The Carboxyl-terminal Domain of RNA Polymerase II Is Not Sufficient to Enhance the Efficiency of Pre-mRNA Capping or Splicing in the Context of a Different Polymerase*

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Eukaryotic messenger RNA precursors (pre-mRNAs) synthesized by RNA polymerase II (RNAP II) are processed co-transcriptionally. The carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II is thought to mediate the coupling of transcription with pre-mRNA processing by coordinating the recruitment of processing factors during synthesis of nascent transcripts. Previous studies have demonstrated that the phosphorylated CTD is required for efficient co-transcriptional processing. In the study presented here we investigated whether the CTD is sufficient to coordinate transcription with pre-mRNA capping and splicing in the context of two other DNA-dependent RNA polymerases, mammalian RNAP III and bacteriophage T7 RNAP. Our results indicate that the CTD fused to the largest subunit of RNAP III (POLR3A) is not sufficient to enhance co-transcriptional pre-mRNA splicing or capping in vivo. Additionally, we analyzed a T7 RNAP-CTD fusion protein and examined its ability to enhance pre-mRNA splicing and capping of both constitutively and alternatively spliced substrates. We observed that the CTD in the context of T7 RNAP was not sufficient to enhance pre-mRNA splicing or capping either in vitro or in vivo. We propose that the efficient coupling of transcription to pre-mRNA processing requires not only the phosphorylated CTD but also other RNAP II specific subunits or associated factors.

In recent years many studies have focused on deciphering the mechanisms by which RNA polymerase II (RNAP II) transcription is coupled with pre-messenger RNA (pre-mRNA) processing, including 5' capping, splicing, and 3' end formation (1–8). These studies have revealed a role of the carboxyl-terminal domain (CTD) of the largest subunit of RNAP II (POLR2A) in the coupling of transcription to pre-mRNA processing. The CTD is unique to POLR2A, as it is not present in homologous subunits of RNA polymerases I and III (9, 10). The highly conserved CTD comprises 25–52 heptapeptide repeats with the consensus sequence YSPTSPS. This sequence can be highly modified in vivo via glycosylation, phosphorylation, and iso-merization of specific residues (11–13). The repetitive nature of the CTD and its ability to be differentially modified has led to the proposal of a CTD code (11, 14). This idea suggests that the modification state of the heptapeptide repeats can serve to coordinate transcription with pre-mRNA processing by recruiting the appropriate processing factors at appropriate stages of the transcription cycle (15).

CTD phosphorylation and dephosphorylation are critical for proper CTD function (15). The hypophosphorylated form of RNAP II, RNAP IIΦ, is associated with the preinitiation complex positioned at the promoter, whereas the hyperphosphorylated form, RNAP IIY, is associated with transcript elongation and pre-mRNA processing (12). The general transcription factor TFIIH phosphorylates Ser5 residues of the CTD soon after transcription initiation. RNAP II with Ser5-phosphorylated CTD repeats undergoes promoter-proximal pausing which is coincident with co-transcriptional capping of the nascent transcript (15–17). Positive transcription elongation factor b, P-TEFb, phosphorylates Ser2 residues, and its activity has been implicated in transcription elongation, splicing, and 3’ end processing (18–20).

Early studies investigating the function of the CTD revealed a requirement of the CTD for efficient pre-mRNA processing (21, 22). Subsequent work designed to examine transcription coupled and uncoupled from pre-mRNA processing demonstrated that CTD phosphorylation only impacted co-transcriptional processing and not post-transcriptional processing, strongly supporting a central role of CTD phosphorylation in coupling (23). Together, these studies established that the phosphorylated CTD is required for efficient co-transcriptional pre-mRNA processing.

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The abbreviations used are: RNAP II, RNA polymerase II; CTD, carboxyl-terminal domain; RNAP III, RNA polymerase III; T7 RNAP, T7 RNA polymerase; POLR3A, largest subunit of RNAP III; POLR2A, largest subunit of RNAP II; Ser5, CTD serine 5 residues; Ser2, CTD serine 2 residues; RPA, RNase protection assay; RT, reverse transcriptase; GST, glutathione S-transferase; CMV, cytomegalovirus; siRNA, small interfering RNA.
Additional evidence supporting a role of the CTD in co-transcriptional pre-mRNA capping and splicing has been provided by several studies. The Ser\(^5\)-phosphorylated CTD recruits 5' -capping enzymes to RNAP II and stimulates capping activity (16, 24–27). Furthermore, the capping enzyme can bind specifically and directly to the CTD (28, 29). In addition, the CTD is required for the recruitment of splicing factors to the sites of transcription (30). Direct contacts between the CTD and splicing factors have been described including interactions with Prp40, PSF, p54\(^{\text{SR}}\)/Non-O, and TCERG1 (31–34). Another splicing factor, the serine/arginine-rich (SR) protein, SRp20, has been shown to be dependent on the CTD for its effect on alternative splicing (35). Moreover, a recombinant CTD fusion protein (GST-CTD) has been shown to enhance splicing of pre-mRNAs recognized via exon definition (36). Together, these data support a role of the CTD in the coupling of transcription to pre-mRNA processing, although the precise mechanisms by which the CTD contributes to coupling remain unclear.

