PERSPECTIVE

Transcription units as RNA processing units

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Several observations made over the past year have forged strong molecular links between transcription by RNA polymerase II (Pol II) and pre-mRNA processing. The key findings support the view that the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II binds directly to protein factors essential for RNA processing. In some cases, there is a clear suggestion that the factors only bind when the polymerase is in the act of transcriptional elongation. As a result, it is now possible to discuss the transcription unit as an RNA processing unit in which the action of RNA Pol II itself brings essential elements of each of these processing steps to the nascent pre-mRNA substrate. Within this RNA processing unit, capping, splicing, and polyadenylation can also regulate one another.

As pre-mRNA is synthesized, it undergoes a number of modifications that determine the protein encoded as well as the stability and translatability of the mRNA. First, the 5' end of the pre-mRNA is modified by capping, in which the terminal phosphate is cleaved and a GMP nucleotide is linked by a 5'-5' triphosphate bridge to produce GpppN. Subsequently, the G is methylated at the N7 position, and this m⁷G constitutes the cap (Shatkin 1976). The m⁷G cap is added shortly after transcriptional initiation within a narrow window of nucleotides (+20 to +40), indicating that the reaction is rapid and efficient (Salditt-Georgieff et al. 1980; Coppola et al. 1983; Jove and Manley 1984; Rasmussen and Lis 1993). The observation that cap-binding complex (CBC) associates with pre-mRNA early in RNA synthesis provides additional evidence that capping can occur during splicing (Visa et al. 1996b). The two subunits of CBC, CBP80 and CBP20, are bound to the cap throughout the RNA's lifetime in the nucleus and have important roles in the identification of the first exon during pre-mRNA transcription, 3'-end formation, and nuclear export (for review, see Lewis and Izaurralde 1997). The CBC dissociates from the cap in the cytoplasm, where the cap is subsequently bound by the translational regulator eIF-4E (Lewis and Izaurralde 1997).

Pre-mRNA splicing is a two-step transesterification reaction that removes intron sequences and ligates exon sequences together. This reaction requires the splicing

small nuclear ribonucleoprotein (snRNPs) as well as a number of non-snRNP splicing factors that assemble with the pre-mRNA to form a multiprotein complex known as the spliceosome (Moore et al. 1993). Cytological studies revealed that complexes begin to associate with proximal splice sites before distal splice sites are synthesized, and that intron removal often occurs while elongating transcripts are still attached to the DNA (Osheim et al. 1985; Beyer and Osheim 1988). The latter observation provided the first convincing evidence that splicing can occur during transcription. Cotranscriptional splicing has subsequently been confirmed in a variety of systems and has been shown to precede polyadenylation (LeMaire and Thummel 1990; Zachar et al. 1993; Bauren and Wieslander 1994; Zhang et al. 1994; Bauren et al. 1996).

Polyadenylation of the 3' end of the RNA is achieved by first cleaving the pre-mRNA downstream of a consensus sequence, AAUAAA, which is the binding site of cleavage and polyadenylation specificity factor (CPSF). Cleavage stimulation factor (CstF) binds a GU-rich sequence downstream of AAUAAA, and cleavage factors I and II are both required for the cleavage event. Subsequently, the new poly(A) tail is synthesized in the nucleus and/or the cytoplasm by poly(A) polymerases (Manley 1995). Because both AAUAAA and GU-rich sequences are also required for termination of transcription by RNA Pol II (Whitelaw and Proudfoot 1986; Logan et al. 1987; Connelly and Manley 1988), polyadenylation cleavage is likely to be integrated with the process of transcription.

Neither capping, splicing, nor polyadenylation occurs efficiently when transcripts are synthesized by RNA Pol I or Pol III (Smale and Tjian 1985; Sisodia et al. 1987; Gunnery and Matthews 1995), suggesting that there is something special about RNA Pol II that permits the integration of these processes. Corden (1990) proposed that the unique feature of RNA Pol II would prove to be the CTD, a domain comprised of a heptad repeat element (YSPTSPS in mouse) conserved throughout evolution but varying in the number of repeats from 26 in yeast to 52 in mouse. The CTD is dynamic with respect to its phosphorylation state during transcription. During pre-initiation complex formation and initiation, the CTD is hypophosphorylated, and this form of the polymerase is known as Pol IIa (Dahmus 1996). The polymerase often pauses after synthesizing only 20–30 nucleotides, and as

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it passes into elongation mode, the CTD is hyperphosphorylated by TFIIF to produce Pol IIo (O'Brien et al. 1994; Dahmus 1996). This reaction occurs precisely when capping occurs (Rasmussen and Lis 1993).

