

Alternative Polyadenylation: Less than meets the eye?

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Abstract:

With the advances in deep-sequencing techniques over the past decade, the study of alternative cleavage and polyadenylation (APA) has shifted from individual gene to whole transcriptome analysis. Findings from such global studies have elevated APA to its currently accepted status as a major player in the regulation of eukaryotic gene expression. Whilst ~70% of human genes have been shown to contain multiple cleavage and polyadenylation sites, the extent of the consequences of APA and its role in regulating physiological processes is still largely unknown. This review aims to summarize the experimental evidence that supports a physiological role of APA and highlights some of the shortcomings that need addressing to substantiate the widely proposed claim that APA is a key player in global gene regulation.

Key-words: Alternative cleavage and polyadenylation, 3'-end processing, gene expression, RNA metabolism, cleavage, post-transcriptional regulation

Abbreviations: 3'UTR, 3'-untranslated region; APA, alternative cleavage and polyadenylation; aUTR, alternative untranslated region; CR-APA, coding-region APA; CPA, cleavage and polyadenylation; UTR-APA, untranslated region APA.

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Alternative Cleavage and Polyadenylation (APA)

Cleavage and polyadenylation is a pre-mRNA processing reaction that matures the 3' end of all pre-mRNAs with the exception of replication dependent histone genes. The reaction is executed by a multi-protein complex that recognises the bipartite poly(A) signal (PAS) present on pre-mRNA. The actual processing reaction can be divided into two steps, the cleavage of the pre-mRNA at the cleavage and polyadenylation (CPA) site and the subsequent polyadenylation of the newly created 3' end of the transcript.

A large proportion of eukaryotic, and in particular mammalian genes, contain multiple CPA sites. The alternative usage of these CPA sites, a process referred to as alternative cleavage and polyadenylation (APA), creates distinct mRNA isoforms that can differ in 3'UTR length, and potentially their coding capacity. Thus, in analogy to alternative splicing, APA can result in multiple distinct transcript isoforms being produced from a single gene, thus increasing the diversity of the transcriptome, and in some cases the proteome.

Depending on the location of the different CPA sites within the gene, APA can be classified as either untranslated-region APA (UTR-APA), or coding region APA (CR-APA) as summarised in Figure 1. UTR-APA occurs when multiple CPA sites are present within the same 3'UTR of a gene. Thus, by default, UTR-APA does not affect the coding capacity of the APA mRNA isoforms, but results in mRNA isoforms that differ solely in 3'UTR length, with the longer

isoform containing an additional alternative UTR (aUTR). The regulatory potential of UTR-APA lies in the presentation of different regulatory modules in this aUTR region, including miRNA target sites and AU-rich elements, that can potentially impact on the stability, translation efficiency and subcellular localisation of that isoform.

CR-APA on the other hand describes APA events that involve the usage of CPA sites that are located upstream of the 3'UTR, therefore altering the coding capacity of the transcript and potentially leading to the production of multiple protein isoforms with distinct C-termini. CR-APA can further be categorised into 3 separate classes: exonic CR-APA, whereby a CPA site is located within the coding region itself; composite intronic CR-APA, where the CPA site is located within an intron and can arise when a 5'-splice site becomes silent; skipped intronic CR-APA, where alternative splicing results in the inclusion of a separate terminal exon containing a CPA site [1] (Figure 1).

Current Opinions on the Importance of APA

APA was described as early as 1980, with initial studies identifying CR-APA in the immunoglobulin heavy chain *IgM* gene, which leads to the production of both membrane-bound and secreted IgM [2]. Individual gene studies have continued from the 1980s to date and describe a large number of APA events which result in the production of APA isoforms with either differential mRNA stability [3,4] or encode different proteins [5,6]. Recent advances in deep sequencing technology have allowed this analysis to move to a global scale, with a plethora of techniques capable of quantitatively sequencing solely the terminal 3' ends of polyadenylated transcripts [7–11].