In this study we investigated whether the CTD was sufficient to enhance the efficiency of pre-mRNA capping or splicing. To address this question, we analyzed the CTD in the context of RNAP III and T7 RNAP. We show that the CTD in the context of RNAP III was unable to enhance pre-mRNA splicing or capping efficiency of constitutively or alternatively spliced transcripts \textit{in vivo}. Purified T7CTD was not sufficient to enhance splicing efficiency, regardless of CTD phosphorylation, when analyzed using a previously described \textit{in vitro} transcription splicing system (37). Furthermore, we found that transcripts synthesized by T7CTD \textit{in vivo} did not exhibit enhanced splicing or capping. These experiments strongly suggest that the CTD is not sufficient to enhance pre-mRNA splicing or capping in the context of a different polymerase. We, therefore, propose that other subunits of RNAP II or RNAP II-associated factors are necessary for the efficient coupling of transcription to pre-mRNA processing.

**Experimental Procedures**

\textbf{Plasmid Construction}—The plasmid DNA constructs used in this study were made by standard cloning techniques and verified by DNA sequence analysis. To construct pcDNA3.1/His-POLR3A, pcDNA3.1/His-POLR3A-CTD, and pcDNA3.1/His-POLR3A-LCTD, cDNAs of POLR3A, CTD, and LCTD were PCR-amplified from a HeLa cDNA library and subcloned into pCR4-TOPO (Invitrogen). The cDNAs were then cloned into pcDNA3.1/HisA (Invitrogen). A carboxy-terminal SV40 T-antigen nuclear localization signal was introduced into the Apal site of pcDNA3.1/His-POLR3A, pcDNA3.1/His-POLR3A-CTD, and pcDNA3.1/His-POLR3A-LCTD. The 5S\(\beta\) reporter plasmids (5S+1+ pA, 5SAl+pA, 5S1ΔpA, and CMV/5S+I) were derived from pSS\(\beta\)A, a gift of B. Sollner-Webb (38). The bovine growth hormone polyadenylation signal of pcDNA3.1 was PCR-amplified and inserted into an XbaI site introduced downstream of β-tubulin exon 3 in constructs containing a polyadenylation site. The GST-elf4E plasmid used for the capping assays was the kind gift of J. Pelletier (39). pBH161-CTD, the vector used for bacterial expression of T7CTD, was a gift of J. Manley (40). pBH161-T7 bacterial expression vector was constructed by digesting pBH161-CTD with XhoI and religating the vector. The pcDNA3.1/HisXpress-T7CTD and pcDNA3.1/HisXpress-T7 mammalian expression vectors were derived from pcDNA3T7CTD, a kind gift of D. Bentley (21). An amino-terminal His tag and an Xpress tag were inserted by annealed oligonucleotide cloning. The β-globin plasmids, pBC12/CMV/β-globin and pBC12/T7/β-globin, used in both the \textit{in vitro} transcription splicing assays and the \textit{in vivo} transfections were described previously (37). Plasmid pBC12/CMV/β-globin was used as the template for PCR amplification of the RNase Protection probe specific to intron 2 and exon 3 of β-globin. The resultant product was cloned into pCR4-TOPO. The reporter plasmid pI12-IIIc has been described previously (41). The RNase protection probe for exon IIIc was generated by digesting pI12-IIIc with XhoI and HindIII and inserting the Xho/HindIII fragment into pBluescript SK+ (Stratagene).

\textbf{Expression and Purification of Recombinant Proteins}—For production of recombinant T7CTD and T7, pBH161-CTD and pBH161-T7 were used to transform Escherichia coli BL21(DE3). Induction of protein expression and subsequent protein purification were carried out as described previously (40). The CTD was cloned as an amino-terminal fusion, as is well established that T7 RNAP does not tolerate carboxyl-terminal extensions. Expression and purification of GST-elf4E were performed as described previously (39).

