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New perspectives on connecting messenger RNA 3′ end formation to transcription

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Recent advances in understanding the molecular mechanism of mRNA 3′ end cleavage and polyadenylation have uncovered an unanticipated involvement of this process in the regulation of the transcriptional apparatus on its chromatin template. Thus, newly defined factors associated with mRNA 3′ end formation are also connected with initiation of transcription, suggesting a close collaboration between the initiation and termination phases of transcription. Furthermore several of these factors are involved in setting up appropriate chromatin structure to facilitate efficient transcriptional elongation and termination.

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Abbreviations

CFIA	cleavage factor IA
CFIm	cleavage factor I
CFIIm	cleavage factor II
CPF	cleavage and polyadenylation factor
CPSF	cleavage and polyadenylation specificity factor
CstF	cleavage stimulation factor
CTD	C-terminal domain
DSE	downstream sequence element
PAP	poly(A) polymerase
Pol II	RNA polymerase II

Introduction

Since the discovery that transcripts synthesised by RNA polymerase II (Pol II) must be extensively processed before they can function as a translatable message, great advances have been made in determining the detailed biochemistry of these different mRNA processing reactions. However, the realisation that mRNA processing occurs as the transcript is being synthesised from its gene template has resulted in a reanalysis of how transcription can affect the efficiency and specificity of mRNA processing. The reverse is also true: transcription itself can be affected by mRNA processing. While mRNA capping and, in particular, splicing have captured much recent interest, the process of mRNA 3′ end formation is also being extensively studied. This latter subject area is the focus of this review. I start by providing an update on the complexity of the biochemistry of polyadenylation, discussing in particular the new insights from yeast systems. I then turn to how polyadenylation is coupled to transcription via the increasing number of factors found to be shared between these two processes. Finally, I discuss how productive transcriptional elongation on chromatin may switch to transcriptional termination at the end of the gene, marked by the polyadenylation process itself.

As well as the connections of mRNA 3′ end processing and transcription described in this review, it is also becoming clear that downstream events are similarly connected. The review by Vinciguerra and Stutz in this issue describes how efficient mRNA 3′ end formation is required for successful release of transcripts from transcription sites and the subsequent export of mRNA from the nucleus to the cytoplasm.

The biochemistry of cleavage and polyadenylation

Our understanding of the biochemistry of polyadenylation is now at a relatively advanced stage. In mammals five separate proteins come together to mediate, first, cleavage of the nascent mRNA 3′ end and, second, coupled polyadenylation: poly(A) polymerase (PAP), cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors I and II (CFIm and CFIIm); see Figure 1. The subunit composition of the two key multimeric mammalian factors CPSF and CstF is well known, as are their contacts to the bipartite AAUAAA and the GU-rich downstream sequence element (DSE) of the poly(A) signal [1,2]. Homologous factors in *S. cerevisiae* are also partially characterised and have been shown to interact with complex sequence elements that constitute the poly(A) signal in yeast (Figure 1). Two multimeric factors called cleavage factor IA (CFIA) and cleavage and polyadenylation factor (CPF) have been purified and shown to share subunits with mammalian CstF and CPSF, respectively [3]. However, both these factors possess additional subunits, which has led to a re-evaluation of the subunit structures of the mammalian proteins. CFIA has four subunits, two of which (Rna14p and Rna15p) are clear counterparts to CstF77 and CstF64, respectively. However, the other CFIA factors, Pcf11p and Clp1p, do not have counterparts in CstF but are found in the less-well-characterised mammalian factor CFIIm [4]. CPF has at least 15 associated polypeptides, including clear homologues to all four CPSF subunits and PAP (Pap1p in *S. cerevisiae*).

