alter gene functions and interfere with pre-existing proteins to break the equilibrium (Flavell et al., 2008). The widespread and acute APA activity in neuronal and immune responses suggests that APA might be a common mechanism in regulating gene networks in response to extracellular stimulations.

Development

In contrast to 3′ UTR shortening in response to extracellular stimulation, global 3′ UTR lengthening was observed during mouse early embryogenesis by analysing a vast amount of EST, microarray and SAGE data (Ji et al., 2009). To confirm these *in silico* results, the 3′ UTR-lengthening process was recaptured *in vitro* in a differentiation model in which mouse C2C12 myoblast cells differentiate into myotubes. In addition, reporter assays showed that a weaker efficiency of polyadenylation at proximal sites was found in the differentiation condition, leading to 3′ UTR lengthening during myoblast differentiation, a situation which is in strong contrast to the 3′ UTR shortening that occurs during T-cell proliferation. The weakening of the polyadenylation efficiency of proximal sites could be caused by the widespread downregulation of genes involved in pre-mRNA 3′ processing during differentiation. Interestingly, a similar trend of 3′ UTR lengthening was also observed during *Drosophila* development

Table 2 Genes functionally regulated by APA based on individual gene studies.

Symbol	Name	Comments	References
IgM	Immunoglobulin M	By switching distal polyA site to proximal site in intron, IgM switches from the membrane-bound form to the secreted form during B cell activation	Alt et al. (1980); Early et al. (1980); Rogers et al. (1980)
e(r)	Enhancer of rudimentary	The long 3′ UTR is specifically expressed in the female germline, which requires the coexpression of the female-specific sex lethal	Wojcik et al. (1994); Gawande et al. (2006)
su(f)	Suppressor of forked	The proximal APA site is located in intron 4, leading to the production of a truncated transcript which may regulate protein accumulation via a negative feedback loop	Audibert and Simonelig (1998)
FGF-2	Fibroblast growth factor 2	Short 3′ UTR isoforms were used in the transformed cell lines with more stability	Touriol et al. (1999)
PhLP, PDCL	Phosducin-like protein	The long 3′ UTR contains multiple AREs, and the transcript with the long 3′ UTR exhibits a much shorter mRNA half-life	Lazarov et al. (1999)
AUF1	Adenosine/uridine-rich element (ARE) binding protein	3′ UTR splice variants have different stabilities involved in the NMD pathway	Wilson et al. (1999; Banihashemi et al. (2006)
MeCP2	Methyl-CpG-binding protein 2	Domain-like conservation pattern of the long 3′ UTR	Coy et al. (1999)
HER-2 (Neu)	Human epidermal growth factor receptor 2	Transcripts with extended 3′ UTRs display increased stability in SKOV-3 ovarian carcinoma cells	Doherty et al. (1999)
CKIAlpha	Casein kinases	The longer 3′ UTR transcripts degrade ∼13 times faster than do the shorter 3′ UTR transcripts in HeLa cells	Yong et al. (2000)
Spin	Spindlin	Differential stability and translational control during the mouse oocyte-to-embryo transition in mouse development	Oh et al. (2000)
RTN3	Reticulon 3	Two transcripts that only differed in 3′ UTR length exist. Similar mRNA stability were observed for these two isoforms, while the longer isoform is more translational efficient	Qu et al. (2002)
COX-2	Cyclooxygenase-2	Truncated variant escapes the binding of tristetraprolin in human colorectal adenocarcinoma cell lines	Sawaoka et al. (2003)
CstF-77 (CSTF3)	Cleavage stimulation factor 77 kDa subunit	Intronic polyadenylation produces short CstF-77 transcripts lacking sequences encoding domains	Pan et al. (2006)
CTNNB1	β-catenin	Three mRNA variants that differ solely in their 3′ UTRs have different cytoplasmic stabilities in Hela cells and may be subjected to differential regulation of ARE	Thiele et al. (2006)
BZW1	Basic leucine zipper and W2 domains 1	Three transcripts that only differed in 3′ UTR length exist in the mouse testis. The shortest isoform is testis specific. The transcript corresponding to the middle APA sites had the lowest translational efficiency	Yu et al. (2006)