\textbf{Cell Culture and Transfections}—293T/17 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin to 100 units and 100 μg per ml, respectively (Invitrogen). Transient transfections were performed with FuGENE 6 (Roche Applied Science). Cells were seeded at a density of 3×10\(^5\) cells per 10 cm\(^2\) well (BD Biosciences) 24 h before transfection. For the RNAP III transfections, cells were co-transfected with both the POLR3A expression plasmids and the 5S\(\beta\)3 reporters. Cells were harvested 48 h post-transfection. For experiments using α-amanitin, cells were treated with 5.0 μg/ml α-amanitin 24 h after transfection of protein expression plasmids. Twelve hours after α-amanitin treatment, cells were re-transfected with reporter plasmids, β-globin, or pI12-IIIc.

\textbf{In Vitro Transcription and Splicing Assay—} \textit{In vitro} transcription and \textit{in vivo} transcription/splicing reactions were performed as described previously (37).

\textbf{RNA Isolation and RT-PCR Assay of Transfected Minigenes}—Total cellular RNA for RT-PCR and RNase Protection was isolated using Trizol reagent (Invitrogen). RT-PCR analysis of the pI12-IIIc minigene was performed with 2 μg of DNase-treated total RNA. The primers used were UexF (5’-GCTGTCGAGTACCCCTAC-3’) and DexR (5’-AGCTTAGCTTTGGCGTTC-3’). PCR products were resolved on nondenaturing 5% polyacrylamide gels at 120V for 3–4 h followed by drying and exposure to PhosphorImager screens. Analysis was performed using Image Quant software (GE Healthcare).

\textbf{Ribonuclease Protection Assay (RPA)—} Antisense [α-\(32\)P]UTP-labeled probes were transcribed from linearized plasmids by T7 or T3 RNA Polymerase Plus (Applied Biosystems). Probes were gel-purified on 8% polyacrylamide, 8 μg urea gels and allowed to elute in high salt column buffer (50 mM Tris, pH 8.0, 400 mM
NaCl, 0.1% SDS) for 4 h at 37 °C with shaking. After elution, probes were phenol/chloroform-extracted, ethanol-precipitated, and resuspended in hybridization buffer. Total RNA from transiently transfected 293T cells (4–20 μg) was hybridized to the probe at 65 °C overnight. As a negative control, yeast tRNA (Sigma) was used. Samples were treated with RNase A/T1 mixture (Applied Biosystems) for 1 h at 37 °C and phenol/chloroform-extracted and ethanol-precipitated using yeast tRNA (Sigma) as a carrier. Samples were washed with 80% ethanol, dried, and resuspended in formamide dye. Before loading onto the gel, reactions were denatured by heating to 100 °C for 3 min. Products were resolved on 8% polyacrylamide, 8 M urea gels at constant power. Gels were exposed to PhosphorImager screens and analyzed using Image Quant software (GE Healthcare).

Western Blot Analysis—Transfected cells were harvested in SDS buffer (58 mM Tris, pH 6.8, 5% glycerol, 1.6% SDS, 1.5% dithiothreitol), incubated at 100 °C for 10 min, and spun down at 14,000 rpm. Cell lysates were spun through Bio-Spin 6 columns (Bio-Rad) to remove SDS. Protein concentration was determined using a Bradford assay (Bio-Rad) or a BCA (bicinchoninic acid) assay (Pierce). Equal amounts of protein were loaded onto a 7.5% SDS-polyacrylamide gel (29:1 acrylamide:bis). For phosphatase experiments, equal amounts of