Two studies reveal the logic behind the correlation between capping and CTD phosphorylation: The capping enzymes themselves associate with the CTD but only when the CTD is phosphorylated (Cho et al.; McCracken et al.; both this issue). In higher eukaryotes, the enzymatic activities for capping reside on one protein for the triphosphatase and guanylyltransferase activities in addition to the methyltransferase (Shuman 1995). In *Saccharomyces cerevisiae*, two separable enzymes, guanylyltransferase (Ceg1) and triphosphatase, exist as subunits of the capping enzyme, and the methyltransferase (Abd1) has also been identified. McCracken and colleagues (this issue) examine capping in human cells whose only active Pol II has a truncated CTD (5 heptad repeats instead of 52) and find that the CTD truncation leads to a fivefold decrease in the level of capping of several test RNAs. The observation that guanylyltransferase activity is eluted from a CTD-GST affinity column, but only if the CTD has been first phosphorylated by the kinase subunit of TFIIF, cdk7-cycH kinase, is consistent with a direct molecular connection between transcription and capping (McCracken et al., this issue). This capping enzyme cDNA was cloned from mouse, and the in vitro translated protein product was shown to bind specifically to the phosphorylated CTD resin. Like the mouse and human counterpart, yeast Ceg1 as well as the methyltransferase Abd1 bind the CTD in a phosphorylation-dependent manner (Cho et al.; McCracken et al.; both this issue). There is some disagreement as to whether each capping component can bind the CTD alone. McCracken et al. (this issue) show that each recombinant protein can bind independently to the phosphorylated CTD column, whereas Cho et al. (this issue) find that bacterially expressed Ceg1 cannot. However, it is clear from both studies that the native capping enzymes bind, and Cho et al. make the additional point that Ceg1 is not detectable in association with Pol II transcription factors or Pol II holoenzyme until the CTD has been phosphorylated by TFIIF.

The association of capping activities with RNA Pol II explains how this modification is specifically targeted to Pol II transcripts, as there are no determinants on the nascent RNA to specify capping. It is a strategy taken by a CTD-less RNA polymerase, that of vaccinia virus, which binds to the capping enzyme by an independent mechanism (Hagler and Shuman 1992). The fact that Pol I and Pol III transcripts are not capped can now be attributed to the lack of the CTD in those polymerases. The requirement that the CTD be phosphorylated prior to capping enzyme binding may be to prevent capping of RNAs that are not destined for elongation. At this time, it is not known whether the CBC also associates with the polymerase, but this might facilitate its rapid binding to the cap (Visa et al. 1996b).

The work reported in this issue falls on the heels of evidence that pre-mRNA splicing and polyadenylation

factors associate with the CTD (for review, see Steinmetz 1997). Importantly, the truncated CTD form of Pol II described above, was insufficient for pre-mRNA splicing, polyadenylation, and transcriptional termination; the effects on polyadenylation and termination were shown to be independent of the effects on splicing (McCracken et al. 1997a). The polyadenylation factors CstF and CPSF were shown to bind TAF (TATA-binding protein-associated factor) components of TFIID as well as the CTD (Dantonel et al. 1997; McCracken et al. 1997a). In other studies that address splicing, Du and Warren (1997) showed that overexpression of the CTD in HeLa cells interfered with splicing, and Yuryev et al. (1996) found that Pol II depletion from HeLa nuclear extract or the addition of CTD peptides could inhibit in vitro splicing. A possible explanation for these data is provided by the observations that splicing snRNPs and members of the SR protein family of non-snRNP pre-mRNA splicing factors associate with the hyperphosphorylated form of Pol II (Chabot et al. 1995; Vincent et al. 1996; Mortillaro et al. 1996; Kim et al. 1997). In addition, Yuryev et al. (1996) detected three as yet uncharacterized proteins associated with the hyperphosphorylated form of Pol II. These proteins are related to SR proteins through their arginine-rich domains but clearly are not canonical SR proteins. It will be of interest to learn how these three proteins function in RNA processing. A more significant challenge will be the development of cotranscriptional splicing systems, which will allow us to fully appreciate the significance of splicing factor association with the CTD on the one hand and permit a mechanistic description of transcription and splicing (as we have for transcription and capping) on the other.