Figure 1

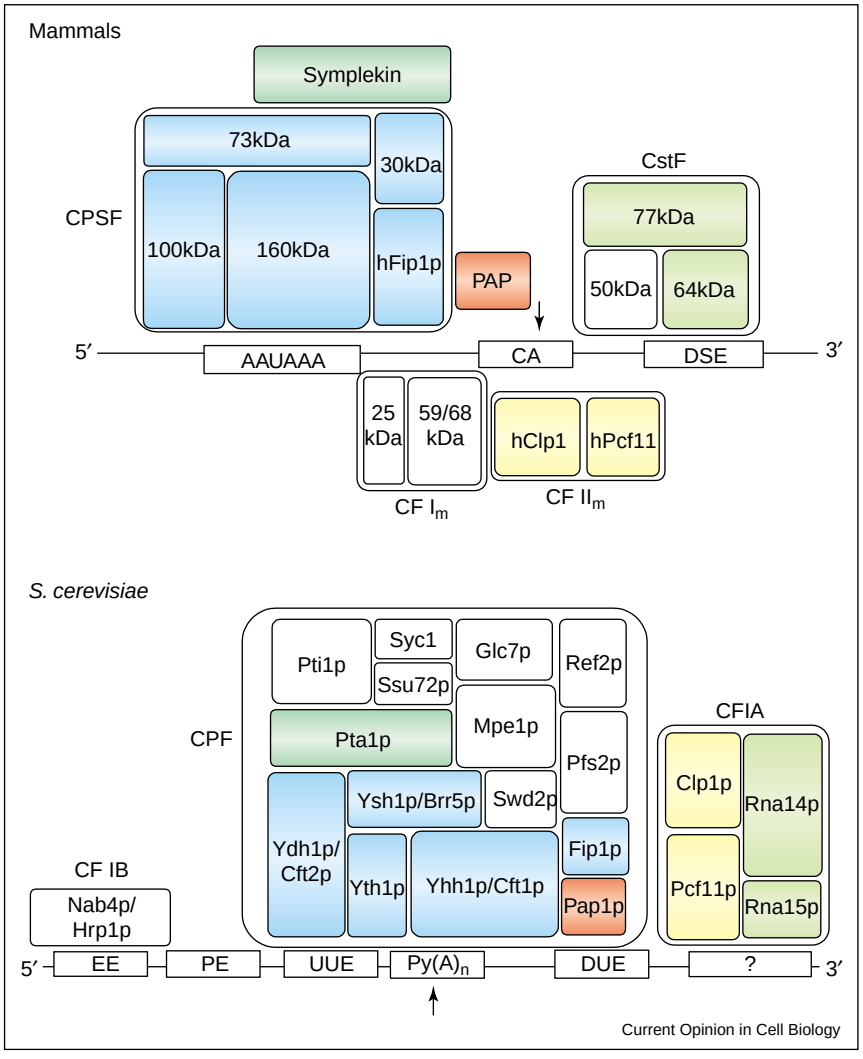


Diagram comparing the subunit composition of mammalian and yeast cleavage and polyadenylation factors and their points of contact with the poly(A) signal. Homologous factors between mammals and yeast are colour coded. Factors that do not have known matches between mammals and yeast are shown in white. For CPSF, 160, 100, 73 and 30 kDa subunits correspond to Yhh1p, Ydh1p, Ysh1p and Yth1p respectively. For CstF, 77 and 64 kDa subunits correspond to Rna14p and Rna15p respectively. The positions of conserved poly(A) signal elements are indicated by white rectangles; in mammals cleavage occurs at a partially conserved CA sequence motif; in *S. cerevisiae*, EE, PE, UUE and DUE denote efficiency, proximal, upstream-U-rich and downstream-U-rich elements, respectively. Cleavage site denoted by black arrow. See [2,3] for more detail. Diagram generously provided by Professor Walter Keller, University of Basel, Switzerland.