![FIGURE 1. POLR3A recombinant proteins are expressed and phosphorylated in vivo. A, a schematic representation of the amino acid sequence alignment between human POLR3A and POLR2A. The sequences were aligned using the pairwise Blast 2 sequences program (NCBI, National Institutes of Health). The shaded and hashed boxes represent two separate regions of homology (percentages are indicated below), and the horizontal line joining the two boxes represents a region without significant homology. The linker region (L), a 110-amino acid region proximal to the CTD, and the CTD (374 amino acids) of POLR2A are also illustrated. B, a schematic of POLR3A, POLR3A-CTD, and POLR3A-LCTD recombinant proteins with carboxyl-terminal V5 and His tags. C, Western blot analysis was performed after transfection of increasing amounts of POLR3A, POLR3A-CTD, and POLR3A-LCTD protein expression plasmids in 293T cells. The blot was probed for PTB as a loading control and anti-V5 antibody to detect increasing amounts of recombinant protein expression. D, Western blot analysis of protein lysates made from 293T cells transfected with either empty vector, POLR3A, or POLR3A-CTD expression plasmids. The blot was probed with anti-hRPC155, an antibody directed against human POLR3A. The sizes of the endogenous POLR3A and the recombinant forms of POLR3A and POLR3A-CTD are indicated. Note that all samples were run on the same gel and exposed for the same time; lanes have been juxtaposed for esthetic purposes. E, Western blot analysis of protein lysates made from 293T cells transfected with protein expression plasmids treated with calf intestinal phosphatase (CIP) as described under “Experimental Procedures.” The blot was probed with anti-V5 antibody to detect recombinant protein expression.
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Cap Binding Assay—GST-eIF4E was expressed in BL21 cells, and the protein was purified using 7-methyl GTP-Sepharose beads (Amersham Biosciences) as described previously (39). Sixty micrograms of purified GST-eIF4E was bound to a packed volume of 25 μl of glutathione beads by end-over-end incubation for ~6 h at 4 °C. The beads were washed a total of 3 times 10 min each with TST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20), and then further purified over a G25 column (Amersham Biosciences). The primer was hybridized to 5.0 μl of glutathione beads by end-over-end incubation at 4 °C for 6 h at 4 °C. The beads were washed a total of 3 times 10 min each with TST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20). RNA was fractionated over the cap binding matrix essentially as described previously (43).

Primer Extension—An oligonucleotide complementary to sequence within exon 3 of the chicken β-tubulin reporter 5Sβ3 PE5 (5’-CACCAGGTCCATGGT-3’) was 5’ end-labeled using T4 polynucleotide kinase (New England Biolabs) and γ-[32P]ATP (PerkinElmer Life Sciences) for 2 h at 37 °C. The labeled primer was purified by phenol/chloroform extraction and ethanol precipitation using glycogen as a carrier. Samples were washed in 80% ethanol, dried, and resuspended in formamide dye. Before loading onto the gel, reactions were denatured by heating to 100 °C for 3 min. Products were resolved on 6% polyacrylamide, 8 M urea gels at constant power. Gels were exposed to PhosphorImager screens and were analyzed using Image Quant software (GE Healthcare).

RNAi Experiments—Two small interfering RNA (siRNA) duplexes complementary to sequences within the 3’-untranslated region of POLR3A were ordered (Dharmacon). The duplexes targeted the following sequences: #1 (5’-CUG-AUGUGAUGUGGAUUA-3’) and #2 (5’-CAAGAAGACAGCAGAUGUA-3’). The nonspecific siRNA duplex C2 described in Wagner and Garcia-Blanco (44) served as a control. All duplexes were resuspended to a concentration of 20 μM. RNA interference experiments in 293T cells were performed as follows. Cells were seeded at a density of 1.5 × 10^5 cells per 10 cm² well (BD Biosciences). The following day cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions at final siRNA concentrations of 20 or 50 nM in culture. At 36 h post-siRNA transfection, cells were transiently transfected with POLR3A expression plasmids and the 5Sβ3 reporters described above. Cells were grown for an additional 36 h post-transfection. At this time cells were harvested for RNA using Trizol (Invitrogen) and for protein using SDS lysis.

Immunofluorescence Staining—Immunofluorescence was performed on fixed, permeabilized 293T cells. Cells were grown on collagen-coated coverslips (BD Biosciences) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 48 h. All subsequent steps were performed at room temperature unless otherwise indicated. Cells were fixed with 3.7% formaldehyde in PBS buffer, pH 7.4, for 15 min, incubated with 20 mM NH₄Cl for 15 min, and treated with 100% methanol for 10 min at −20 °C. Cells were then washed with PBS and blocked in PBS containing 1% normal donkey serum (Chemicon) and 0.1% (v/v) Tween 20 for 30 min. Cells were incubated...
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POLR3A Recombinant Proteins Were Expressed and Phosphorylated in Vivo—To determine whether the CTD was sufficient to enhance pre-mRNA processing efficiency, we constructed fusions of the largest subunit of RNAP III, POLR3A. POLR3A and POLR2A are highly homologous (Fig. 1A), but the latter has an additional 580-amino acid extension in the carboxyl terminus which includes the 52 repeats known as the CTD (9, 10). We constructed cDNAs capable of expressing POLR3A, POLR3A-CTD, which had the CTD appended to the carboxyl terminus of POLR3A, and POLR3A-LCTD, which had the 110-amino acid linker region upstream of the CTD (L) and the CTD appended to the carboxyl terminus of POLR3A (Fig. 1B).