An important question for the model (Fig. 1) is whether RNA Pol II is constitutively associated with processing factors in a multifunctional "holo-particle" or whether complexes are formed in a more dynamic way in which the processing factors associate only once polymerase is elongating. The work on capping and polyadenylation clearly favors the preferential association of factors with the elongating polymerase. Both groups studying the capping enzymes find that these molecules only have affinity for the hyperphosphorylated CTD (Cho et al.; McCracken et al.; both this issue). Interestingly, the polyadenylation factors CPSF and CstF have affinity for both phosphorylation forms of the CTD (McCracken et al. 1997a). However, a recent study shows that CPSF is brought to the transcription unit by TFIID, CPSF binding is only transferred to the CTD after elongation begins, and TFIID is left behind at the promoter (Dantonel et al. 1997). Thus, in the case of CPSF, binding to the CTD may not be determined solely by phosphorylation but also by the sequestration of the factor(s) in other functional complexes. Importantly, the association of this factor with TFIID also ensures CPSF targeting to RNA Pol II transcripts.

How do factors associate with the CTD? Greenleaf (1993) first proposed that arginine-rich domains of splicing factors would interact with the phosphorylated CTD through electrostatic interactions. Many metazoan splic-

Figure 1. Transcription of an intronless gene. (*Top*) The dynamic assembly of factors on the CTD as well as the RNA substrate. (*Left to right*) As RNA polymerase II begins elongation, the CTD is hyperphosphorylated and capping enzymes associate. The nascent RNA is capped and capping enzymes diffuse away. Subsequently, CPSF and CstF associate with the CTD and bind their target RNA sequences upon their synthesis. The binding of these factors leads to recruitment of the cleavage factors, which carry out polyadenylation cleavage. Transcription is terminated, and the naked polymerase awaits reentry into the cycle. (*Bottom*) A holoparticle of the polymerase associated with all of the possible factors needed for processing, including SR proteins even though this gene has no introns. As the RNA is synthesized, it moves through the particle for modification. When additional factors are needed (e.g., the cleavage factors), they add to the complex at the polymerase.

ing factors, including the SR proteins, contain such alternating-arginine domains comprised almost entirely of arginine alternating with glutamate, aspartate, and phosphoserine (Neugebauer et al. 1995). However, the overall positive charge of these domains cannot be assumed, as many of the intervening amino acids (aspartate, glutamate, and phosphoserine) are negatively charged. Although splicing factor candidates observed to associate with the CTD were alternating-arginine proteins (Yuryev et al. 1996), the same study showed that the protein domains apparently responsible for CTD binding were distinct from the alternating-arginine domains. Thus, alternating-arginine proteins, including the SR proteins, have a tendency to associate with the CTD, but this may not be due to the alternating-arginine domain itself. However, none of the capping or polyadenylation factors studied thus far have arginine-rich domains typical of splicing factors (Manley 1995; McCracken et al., this issue). It will be important to determine which regions of these latter factors are required for CTD binding.

The results of McCracken et al. (1997a and this issue) show that five heptad repeats of the CTD are sufficient for transcription but not for RNA processing. One wonders whether the RNA processing functions have similar minimal requirements for CTD heptads and whether this requirement can be understood in terms of the stoichiometry of factor association with RNA Pol II. Interestingly, truncation mutants of the CTD reveal that organisms can survive with as many as one-half of the CTD deleted (Allison et al. 1988; Bartolomei et al. 1988). Zehring et al. (1988) found that although a truncation of the CTD in *Drosophila* Pol II from 42 to 20 heptad repeats was lethal in the homozygote, this purified truncated polymerase was fully active for transcription in vitro on a variety of promoters. This suggests that the

lethality may have been due to RNA processing phenotypes rather than a block of transcription. Now that the connection between the CTD and RNA processing has been made, perhaps it will be possible to re-examine the molecular phenotypes of these truncation mutants.

The possibility that factors associate in a dynamic way with the CTD of the elongating polymerase is attractive with respect to splicing in particular, because the nascent RNA itself could play a role in the binding specificity of the processing factors. Though nearly every mRNA is capped and polyadenylated, not every transcript is spliced. In yeast, only 235 genes of at least 5885 are spliced (Goffeau et al. 1996), making the association of snRNPs with every transcription unit a highly inefficient prospect. In contrast, mRNA-type splicing can also occur when RNA is transcribed by RNA Pol III, as in the case of U6 snRNA synthesis (Koehrer et al. 1990; Tani and Oshima 1991). Consistent with a role for the RNA in splicing factor recruitment, Huang and Spector (1996) found that splicing factor association with transfected expression constructs was dependent on the presence of an intron. Transcripts containing many exons and introns may “grab” splicing factors from the CTD for spliceosome assembly (Fig. 2). In this scenario, the empty CTD must then be reloaded to supply the next splice site with factors. Overall, it would make sense that factors could assemble with the CTD during the process of transcription and processing, in the manner suggested by the term “loading site” (Corden 1990). In this model, the CTD would stabilize factor binding to an RNA sequence for which the factor already has some specificity.