Indeed, CPF-associated Fip1p, which was originally identified through its interaction with Pap1p, appears to be a fifth, hitherto undiscovered component of mammalian CPSF. Here it may act to tether PAP to CPSF in the initial cleavage stage of polyadenylation. PAP is thus ready to add the poly(A) tail to freshly generated mRNA 3′ ends [5]. The CPF-associated factor Pti1p has been noted to display significant homology to Rna15p and may also exist as a variant component of CFIA [6]. A significant part of the unexplained complexity of CPF may be that another class of transcripts generated by Pol II, snRNAs and snoRNAs also make use of CFIA and CPF to generate mature 3′ termini [7,8•]. Whether these small Pol II transcripts have a dedicated 3′ end formation apparatus or whether they essentially share the mRNA 3′ end factors remains to be established. However, the CPF-associated factors Pti1p, Ref2p, Swd2p and Ssu72p have all been implicated in snRNA and snoRNA 3′ end formation and may exist as part of a CPF sub-complex [9–11]. Some of the additional subunits in CPF are also thought to be associated with connecting functions between mRNA 3′ end processing and transcription, as discussed below.

A biochemical embarrassment in the above analyses is that the molecular nature of the endoribonuclease activity

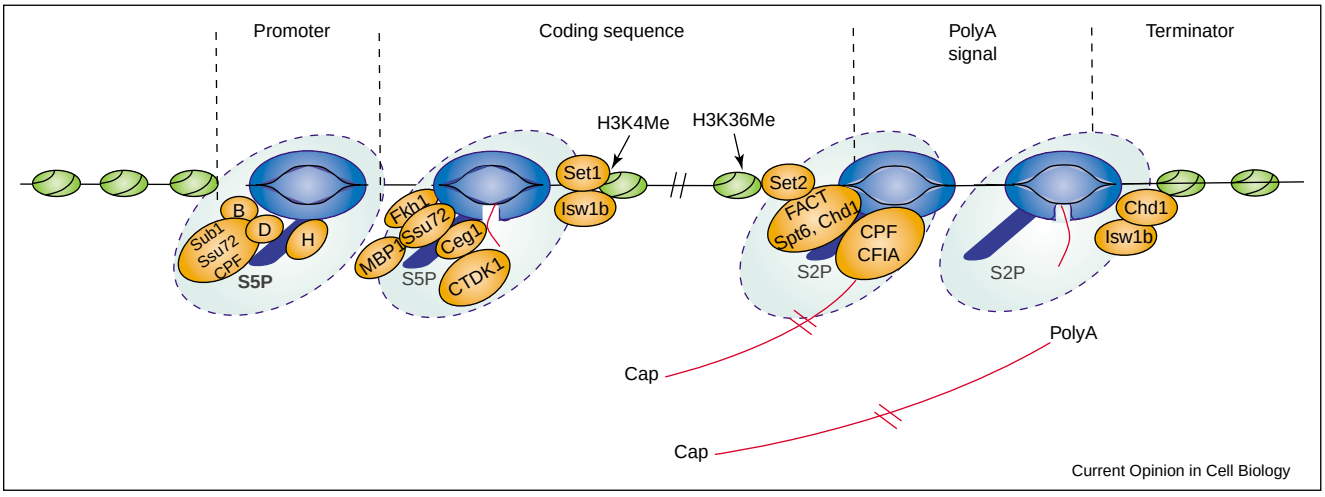
that generates the mRNA 3′ end is still unknown. However, a recent study points to the CPSF73 subunit as a likely candidate, as it possesses a potential nuclease domain that is predicted to be Zn^{2+}-specific [12•]. The cleavage activity of the whole complex has now been shown to require Zn^{2+} and, furthermore, specific amino acids in this conserved domain in the *S. cerevisiae* homologue, Ysh1p, are particularly sensitive to mutation [13••]. CFIm, a dimeric protein, has recently been implicated as a regulator of poly(A) site selection [14]. Although it is an obligatory component of the cleavage/polyadenylation apparatus, it may also act to selectively block inappropriate poly(A) site use. In this respect it may be functionally equivalent (although not homologous) to CFIB in *S. cerevisiae*, which is likewise required to prevent inappropriate polyadenylation at sites on the mRNA upstream of the authentic mRNA 3′ end [15].