We wanted to verify that the POLR3A recombinant proteins could be expressed in vivo. We transiently transfected our constructs into 293T cells and analyzed protein production via immunoblotting for the V5 tag (Fig. 1C). The results demonstrated that all three recombinant proteins expressed in 293T cells. The recombinant POLR3A accumulated to levels similar to those of the endogenous POLR3A (Fig. 1D and supplemental Fig. 1A).

The POLR3A-CTD and the POLR3A-LCTD fusions migrated as two bands. We speculated that the slower migrating bands might be recombinant proteins with hyperphosphorylated CTDs. Indeed, treatment of our protein samples with calf intestinal phosphatase led to the disappearance of the slower migrating bands and to an increase in the faster migrating species (Fig. 1E). We concluded that not only are

with primary antibodies at appropriate dilutions in PBS containing 1% normal donkey serum and 0.1% (v/v) Tween 20 for 1 h and subsequently washed with PBS and refixed with formaldehyde. Cell nuclei were identified by staining with Hoechst stain at 1:5000 dilution for 5 min. The coverslips were then mounted onto glass slides using gel/mount media (Biomeda). T7 RNA polymerase monoclonal antibody (Novagen) and anti-V5 antibody were used as the primary antibodies and were labeled with Zenon Alexa Fluor 488 or Hoechst stain at 1:5000 dilution for 5 min. The coverslips were then mounted onto glass slides using gel/mount media and the endogenous CTD of RNAP II, the CTD of POLR3A-CTD and POLR3A-LCTD was extensively phosphorylated. In addition, we determined the localization of the POLR3A recombinant proteins by immunofluorescence and observed that all three recombinant proteins exhibited both nuclear and cytoplasmic localization (supplemental Fig. 2).

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scripts derived from cryptic RNAP II promoters. In agreement with previous studies, we did not detect spliced product when the 5S+1 reporter was co-transfected with empty vector or with the POLR3A recombinant protein-producing vector (Fig. 2B). When the 5S+1 reporter was co-transfected with either the POLR3A-CTD or POLR3A-LCTD constructs, we did not detect an increase in spliced product compared with transcripts synthesized by the recombinant POLR3A. Overexpression of any of the POLR3A constructs led to higher expression of the 5Sβ3 transcripts over background (Fig. 2B).

Because strong polyadenylation sites have been demonstrated to promote splicing of 3’-terminal exons (46), we designed our reporters with and without polyadenylation signals to determine whether the cross-talk between polyadenylation and splicing factors would affect the splicing of 5Sβ3 transcripts. The reporters with and without polyadenylation signals are designated 5S+1+pA and 5S+1ΔpA, respectively. We observed that the presence of a strong polyadenylation signal did not enhance splicing efficiency (Fig. 2B).

To decrease the activity of endogenous RNAP III, we lowered levels of endogenous POLR3A using two siRNAs directed at the mRNA encoding this protein (#1 and #2 in supplemental Fig. 1A). Under conditions where we lowered levels of endogenous POLR3A by at least 2-fold, we obtained the same results described above (supplemental Fig. 1B). Together, these results indicate that the CTD in the context of RNAP III was not sufficient to enhance pre-mRNA splicing of the 5Sβ3 transcripts.

The CTD in the Context of RNAP III Was Not Sufficient to Enhance the Efficiency of Pre-mRNA Capping—In addition to examining the ability of POLR3A-CTD and POLR3A-LCTD to support splicing, we tested the ability of the fusion proteins to facilitate co-transcriptional capping of the 5Sβ3 transcripts. In this assay purified GST-elf4E was immobilized on glutathione-agarose beads to create a cap binding matrix (39, 43). To control for column specificity and function, RNase protection probes specific for human β-tubulin and 5S rRNA were generated. Human β-tubulin transcripts are synthesized by endogenous RNAP II and are expected to be capped, whereas 5S rRNAs are synthesized by endogenous RNAP III and are predicted to be uncapped. Our RNase protection results indicate that the β-tubulin transcripts were enriched in the bound fraction, and the 5S rRNAs were located exclusively in the free fraction (Fig. 3A), confirming that the cap binding matrix was functioning properly. We also had an additional internal control labeled CMV/5S+1 (Fig. 3, A and B). These cells were transfected with a 5Sβ3 reporter constructed with a CMV promoter upstream of the 5S promoter. This reporter can be transcribed by both endogenous RNAP II and RNAP III, and the origin of the transcripts can be deduced from the length of the primer extension product. Our primer extension results indicated that the CMV/5S+1 reporter was indeed transcribed by both endogenous polymerases (Fig. 3B, lanes 7–9). This internal control demonstrates that when transcribed by RNAP II, the 5Sβ3 transcripts were mostly uncapped and found in the free fraction. In contrast, when transcribed by RNAP II, these transcripts were enriched in the bound fraction, indicating they were capped.