CCND1	Cyclin D1	Shorter 3′ UTR leads to an additional 1.6-fold increase in protein expression and correlates with both increased proliferation of the lymphoma cells and decreased overall survival of patients	Mayr and Bartel (2009); Rosenwald et al. (2003)
StAR	Steroidogenic acute regulatory protein	The abundance of the transcript with the long 3′ UTR increases and declines much more rapidly by cAMP stimulation in rodent steroidogenic cells; the transcript with the long 3′ UTR is less stable than is the short one	Duan and Jefcoate (2007)
HMGA2	High mobility group A2	Shorter 3′ UTR generated from chromosomal translocations promotes anchorage-independent growth for escaping repression by the miRNA let-7 in NIH3T3 cells	Lee and Dutta (2007); Mayr et al. (2007)
BMP2	Bone morphogenetic protein 2	Shorter mRNAs were more abundant than were longer mRNAs. A 72-nucleotide region downstream of the distal polyA sites contains two novel cis-acting elements	Liu et al. (2008)
BDNF	Brain-derived neurotrophic factor	Different localization of mRNAs with short and long 3′ UTRs in the neuron body and axon	An et al. (2008)
ABCG2	ATP-binding cassette sub-family G member 2	Shorter 3′ UTR isoform was used in S1MI80 cells, which is resistant to the binding of hsa-miR-519c	To et al. (2008)
Actb1	β-actin	The longer 3′ UTR was expressed at a relatively lower level and conferred higher translational efficiency in mouse neuronal cells; it may be regulated by mmu-miR-34a/34 b-5p	Ghosh et al. (2008)
GluR2	Glutamate receptor 2	Two 3′ UTR isoforms exist in the mammalian brain; the long 3′ UTR isoform is translationally repressed but is depressed after seizure inductions	Irier et al. (2009)
HuR	Human antigen R	Different polyadenylation variants have different expression abundance regulated by AU-rich elements (ARE)	Al-Ahmadi et al. (2009)
CCND2	Cyclin D2	The shorter 3′ UTR mRNA leads to more cells in the S Phase	Mayr and Bartel (2009)
IMP-1 (IGF2BP1)	Insulin-like growth factor 2 mRNA binding protein 1	Expression of the short 3′ UTR isoform greatly promoted cell transformation, and much of this transformation was attributable to the loss of miRNA let-7 targeting sites	Mayr and Bartel (2009)
DICER1	Dicer 1	Short 3′ UTR isoforms were used in the transformed cell lines	Mayr and Bartel (2009)
IMPA1	Myo-inositol monophosphatase 1	Long 3′ UTRs would fold as hairpin loops and may interact with ribonuclear particles (RNPs)	Andreassi et al. (2010)
PRMT2 (HRMT1L1)	Arginine N-methyltransferase 2 protein	An intron-retaining transcript by alternative polyadenylation has distinct intra-cellular localization	Zhong et al. (2011)
Polo	POLO cell cycle kinase	Lack of the distal poly(A) signal causes a significant decrease in Polo protein levels, leading to a failure of the proliferation of the precursor cells of the abdomen at the onset of metamorphosis in *Drosophila*	Pinto et al. (2011)
Pax3	Paired box 3	APA generates shorter 3′ UTRs that render the mRNA resistant to regulation by miR-206 in muscle quiescent SCs (QSCs) and in myogenic progenitors during development	Boutet et al. (2012)

Table 3 Biological processes with a global APA switch.

Biological process	Genome-wide APA pattern	Technique	Reference
T-cell activation (mouse)	Tandem 3′ UTR shortened	Mouse Exon 1.0 ST microarrays	Sandberg et al. (2008)
Hippocampal neuron stimulation (rat)	Shortened 3′ UTR and truncated mRNAs	RAE230.2 microarray	Flavell et al. (2008)
Mouse embryogenesis	Tandem 3′ UTR progressively lengthened	EST, microarray and SAGE	Ji et al. (2009)
Drosophila embryogenesis	Tandem 3′ UTR lengthened	Microarray and RNA-seq	Hilgers et al. (2011)
C. elegans development from embryo to adult	3′ UTR progressively shortened	PolyA capture	Mangone et al. (2010)
Lymphomas (mouse)	Shortened 3′ UTR and truncated mRNAs	Mouse genome 430v2 microarrays	Singh et al. (2009)
Breast cancer (human cell lines)	Tandem 3′ UTR shortened in non-metastasis cell line but lengthened in metastasis cell line	SAPAS	Fu et al. (2011)

(Hilgers et al., 2011), indicating the functional conservation of APA regulation in animal development. Indeed, the 3′ UTR lengthening could be functionally important, and differential impacts of miRNA-mediated regulations for mRNAs with different 3′ UTR lengths have been addressed for several critical genes during *Drosophila* development (Thomsen et al., 2010).