Interconnections between mRNA 3′ end processing factors and RNA polymerase II

Forming the 3′ end of a transcript generated by Pol II is a multi-step process. The mRNA 3′ ends must be generated by coupled cleavage and polyadenylation, and in addition the Pol II elongation complex must be halted and the Pol II enzyme terminated to allow recycling back to productive transcriptional initiation at the promoter. A detailed account of the mechanism of Pol II termination was previously presented [16•]. Early studies showed that the poly(A) signal on the transcript is also in effect a termination signal for Pol II, since its mutation leads both to loss of mRNA (through defective polyadenylation) and to the continued nascent transcription of the 3′ flanking regions of genes (through defective termination) [17,18]. Indeed failure to terminate transcription through loss of poly(A) signals may cause transcription read-through into downstream genes which then results in their inactivation by transcriptional interference, as access of Pol II initiation complexes to the promoter of the downstream gene is blocked [19]. With the advent of biochemical and genetic evidence for multiple cleavage/polyadenylation factors, it became a straightforward matter to determine which of these factors also acted as termination factors. In the case of cleavage/polyadenylation factors in *S. cerevisiae*, mutant strains defective in the expression of either CFIA or CPF subunits were obtained and these were tested for defects in Pol II termination by transcription run-on analysis. From these initial studies, components of CFIA were found to be required for Pol II termination whereas CPF components were not [20]. However, more recently, at least one major CPF component, Yhh1p, which is the homologue of mammalian CPSF160, was shown to be required for Pol II termination [21••]. Much insight was provided by the discovery of the C-terminal domain (CTD) of the largest subunit of Pol II, which is positioned outside the overall globular Pol II 3D structure but just below the RNA exit channel; it is an unstructured domain comprising multiple heptad repeats (52 in mammals and 26 in *S. cerevisiae*). A key feature of these heptad repeats is the presence of two critical serine residues (ser5 and ser2), which are subjected to phosphorylation by specific cyclin-associated kinases, called in mammals Cdk7/cyclinH for ser5 (part of TFIIH, Kin28p/Ccl1p in *S. cerevisiae*) and Cdk9/cyclinT for ser2 (also known as PTEFb, CTDK1 in *S. cerevisiae*). Ser5 phosphorylation is an early event in the transcription cycle, promoting promoter release, the formation of a Pol II elongation complex and mRNA capping. By contrast, ser2 phosphorylation subsequently promotes efficient elongation and ultimately termination [16•,22,23]. Several CTD phosphatases have also been identified, including Fcp1p [24], SCP1 [25] and Ssu72p [26], that may play a direct role in Pol II elongation/termination or the subsequent recycling of terminated Pol II back to initiation by promoting CTD dephosphorylation. Fcp1p and SCP1 appear to be specific for ser2 and ser5 phospho-CTD, respectively. In the case of Ssu72p its presence in CPF as well as its role in transcription initiation is likely to relate to its CTD-phosphatase activity (see below and Figure 2).

Initially, deletion of the Pol II CTD in mammalian cells was shown to have a detrimental effect on all three major mRNA processing activities (capping, splicing and polyadenylation) [27]. Furthermore, phospho-CTD interacts with components of all these RNA processing mechanisms and directly activates the reactions [3,16•,23]. Direct contact between yeast cleavage/poly(A) factors and CTD has now been demonstrated both biochemically and genetically. First, CFIA specifically interacts with phospho-CTD through contacts between Pcf11p and Rna14p subunits [28]. Subsequently, both CPF components (Yhh1p and Ydh1p) were shown to interact with phospho-CTD [21••,29]. It is noteworthy that some domains of these cleavage/poly(A) factors are devoted to termination and/or phospho-CTD binding whereas others are specific to poly(A) signal binding. Thus, Rna15p and its *S. pombe* counterpart *ctf1* contain a C-terminal domain (separate from the RNA binding region) which when deleted causes a loss of termination, but not of polyadenylation [30]. Furthermore, Yhh1p directly binds phospho-CTD via a domain that is separate from its RNA-binding region. This latter interaction means that one polypeptide spans both Pol II CTD and the poly(A) signal of the nascent transcript, as it appears from the RNA exit channel [21••]. Pcf11p has a specific CTD-binding domain in which mutations block termination but not mRNA 3′ end cleavage [31]. This domain is shared with a family of proteins including Nrd1p, which is a known termination factor for snoRNA and snRNA genes in yeast [28,32]. Pcf11p has also been shown to preferentially bind ser2 phospho-CTD, consistent with CFIA playing a role in the termination phase of transcription [33]. New results confirm this role by describing the effect of inactivating the ser2 kinase in yeast by gene knockout (Ctk1p of CTDK1) [34•] or in *Drosophila* by drug inactivation of