Having confirmed that the cap binding matrix functioned properly, RNA isolated from cells co-transfected with the POLR3A recombinant proteins and the 5Sβ3 reporters was fractionated over the cap binding matrix and analyzed by primer extension. The data show that there was no increase in capped 5Sβ3 transcripts in cells expressing POLR3A-CTD or POLR3A-LCTD (Fig. 3B). We concluded that the CTD was not sufficient to enhance pre-mRNA capping efficiency in the context of RNAP III.

The CTD in the Context of T7 RNAP Was Not Sufficient to Enhance Pre-mRNA Splicing Efficiency In Vitro—Previously, we reported on the development of an in vitro system that could be used to analyze coupled transcription splicing of both constitutively and alternatively spliced transcripts (37, 47). Using this system we observed that transcripts synthesized by T7 RNAP in HeLa nuclear extract, although efficiently transcribed, exhibited very low splicing efficiency compared with those transcripts synthesized by the endogenous RNAP II. We, therefore, anticipated that if any enhancement of splicing occurred as a result of the CTD in
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To assess whether the CTD was sufficient to enhance pre-mRNA splicing efficiency in the context of T7 RNAP, we first expressed and purified T7 RNAP-CTD (T7CTD) and T7 RNAP (T7) fusion proteins (40) (Fig. 4A). Levels of T7CTD and T7 were evaluated by immunoblotting with anti-His antibody (Fig. 4B). The RNA synthesis activities of T7 and T7CTD were indistinguishable (data not shown).

Incubation of the T7CTD fusion protein with HeLa nuclear extract and ATP caused a shift in mobility, which we attributed to CTD phosphorylation (Fig. 4C). This was verified by immunoblotting with H14, an antibody specific for Ser5 phosphorylation of the CTD (data not shown). Mock-phosphorylated and phosphorylated preparations of T7CTD and T7 were made by incubating the purified proteins with HeLa nuclear extract in the absence or presence of ATP, respectively. The mock-phosphorylated and phosphorylated T7CTD and T7 proteins were subsequently re-purified using nickel affinity chromatography and used as described below.

We performed *in vitro* transcription splicing assays using both mock-phosphorylated and phosphorylated protein preparations of T7CTD and T7. The human β-globin DNA templates fused to either the T7 promoter or the cytomegalovirus promoter used in this experiment are illustrated in Fig. 5A. We used reverse RNase protection to analyze our results. This detection method allowed us to quantify both partially spliced transcripts (11100012) as well as fully spliced transcripts (11100012/11100013). The positive control, RNAP II synthesized β-globin pre-mRNA (Fig. 5B, lane 1) was spliced efficiently (~13% for 11100012 spliced product and ~4% 11100012/11100013 spliced product). In contrast, β-globin pre-mRNA synthesized by either T7CTD or T7 was not spliced (Fig. 5B, lanes 2–13). Moreover, the phosphorylation state of T7CTD did not impact its effect on splicing. We concluded

FIGURE 5. The CTD in the context of T7 RNAP is not sufficient to enhance pre-mRNA splicing efficiency when analyzed using an *in vitro* transcription splicing assay. A, schematic diagram of the human β-globin constructs used for *in vitro* transcription splicing assays. Boxes represent exons, and horizontal lines connecting the boxes indicate introns. In addition, the arrows immediately after either the T7 promoter (pT7) or the cytomegalovirus promoter (pCMV) indicate the transcriptional start sites. B, RPA analysis of products from *in vitro* transcription splicing assays. *In vitro* transcription splicing assays were performed over a range of enzyme concentrations using mock-phosphorylated and phosphorylated forms of purified T7 and T7CTD. Mock-phosphorylated and phosphorylated forms of T7 and T7CTD are labeled mP and P, respectively, at the top. The ladder and sizes in bp are indicated at the left. In addition, specific size controls for β-globin labeled 1 + 2 for a partially spliced product consisting of exons 1 and 2 and 1 + 2 + 3 for a fully spliced product consisting of exons 1, 2, and 3 are also shown. Lane 1 serves as a positive control for splicing. The expected products from the RPA are indicated at the right and include both unspliced and spliced products.
from this that the CTD in the context of T7 RNAP, regardless of CTD phosphorylation, was not sufficient to enhance pre-mRNA splicing efficiency in vitro in a coupled transcription-splicing assay.