These techniques have been used for both the mapping of CPA sites within an organism, as well as quantitatively measuring the relative usage of CPA sites between different cell states or cell types. Mapping studies have found that ~70% of all human genes contain multiple CPA sites in their 3'UTRs [12], and large-scale shifts in these observed APA profiles have been seen between tissues [12–14], in cancer [15–18], cellular proliferation [19] and differentiation [20,21]. From these observations it was extrapolated that global APA may contribute significantly to the establishment of the appropriate gene expression profiles associated with different cell states and disease phenotypes.

These bioinformatic studies provide invaluable data to help understand the key concepts behind APA. However, one crucial caveat of these studies is that it is difficult to determine cause from consequence. The fact that these shifts occur does not necessarily qualify them as key driver events. Furthermore, concerns have recently been raised that global studies may focus too much on the bioinformatic correlations and give too little consideration to their biological significance [22].

Evidence for Effects of UTR-APA on transcript Stability

In UTR-APA, the lengthening or shortening of the 3'UTR through APA can potentially impact upon the post-transcriptional regulation of the APA mRNA isoforms through altering their relative stability, localisation and translational efficiency. As the 3'UTR is generally considered to have an overall repressive nature [16], use of the proximal CPA site was originally thought to result in increased mRNA stability.

This principle has been directly demonstrated in quiescent muscle stem cells, where 3'UTR shortening by APA in the myogenic regulator *PAX3* mRNA results in the removal of a miR-206 target site and consequently enables *PAX3* to escape miR-206 mediated repression [23]. Conversely, 3'UTR lengthening in the DNA repair enzyme *MGMT* in glioblastomas serves as an example where 3'UTR lengthening results in the inclusion of several miRNA-target sites and the subsequent repression of the *MGMT* expression [4].

Although many of these individual events have been shown to be physiologically significant the pervasiveness of UTR-APA effecting stabilisation at a global scale is much less evident. If the large-scale shifts in UTR-APA described in the numerous global studies performed were to have a significant impact on the RNA stability, it would be expected that the overall mRNA abundances of these genes would either increase in the case of 3'UTR shortening, or decrease on 3'UTR lengthening. However, this expected pattern is not consistently seen. de Klerk *et. al.* found ~45% of genes that showed general 3'UTR shortening were accompanied by changes in transcript levels, but only a slight bias towards overall upregulation is observed [24]. Similarly, in several other studies only modest, if any, changes in transcript levels were observed in the genes that show a shift in CPA site choice [16,25,26].

Finally, a recent analysis that directly assesses the relative mRNA stabilities of APA isoforms globally draws similar conclusions. In NIH 3T3 mouse fibroblast cells, of 3463 detected genes that produce several APA isoforms, only around 500 differ in relative mRNA stability [27]. Interestingly, in ~40% of these APA events where significant changes in stability are seen, the longer isoform is actually more stable than the shorter transcript, suggesting the presence of stabilising *cis*-elements in the aUTR. A similar association between longer UTRs and increased stability has also been described in human adipocyte stem cells [28]. Global studies in yeast however, suggest that here APA can have a more profound impact on the stability of transcript isoforms, even in isoforms differing by a single nucleotide [29,30].

Nevertheless, in mammalian cells and tissues, even though APA is widespread, relatively few events appear to have a significant impact on overall mRNA stability. Furthermore, with few exceptions, there is little evidence that these changes in mRNA isoform stability affect the final protein output to a point that could have a physiological impact.

Differential Translational Efficiencies of APA mRNA isoforms

Like stability, a number of studies have identified examples where APA events lead to alteration in translational efficiency [15,31–34]. With the help of reporter assays, protein output of short and long UTRs of *CCND2*, *IMP-1* and *DICER* were compared and 3'UTR shortening was found to be generally associated with increased translational efficiency, and thus increased expression [15]. However, this view has become more complex in recent years as opposite effects have also been seen where the distally polyadenylated transcript is actually translated with a higher efficiency [27,31,33].