Figure 2



Schematic arrangement of Pol II complexes showing the different stages of initiation, early elongation (checkpoint), late elongation and termination. The polymerase is dark blue with the CTD shown as a protrusion while the large complex of associated elongation factors are indicated by an outer pale blue area in which specific factors mentioned in the text are placed in brown circles. The relative positions of these factors do not necessarily imply molecular association. The transcript (in red) is shown extruding from the Pol II RNA exit channel. Nucleosomes are indicated in green. The CTD phosphorylation and nucleosome methylation states are indicated. This diagram was drawn by Natalia Gromak.

PTEFb [35•]. Interestingly, both studies found that the inactivation has little effect on elongation, but does cause a substantial reduction in the level or fidelity of mRNA 3′ formation. Also, in the yeast studies, loss of cleavage/polyadenylation factor recruitment to the end of genes was observed [34•]. From these data it is apparent that the act of cleavage of the nascent RNA does not elicit termination. Instead, complex molecular interactions must occur between the CTD-associated-cleavage/poly(A) factors and the poly(A) signal on the nascent transcript that somehow initiate the termination mechanism. Presumably this comes about through conformational changes to this complex as it 'gets its teeth' into the poly(A) signal.

The C-terminal domain of Rna15p does not directly contact the Pol II CTD but instead interacts with specific transcription factors [3]. One such factor is Sub1p or PC4 (in mammals) [36]. PC4 was originally associated with promoter co-activator function [37] and was subsequently found to interact with TFIIB [38]. As Sub1p appears to have an anti-termination activity at the 3′ end of genes [36], it is apparent that PC4 has dual roles in initiation and termination. The CPF-associated factor Ssu72p also has functions in transcription initiation and mRNA 3′ end formation, making similar contacts to those of PC4. Indeed these two polypeptides appear to compete for binding both to TFIIB and to Pta1p, a component of CPF [39,40••]. Ssu72p is directly required for mRNA 3′ end cleavage [40••] and may have a role in Pol II termination [41], especially for snRNA and snoRNA genes [10]. An early connection between general transcription initiation factors and mRNA 3′ end formation was made when CPSF was demonstrated to be a component of some active TFIID preparations. TFIID-associated CPSF is transferred from the initiation complex to the elongation complex following transcriptional activation [42].

Two other transcription factors, Mbp1p and Fkh1p, are also now known to have a role in transcriptional termination. Both have specific but different roles in cell cycle regulation, with Mbp1p required for G1–S phase gene activation and Fkh1p for G2–M phase gene activation. Mbp1p was originally implicated in Pol II termination through its interaction with the Rna15p/Ctf1 CTD binding domain [30]. By contrast, Fkh1p was shown to bind to chromatin within the coding region of a range of genes; these genes are different to those it regulates in the cell cycle. Yeast strains deleted for *FKH1* show multiple elongation defects including loss of both ser5 and ser2 CTD phosphorylation as well as uncontrolled elongation past the normal termination region of the genes tested [43•]. Fkh1p is thought to be part of a protein complex involved in a checkpoint process in early elongation (Figure 2). This is proposed to constrain the elongating polymerase, allowing time for modification of Pol II (by CTD ser5 phosphorylation), histone tail methylation (H3 lysine 4) and co-transcriptional capping to occur. Inactivation of Fkh1p results in rampant, uncontrolled transcription throughout and beyond the normal 3′ terminus of the gene [43•].