The CTD in the Context of T7 RNAP Was Not Sufficient to Enhance Pre-mRNA Splicing Efficiency in Vivo—To analyze the ability of the T7CTD fusion protein to enhance pre-mRNA splicing efficiency in vivo, we generated amino-terminal His/Xpress-tagged versions of T7CTD and T7 (Fig. 6A). We expressed these recombinant proteins in 293T cells and evaluated their expression by immunoblotting with an anti-His antibody (Fig. 6B). We also performed immunoblotting with a monoclonal antibody to T7 RNA polymerase (Novagen) and found that this antibody was unable to detect the slower migrating species of T7CTD in vivo (data not shown). Importantly, we noted that T7CTD was expressed at lower levels compared with T7, and accordingly, we adjusted the levels of transfected plasmid to equalize the levels of protein expression. We also tested whether T7CTD and T7 were phosphorylated in vivo by incubating protein lysates from transfected cells in the absence or presence of calf intestinal phosphatase. Our data indicate that T7CTD was phosphorylated in vivo as indicated by a mobility shift upon treatment with phosphatase (Fig. 6B). We also confirmed the nuclear localization of T7CTD and T7 by immunofluorescence (supplemental Fig. 3).

Having confirmed the expression, phosphorylation, and nuclear localization of T7CTD and T7, we were able to address the question of whether the CTD in the context of T7 RNAP was sufficient to enhance pre-mRNA splicing efficiency in vivo. First, we examined the splicing of human β-globin transcripts. The probe for the RPA was designed to detect sequences spanning from intron 2 into exon 3, which allowed for the analysis of both unspliced and spliced β-globin transcripts. The timeline of the experiment is shown in supplemental Fig. 4A. In agreement with our in vitro data, we observed efficient splicing of the β-globin reporter synthesized by endogenous RNAP II but observed no detectable splicing of the β-globin reporters transcribed by either T7CTD or T7 (supplemental Fig. 4B). These results corroborate our in vitro findings that indicate that the CTD in the context of T7 RNAP was not sufficient to enhance splicing efficiency.

We further investigated the function of T7CTD by examining its ability to enhance the pre-mRNA splicing efficiency of the minigene reporter, pI12-IIIc, which was used to study the alternative splicing of fibroblast growth factor receptor 2 transcripts (Fig. 7A). This minigene recapitulates the alternative splicing of exon IIIc in 293T cells, which efficiently include exon IIIc (41). The timeline of the experiment is shown in Fig. 7B. As a positive control for splicing, pI12-IIIc was transfected into cells in the absence of α-amanitin. The RPA data show that, as expected, the pI12-IIIc reporter, when transcribed by endogenous RNAP II, accumulates as spliced product (Fig. 7C, lanes 1–3). The pI12-IIIc reporter was also transfected in the presence of α-amanitin, which served as our negative control (Fig. 7C, lanes 4–6). When T7CTD and T7 were expressed in the presence of α-amanitin and the pI12-IIIc reporter, we observed mostly unspliced transcripts, although a low level of spliced product was also observed (Fig. 7C, lanes 7–18). These data indicate that the CTD in the context of T7 RNAP was unable to enhance the overall splicing efficiency of exon D in the pI12-IIIc minigene in vivo.

In addition, we wanted to investigate the splicing pattern of the low level of transcripts that were spliced by T7CTD and T7 and whether transcripts synthesized by T7CTD displayed differences in splicing patterns compared with those synthesized by T7. We transfected pI12-IIIc into 293T cells, isolated RNA, and performed RT-PCR. The RT-PCR analysis allowed us to examine IIIc inclusion and skipping products. When pI12-IIIc was transfected in the absence of α-amanitin and transcribed by endogenous RNAP II, we observed mostly IIIc inclusion (Fig. 7D, lanes 1–3). In contrast, when transcripts were synthesized by either T7 or T7CTD, the majority of the observed products did not include exon IIIc (Fig. 7D, lanes 7–18). Our data indicate that the CTD did not enhance overall splicing efficiency nor did it impact alternative splicing decisions in the context of T7 RNAP.