The fact that splicing in metazoans is regulated, producing alternative splicing, raises questions about how particular splicing factors associate with various transcription units. The SR proteins have been shown to

Figure 2. Transcription and splicing of an RNA containing multiple introns and exons. An elongating RNA is already capped and the CTD is bound to splicing factors, including SR proteins, other non-snRNP splicing factors (e.g., U2AF), and snRNPs that recognize exons and splice sites. As the first exon is synthesized, factors recognizing the first 5' splice site of the first intron are bound to the RNA. Subsequently, other factors bind to the 3' splice site (3' SS) of that intron, and SR proteins associate with exon 2. A spliceosome is assembled. This depletes the the polymerase of the splicing factors it initially carried, and these splicing factors are replenished from soluble, nucleoplasmic pools. The cycle is repeated, depending on the number of introns and exons in the gene.

have distinct functions in determining exon usage (Fu 1993; Zahler et al. 1993; Caceres et al. 1994), and current evidence indicates that this is due, at least in part, to differences among the SR proteins in RNA-binding specificity (e.g., see Manley and Tacke 1996). The observation that particular SR proteins are heterogeneously distributed at active sites of transcription suggests that distinct factors may be specifically recruited to nascent transcripts (Neugebauer and Roth 1997). Furthermore, there are seemingly too many snRNP and non-snRNP splicing factors in the metazoan cell nucleus for them all to associate with each CTD all of the time. The canonical SR proteins belong to a larger family of ~25 alternating-arginine proteins, including the essential splicing factors U2AF⁶⁵ and U2AF³⁵ (Neugebauer et al. 1995). Furthermore, the spliceosome is a dynamic association of proteins and RNAs that evolves from E to A to C complex during the spliceosome cycle; 60 polypeptides are present in C complex alone (Moore et al. 1993; Gozani et al. 1994). Certainly, the greater number of factors required for regulated splicing in metazoans could help explain the correlation between the length of the CTD and the complexity of the organism (Corden 1990). In the future, it will be important to distinguish between a model in which all of the components of the spliceosome are brought to the substrate by the CTD from a model in which only selected splicing factors bind to the CTD and promote the assembly of the spliceosome from mostly nucleoplasmic components.

It is possible that the idea of the RNA participating in determining the specificity of factor binding can be generalized to other processes, such as polyadenylation and transcriptional termination. Both CPSF and CstF have binding sites on the nascent RNA. The CTD may play an important role in stabilizing these sequence-dependent interactions, however, as the RNA sequences bound (AAUAAA and GU-rich tracts) are likely to be present in Pol I and Pol III transcripts that are not polyadenylated. Conversely, transcription of rDNA by Pol II yields a pre-rRNA that is processed appropriately to large rRNAs,

demonstrating that the function of the CTD in processing is not dominant over other processing signals (Nogi et al. 1991). Moreover, the dual binding site model allows for the possibility that CPSF and/or CstF binding to the nascent RNA signals termination to the polymerase through the CTD. Alternatively, a distinct Pol II termination factor may be recruited to the transcription unit by the polyadenylation complex with or without the aid of the CTD.

The synchronization of transcription and RNA processing events surely has significance beyond the targeting of RNA modifications to Pol II transcription units. For many years now it has been appreciated that capping, splicing, and polyadenylation are interrelated processes, suggesting that some of the effects detectable by genetic manipulation of these close-knit processes, such as inhibition of pre-mRNA splicing, may actually be indirect effects of having disturbed the overall processing pathway. This may be understood by considering that the cap defines the 5' end of the pre-mRNA—the first exon—whereas the poly(A) site defines the 3' end—the last exon. Pre-mRNA splicing is blocked when capping activity is inhibited genetically in yeast (Fresco and Buratowski 1996) or when substrate pre-mRNAs lacking a cap are spliced in vitro (Ohno et al. 1987). This suggests that the cap is part of a multistep mechanism for identifying the first exon in the pre-mRNA. Recognition of the cap by the CBC has been shown to stimulate the formation of the earliest spliceosomal complex, complex E (Colot et al. 1996; Lewis et al. 1996). Berget and colleagues found that the presence of an intron upstream of a polyadenylation signal stimulated cleavage (Niwa et al. 1990) and, conversely, that wild-type polyadenylation signals are required for efficient splicing of the last exon (Niwa and Berget 1991). This interesting interaction between polyadenylation and splicing is important, because many alternative splicing events involve a choice of alternative terminal exons. Recently Lou et al. (1996) have characterized an intronic enhancer of polyadenylation cleavage in the calcitonin/CGRP (calcitonin gene-