One of the most well characterised examples of the latter is the anxiety-related gene *SERT*. Here, hnRNPK binding in the aUTR region of the distally polyadenylated *SERT* transcript prevents miR-16 binding and promotes the translation of the distal isoform relative to the proximal isoform [31]. A similar situation is also seen in the *Drosophila* cell cycle gene *polo*, where its longer APA isoform is translated at three times the rate of the shorter isoform [33].

More global studies have found similar conflicting trends regarding correlations between APA mRNA isoforms and translational efficiency. Studies in HEK293T cells show a bias towards a greater polyribosomal association of the shorter UTR-APA mRNA isoforms [35]. This is somewhat in contrast to a recent study using ribosomal profiling assays performed in mouse NIH 3T3 cells, where increases in ribosomal association of the distal APA isoform relative to their proximal counterpart were seen [27]. However, these effects were minimal, with a median <3% increase in ribosomal association of the distally polyadenylated transcript. This latter study concludes that the large majority of the APA events are unlikely to have a physiologically significant effect on either transcript stability or translational efficiency, at least in NIH 3T3 cells.

Differential Localisation of APA mRNA isoforms

Only a small number of examples that demonstrate a role of UTR-APA in regulating the subcellular localisation of mRNA isoforms have been presented so far. Inclusion of the inverted *Alu* repeats in the distal APA isoform of *NICN1* has been shown to lead to its nuclear retention, whereas the proximal APA isoform, which lacks these sequences, is freely exported to the cytoplasm [36]. The general role of 3'UTRs in the localisation of mRNAs in highly polarised cells, is well known [37] and differential localisation of APA mRNA isoforms in neurons has been reported. aUTRs play a critical role in regulating the localised translation of *BDNF* APA mRNA isoforms in neurons where the distal APA isoform localises to the dendrites and the proximal APA isoform is restricted to the somata [38]. 3'UTR lengthening in the brain is one of the most striking examples of tissue-specific APA [13], which may act to serve as a platform to control the localisation of these transcripts. However, so far there have been no global studies that look at the effect of APA on subcellular localisation so, whilst it appears that APA may control differential APA isoform localisation in the brain, its prevalence is currently unknown.

Conservation of APA

Comparative genomics have been used in a number of studies to assess the conservation of APA between species. Global features, such as the general sequence composition surrounding the cleavage site, and the presence of multiple CPA sites at a significant portion of genes are conserved between human and mouse [39]. However, when the conservation of individual CPA sites between mouse and human are considered, only ~500 genes contain conserved tandem CPA sites, and consequently it was suggested that “in general, alternative poly(A) sites are species-specific and involve minor, non-conserved sites that are unlikely to play essential roles” [40].

Derti *et. al.* see a similar lack of conservation of CPA sites [12]. Of the 439,390 CPA sites they identified in humans, only 2590 (0.6%) of them were found to have CPA sites within the equivalent 30 base region in all four other mammals considered (Rhesus, Dog, Mouse, Rat) [12]. Interestingly, it was found that of the conserved CPA sites, the read numbers were more similar in equivalent tissues across species, rather than between tissues in the same species [12]. However, this approach is looking at absolute read numbers over individual CPA sites and not looking at the relative usage of tandem CPA sites, so this may simply reflect a conservation of tissue specific gene expression rather than the conservation of APA patterns itself.

Of the tandem CPA sites that are conserved, it is the distal APA sites that are conserved at a higher level than the proximal APA sites [41], and in general, it is these sites that have the highest processing efficiency [42]. This may therefore suggest a mechanism by which the distal CPA sites serve as the default CPA site and functions to ensure efficient 3' end processing and transcription termination, while the proximal CPA site functions as a regulatory site that can potentially be activated to remove any downstream regulatory *cis*-elements.

Conclusions and Perspectives

With the recent wave of global studies, APA is now considered to be a key regulator of gene expression, but there are several issues that need to be addressed in future studies. Most importantly, more direct physiological evidence needs to be presented that supports the proposed global role of APA in establishing tissue and proliferation specific gene expression signatures.