However, this 3′ UTR lengthening was not observed in the development of *C. elegans*. With the polyA capture method that we have described above, Mangone et al. (2010) determined alternative 3′ UTR isoform changes in *C. elegans* from the embryonic stage to the adult stage (embryo, L1, L2, dauer, L3, L4, adult, male). They found that the average 3′ UTR length progressively decreased throughout development, although the extent of this decrease was not significant. This result was not, however, inconsistent with the result from mouse because no significant change of 3′ UTR length was discovered in the postnatal development of mice (Ji et al., 2009). The 3′ UTR dynamics in nematode embryogenesis, however, still need to be elucidated. Moreover, the underlying mechanism that modulates APA and the functional consequence of differential 3′ UTR usage in the development of invertebrates and vertebrates remains largely unknown.

Tumorigenesis

It has been possible for years to classify different tumour subtypes using miRNA profiles (Calin et al., 2004), and aberrant expressions of miRNAs were associated with cancer (Esquela-Kerscher and Slack, 2006). As the direct targeted region of miRNAs, the 3′ UTRs also showed distinct features in primary cancer samples, as characterized in particular by the global shortening of 3′ UTRs both *in vitro* (Fu et al., 2011) and *in vivo* (Singh et al., 2009). With the characterization of alternative 3′ UTRs, tumour subtypes with various survival characteristics could be distinguished with an accuracy of >74% (Singh et al., 2009). Shorter 3′ UTRs have functional consequences, leading to greater mRNA stability and increased protein output (Mayr and Bartel, 2009). Phenotypic consequences were observed by forcing the expression of shorter 3′ UTR isoforms. By expressing either shorter or longer 3′ UTR isoforms of *Cyclin D2* in MCF7 cells, a significantly higher fraction of cells in the S phase were found in cells expressing the short isoform compared with those expressing the long mRNA (Mayr and Bartel, 2009). Moreover, the expression of the short isoform of *IMP-1*, an RNA-binding protein overexpressed in various cancers, led to significantly more oncogenic transformation, whereas the expression of the long isoform did not (Mayr and Bartel, 2009). These

results strongly suggest a fundamental role of APA underlying tumourigenesis.

Based on studies of individual genes, two mechanisms underlying the preferential usage of short 3′ UTRs in cancer cells/patients have been proposed: genome aberrations and APA regulation. In mantle cell lymphoma tumours, cyclin D1 (*CCND1*) mRNA levels are always exceptionally highly expressed, and short *CCND1* mRNA isoforms have been detected (Rosenwald et al., 2003). Wiestner et al. (2007) found that these short *CCND1* mRNA isoforms only differ in the 3′ UTRs, resulting from either genomic deletions of the *CCND1* 3′ UTR region or from point mutations that create *de novo* polyA signals, leading to premature cleavage and polyadenylation. Another example of genomic aberration is the *HMGA2* gene, which swaps its 3′ UTR with another gene by chromosomal translocation, resulting in the loss of miRNA *let-7*-binding sites and an escape from *let-7*-mediated repression (Lee and Dutta, 2007; Mayr et al., 2007). In contrast, the aberrant usage of the short 3′ UTR could also be produced by APA regulation, uncovering an epigenetic mechanism that achieves the same effect but leaves the genome intact (Mayr and Bartel, 2009).

Nevertheless, the mechanism behind the alteration by APA of the global 3′ UTR pattern in cancer cells is still unclear. The only hint is that 3′ UTR shortening is associated with cell proliferation, such as in T-cell activation or early embryogenesis (Ji et al., 2009; Sandberg et al., 2008). Making the problem more complicated, a recent study showed that unlike primary cancer, there existed significantly more genes with lengthened 3′ UTRs in a metastasis cell line (Fu et al., 2011). These findings raise the question of whether there is a dynamic deregulation of APA during the life cycle of cancer cells; more details are needed to elucidate this system.