related peptide) gene that results in the alternative processing of the pre-mRNA. Interestingly, this intronic enhancer associates with the U1 snRNP, SR proteins, and another splicing regulator, PTB (polypyrimidine tract-binding protein) (Lou et al. 1996). Finally, the cap even appears to affect polyadenylation. Several studies have found that efficient polyadenylation cleavage requires the cap structure (Gilmartin et al. 1988; Cooke and Alwine 1996), and that this effect is also mediated by the CBC (Flaherty et al. 1997). In yeast, however, inhibition of capping has no detectable effect on polyadenylation (Fresco and Buratowski 1996). That polyadenylation signals differ between yeast and metazoans perhaps is another difference reflecting the reduced complexity of the yeast transcription unit.

One nuclear function that may be connected to transcription—but has not been yet—is nuclear export. Many Pol II transcripts are destined for the cytoplasm, and they are generally escorted to the nuclear pore by protein factors in a particle, the messenger RNP (mRNP; Daneholt 1997; Ullman et al. 1997). It is known that the cap promotes RNA export, and this effect is probably mediated by the binding of CBC (Hamm and Mattaj 1990; Izaurralde et al. 1995; Visa et al. 1996b). Interestingly, intron-containing RNAs seem to be specifically retained in the nucleus (Hamm and Mattaj 1990). Some factors that have been observed in association with RNAs in the process of export include the CBC, heterogeneous nuclear RNPs (hnRNPs), and SR proteins (Piñol-Roma and Dreyfuss 1992; Visa et al. 1996a; Visa et al. 1996b; Daneholt 1997). As discussed above, association of some of these factors with RNA is perhaps guided by the polymerase itself, indirectly connecting export with transcription. It will be of interest to learn whether hnRNP protein association with RNA is in any way facilitated by the CTD. An additional possibility is that other export factors bearing nuclear export signals (NESs; for review, see Ullman et al. 1997) may also associate with the CTD, such that the local concentration of the export factors is highest where the mRNP is being assembled. In particular, do recently identified components of the nuclear export pathway, such as exportin 1, have any affinity for RNA Pol II that synthesizes the RNAs for transport?

Most of our concern thus far has been directed toward understanding how RNA processing follows from the initiating event—transcription. If RNA processing factors bind to the polymerase, it is likely that processing can influence transcription, just as we now know the reverse is true. To what extent do processing factors affect the activity of the polymerase? Several studies in nematodes and mice have shown a dependence on the presence of introns for gene expression driven by a variety of promoters (Brinster et al. 1988; Okkema et al. 1993). In particular, transgenic mice harboring intron-containing or intron-less genes behind natural promoters show intron-dependent expression of those genes (Brinster et al. 1988). Moreover, the change in RNA abundance exactly parallels measured changes in transcriptional rates for the intron-containing constructs. This

suggests a positive feedback mechanism between RNA processing, in this case splicing, and transcriptional activity. In this scenario, the presence of an intron might stimulate the accumulation of splicing factors that bind the CTD.

In the last year, our concept of the transcription unit has changed from that of a place in which RNA is synthesized and processed very rapidly through a series of independent biochemical events to one in which RNA synthesis is coordinated with processing. If elongating RNA Pol II can act to increase the local concentrations of processing factors specific for these transcripts, then nuclear organization is, at least in part, determined by gene activity. It suggests that the transcription unit, once fired, is a self-assembling functional unit. This concept is very different from models put forth in the last decade in which different functions in mRNA biogenesis are proposed to be compartmentalized into distinct domains within the nucleoplasm (e.g., see Moen et al. 1995; de Jong et al. 1996). Examination of transcription units in cytological preparations from amphibians and insects has revealed that processing factors are highly concentrated at active sites of transcription (Gall 1991; Bauren et al. 1996; Daneholt 1997). However, we have been reluctant to extrapolate these observations to all nuclei (Mattaj 1994). Recent progress in high-resolution light microscopy, immunocytochemical methods, and in situ hybridization techniques indicate that we can examine these transcription units in mammalian interphase nuclei as well. Already, we have seen that active sites of RNA Pol II transcription are distributed throughout the nucleoplasm (Wansink et al. 1993) and that they are the sites of high concentrations of RNA Pol II, SR proteins, and CstF (Iborra et al. 1996; Schul et al. 1996; Neugebauer and Roth 1997; Zeng et al. 1997). This cell biological approach is bound to complement the biochemical and genetic approaches already ongoing to understand the rules and regulations of transcription unit assembly.

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