A clear distinction needs to be drawn between cause and effect of APA. Since APA mRNA isoforms potentially have varying levels of stability, observed shortening or lengthening of 3'UTRs could be the result of two separate mechanisms: i) a change in CPA site choice, resulting in an increased production of the shorter transcript; ii) an increase in post-transcriptional regulation targeted to the aUTR, which would result in the selected degradation of solely the longer transcript [43]. This introduces potential complications in studies that aim to address the mechanism of APA regulation at the point of CPA site choice.

It is still largely unclear how APA is regulated and how CPA site choice is determined. This is highly critical in order to distinguish between stochastic events that may result from fluctuations in the levels of key CPA factors, and APA events that are indeed the result of targeted regulation. A number of knockdown studies have identified several key polyadenylation and splicing factors whereby APA profiles are altered by their depletion, notably CFI_m68 [41], CFI_m25 [44,45], PABPN1 [46] and U1 snRNP [47]. However, linking these potential regulators to physiological processes that are driven by changes in CPA site choice is far from complete. It is not clear how regulation of APA through such global factors could achieve specificity.

However, it is important to note that mechanisms of APA regulation by several more specific, RNA-binding factors are emerging, such as the case for the cytoplasmic polyadenylation binding protein 1 (CPEB1) [48]. This RNA-binding protein has been shown to shuttle to the nucleus, where, through its co-localization with splicing factors, regulates the 3'UTR shortening of specifically mRNAs which contain cytoplasmic polyadenylation elements (CPEs), the CPEB1 binding sites [48]. Similarly, the cold-induced RNA-binding proteins Cirbp and Rbm3 have also been linked to the control of circadian gene expression through the modulation of APA events [49]. Furthermore, the RNA-binding proteins FPA and FCA have been shown to play an essential role in the control of seasonal flowering in *Arabidopsis* through the modulation of 3' end formation [50].

There is no doubt that APA can play a key regulatory role in contributing to the control of gene expression. The most compelling evidence that modulation of APA can indeed affect gene expression in a physiological context is a recent study where CFI_m25 levels in glioblastomas

were found to influence the 3'UTR length at a subset of genes and ultimately glioblastoma tumorigenicity [45].

However, the current view that APA is playing a global role in regulating gene expression in association with tissue specificity and proliferation has been seriously challenged [27]. Furthermore, considering that, on average, >90% of overall reads come from the dominant (most utilised) CPA site of a gene [12], it is challenging to explain how minor changes in stability and translational efficiency of mRNA isoforms may be of biological significance.

Perhaps APA will follow a similar path to alternative splicing, whereby even though >75% of genes are capable of alternative splicing, most of these events may be a result of stochastic noise and may be of no functional significance [51]. Overall, future studies on global APA analysis should concentrate more on the biological significance of observed APA events, rather than statistical significance. Only then will we uncover the true physiological consequences of APA.