Transcriptional termination in the context of a dynamic chromatin template

In all eukaryotes, the Pol II transcription template is nucleosomal in nature. Consequently, much effort has been put into gaining a molecular understanding of how

nucleosomes contribute to transcriptional regulation [44,45]. Although the need to expose gene promoters from their chromatin covers is a well-studied phenomenon, it is also now apparent that nucleosomal structure plays a key regulatory role in both transcriptional elongation and termination [46]. Nucleosomes can either be covalently modified, particularly on their exposed H3 and H4 amino termini (so called histone tails), or can be repositioned by complex chromatin-remodelling enzymes. Although acetylation of histone tails has long been correlated with promoter activation, it appears that methylation of these same tails may have greater relevance to elongation and termination. H3 lysine 4 methylation mediated by the multi-subunit Set1p methylase (also known as COMPASS) is a marker for early elongation, correlating with ser5 phosphorylation of the Pol II CTD [47]. Intriguingly, one of the unaccounted-for CPF subunits Swd2p is also a component of Set1p, again making a molecular contact between the beginning and end of the gene [48]. By contrast, H3 lysine 36 is methylated by Set2p, which appears to be a nucleosomal mark for late elongation and termination and which correlates with CTD ser2 phosphorylation. Both Set1p and Set2p are thought to associate with the elongating Pol II complex (Figure 2) [46,49].

Of particular relevance to a consideration of mRNA 3′ end formation, it was recently discovered that the chromatin-remodelling enzyme Chd1p is required for termination of at least some genes in yeast [50••]. Chd1p appears to define a specific nucleosome structure in the termination region of the genes tested and this process occurs before gene activation. Therefore evidence exists for a predefined chromatin transcription unit with both the promoter and terminator region set up before transcription commences. However, Chd1p is also involved in the elongation process as it is known to interact with elongation factors and may directly bind to methylated nucleosomes through a specific region of the protein called the chromodomain [46,51]. One of these interacting elongation factors is FACT, a protein that realigns nucleosomes following passage of the elongating Pol II complex across the gene [52•]. Significantly, lack of either FACT (or of its yeast counterpart, Spt16/Pob3) or of another related elongation factor, Spt6, causes inaccurate transcriptional initiation [53,54]. This is presumably because the failure to put nucleosomes back in place after passage of the Pol II elongation complex means that internal gene sequence is exposed to predatory initiation factors, which are otherwise restricted to only the authentic (remodelled) promoter. The exact molecular role(s) of Chd1p in elongation and termination remains to be established. However, further complexity in the relationship between chromatin and transcription elongation is revealed by analysis of a second class of chromatin-remodelling enzyme called ISW [55]. Isw1a and Isw2 may both have negative roles in promoter regulation by setting up repressive chromatin structures. By contrast, Isw1b performs a positive role in transcriptional elongation and termination. Indeed, mutational inactivation of the central ISWI ATPase subunit causes loss of controlled elongation and subsequent lack of Pol II termination just as observed with strains defective in Fkh1p expression. Furthermore, both proteins interact to control both Set1p/2p nucleosomal modification and ser5/2 CTD phosphorylation [56••]. A picture emerges of tight interconnections between the factors and processes controlling all stages of elongation: promoter escape, early elongation checkpoint, continued elongation and termination (Figure 2). The fact that promoter and poly(A) factors show up at either end of a gene and that events in early elongation impact on later termination suggests that a plethora of molecular connections may be available to regulate transcription at the elongation and termination stages.

Conclusions

The biological processes of mRNA 3′ end formation and concomitant Pol II termination continue to provide a fascinating window into the complexities of eukaryotic gene expression. Why is the basic cleavage/polyadenylation process so complex, involving scores of factors? We now see that this complexity is in part necessitated by the fact that all gene expression mechanisms are interconnected; checks thus are continually made to ensure that mRNA is appropriately processed and that genes are expressed accurately and efficiently. This necessitates a high level of cross-control that in turn requires many additional regulatory functions. As described here, these factors are arranged in a spectrum from the basic mRNA processing apparatus to those that connect with the elongating polymerase and therefore must also impact on the chromatin architecture of the gene. Indeed it is arguable that setting up an active promoter represents only a part of gene regulation in many biological situations. Transcriptional elongation/termination and its coupling to mRNA processing may also prove to be major targets for gene regulation.

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