Perspectives

Derived from the second- or third-generation sequencing technologies, genome-wide profiling methods of polyA sites have provided us global view of APA events. However, technical challenges still exist. For example, a certain amount of total RNA or polyA$^+$ RNA are needed for the sequencing library preparation, which would be a difficult task for those samples with low RNA quantities such as paraffin section samples and single cells. Therefore, new methods requiring less RNA are in great demand.

The functional consequences of using different 3′ UTR lengths could be different from one gene to another, and could greatly depend on cellular environment. The most common consequence is that mRNAs with shorter 3′ UTRs produce more protein output, benefiting from lacking specific *cis*-elements targeted by *trans*-acting factors for post-transcriptional or translational repression (Sandberg et al., 2008; Mayr and Bartel, 2009). This functional consequence feeds perfectly well into various physiological processes that we mentioned above, in which 3′ UTR shortening has been found to be associated with cell proliferation in immune response (Sandberg et al., 2008), the earliest stage of embryogenesis (Ji et al., 2009) and cancer transformation (Mayr and Bartel, 2009). Because cell proliferation is a process that needs relatively high rate of protein synthesis (Zetterberg and Killander, 1965), the usage of shorter 3′ UTRs accelerates the process of protein synthesis on a global scale. These observations suggest that a great proportion of these genes shortened the 3′ UTRs in order to up-regulate protein expression.

However, short 3′ UTR length is not always associated with high levels of protein product. Pinto et al. (2011) found that the *Polo* mRNA with full length 3′ UTR is much more translationally efficient than that with short 3′ UTR. More interestingly, when the *Polo* protein was overexpressed, isoforms with the short 3′ UTR were preferentially transcribed *in vivo*, resulting in a negative feedback loop that fine-tunes *Polo* expression (Pinto et al., 2011). Thus, APA could confer another regulatory layer of buffering gene expression, and could possibly explain why 3′ UTR isoforms should be regulated in a tissue-specific manner (Zhang et al., 2005; Wang et al., 2008).

Although it is still unclear how different APA usage is regulated, several possible mechanisms have been proposed. In some previous studies, the APA regulation has been found to occur in gene transcription processing and to be affected by gene promoters and the RNA polymerase II complex (Huang et al., 2012; Martincic et al., 2009; Moreria, 2001; Nagaike et al., 2011). This viewpoint was well summarized in a recent review (Kuehner et al., 2011). In other studies, the use of a proximal or distal polyA site could be due to combining the capacities of different polyA signals (weak or strong) and expression levels of polyA-binding factors, such as CstF-64 in pre-B cells (Takagaki and Manley, 1998). The high concentration of CstF-64 was associated with a proximal polyA signal in B cells (Takagaki and Manley, 1998). After stimulation by LPS, the expression level of CstF-64 was upregulated, and short 3′ UTR usage was observed (Shell et al., 2005). In most signalling pathways, transcription factors (such as NFκB) are ultimately affected by immune stimulation. It is interesting that the immune stimulation eventually changed not only the initiation of transcription but also its termination and polyadenylation. It will be exciting to uncover the relationships between immune signal transduction and APA regulation. In all, the involved signalling pathways and molecular mechanisms that regulate mRNA 3′-end processing and their role in health and disease remain enigmatic.

Benefiting from recent genome-wide APA studies, a number of new motifs were discovered in 3′ UTRs, and several of which were speculated to affect the choosing of polyA site. For instance, the TTGTTGGG motif, which was found to surround non-activity-regulated sites of polyA tail in active neurons, could direct activity-dependent polyadenylation to specific sites (Flavell et al., 2008). At the same time, some genes' epigenetic alterations, such as gene imprinting of the mouse gene H13 and chromatin architecture changes by histone modification, may correlate with differential APA usage (Lian et al., 2008; Spies et al., 2009; Wood et al., 2008). However, some of these proposed mechanisms were based on bioinformatics analysis, while others were only proved in special cell lines and genes. Thus, whether there is a global pattern of APA usage for gene expression regulation is still a puzzle. Taken together, understanding the mechanisms behind APA regulation in eukaryotes is only at its initial stage, and many more novel discoveries remain to be made.

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