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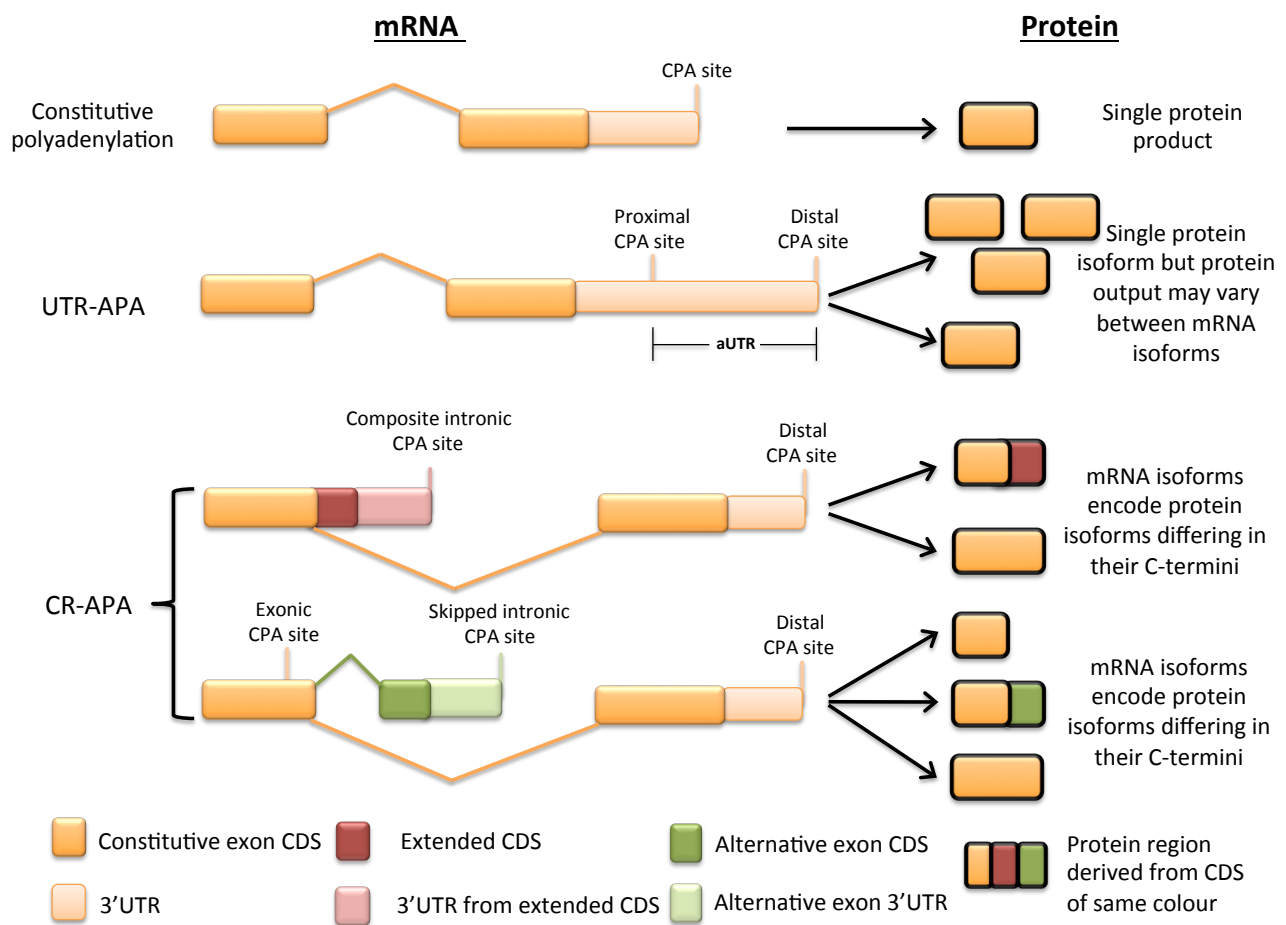


Figure 1 – Schematic Representation of Types of Polyadenylation

Adapted from Di Giammartino et al (2011) [52]. Top: Constitutive polyadenylation, which occurs when a gene contains a single cleavage and polyadenylation (CPA) site resulting in one mRNA isoform and one protein isoform. Middle: Untranslated region alternative cleavage and polyadenylation (UTR-APA), which occurs when several CPA sites occur within the same 3'untranslated region (3'UTR) resulting in several APA mRNA isoforms. Since these mRNA isoforms only differ in their 3'UTR length, they encode the same protein but may have altered mRNA stability or translational efficiency, potentially resulting in differential protein output. The region located between the proximal and distal CPA sites is known as the alternative UTR (aUTR). Bottom: Coding-region APA (CR-APA), which occurs when a CPA site are located upstream of the terminal 3'UTR. This can occur upon intron retention, in the case of composite-intronic CR-APA, or within an alternatively spliced exon in the case of skipped-intronic CR-APA. Exonic CR-APA occurs when a CPA site is located within the coding sequence (CDS) of a gene. CR-APA results in either C-terminus truncation of the resulting protein in the case of exonic APA, or a different C-terminus, in both composite and skipped intronic CR-APA.