The CTD in the Context of T7 RNAP Was Not Sufficient to Enhance Pre-mRNA Capping Efficiency in Vivo—Upon determining that the CTD in the context of T7 RNAP did not...
have an effect on pre-mRNA splicing efficiency, we tested whether or not it had an effect on pre-mRNA capping efficiency. We performed the cap binding experiment as described above and again confirmed that the cap binding matrix was functioning properly (Fig. 8A). We performed RPA using a probe specific for the pI12-IIIc minigene and observed an enrichment of spliced transcripts synthesized by endogenous RNAP II in the bound fraction (Fig. 8B, lanes 1–3), whereas transcripts synthesized by T7 and T7CTD remain largely unspliced and are almost entirely uncapped (Fig. 8B, lanes 7–18). Our results demonstrate that the CTD in the context of T7 RNAP did not enhance pre-mRNA capping efficiency. Furthermore, the CTD in the context of T7 RNAP did not appear to affect splicing decisions indicated by the similar splicing patterns of the small percentage of transcripts synthesized by T7 and T7CTD that are capped (Fig. 8C). We conclude from these experiments that the CTD in the context of T7 RNAP was not sufficient to enhance pre-mRNA capping efficiency.

DISCUSSION

In this study we sought to determine whether the CTD of the largest subunit of RNAP II was sufficient to enhance the efficiency of pre-mRNA capping or splicing in the context of two distinct polymerases: mammalian RNAP III and bacteriophage T7 RNAP. The data presented here from both in vitro and in vivo assays revealed that the CTD in the context of either RNAP III or T7 RNAP was not sufficient to enhance pre-mRNA splicing efficiency, to alter splicing decisions, or to enhance pre-mRNA capping efficiency. Combined, these data suggest that the coupling of transcription to pre-mRNA processing is not solely coordinated by the presence of the CTD but requires other RNAP II subunits and/or associated factors. These additional subunits and factors could be recruited by specific cis-acting elements found in the RNAP II promoter (e.g. the binding site of TFIIH). Alternatively, the chimeric constructs used here may not...
reconstitute elongation and termination complexes properly as has been seen for RNAP III transcripts that can be polyadenylated only if an appropriate polyadenylation signal and RNAP III terminator are present (45).

One possible explanation for our results is that a RNAP II-specific promoter may be required for efficient processing. For instance, RNAP II promoters recruit the RNAP II-specific general transcription factor, TFIIF, which assembles at the promoter as part of the transcription preinitiation complex (48). The kinase activity of TFIIF phosphorylates the Ser\textsuperscript{5} residues of the CTD (18, 25), and the Ser\textsuperscript{5} phosphorylated form of the CTD binds to capping enzyme permitting efficient co-transcriptional capping of RNAP II nascent transcripts (15, 16, 28).

Moreover, it is possible that the CTD is not sufficient to couple transcription to pre-mRNA processing because other subunits of RNAP II are required to recruit factors necessary to enhance co-transcriptional pre-mRNA processing. Intriguing experiments performed by Buratowski and co-workers (49) have shown that in yeast strains depleted of the RNAP II subunit, Rpb4, co-transcriptional 3’/5’ end processing is impaired. Another subunit of RNAP II, Rpb7, which interacts with the emerging nascent RNA (50), has been shown to bind directly to Seb1, a protein predicted to be involved with 3’ end formation (51).

The data presented here suggest that the CTD is not sufficient for pre-mRNA capping and splicing. This tentative conclusion does not fit well with prevailing models, which depict the CTD as determining the processing fate of pre-mRNAs. Our results strongly suggest that properties specific to RNAP II transcription are required for CTD-mediated functional coupling of transcription to pre-mRNA processing and underscore the need for further characterization of the mechanisms connecting these processes.

**FIGURE 8.** The CTD in the context of T7 RNAP is not sufficient to enhance pre-mRNA capping efficiency. A, RPA analysis of total cellular RNA isolated from transfected 293T cells fractionated over a cap binding matrix as described under “Experimental Procedures.” 293T cells were transfected with empty vector (Mock), T7, or T7CTD protein expression plasmids as indicated at the top. All samples were transfected with the pI12-IIIc minigene reporter. Input (I), free (F), and bound (B) fractions were analyzed by RPA. Lanes corresponding to total RNA isolated from cells treated in the absence/presence of α-amanitin are labeled −/+/α-am. The ladder and sizes in bp are indicated to the left. The lane labeled RNA+Rnase serves as the negative control for the RPA. Expected sizes of protection products of endogenous β-tubulin and endogenous 5 S rRNA are indicated to the right. B, RPA analysis of the same fractions as in A. The ladder and sizes in bp are indicated to the left. The expected sizes of unspliced and spliced protected products are indicated to the right. C, RT-PCR analysis of the same fractions as in A. Lanes labeled with −RT were performed without reverse transcriptase and served as negative controls. The expected products are indicated to the right. U and D are the upstream and downstream exons of pI12-IIIc transcripts.
The CTD Is Not Sufficient for Pre-mRNA Capping or Splicing

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REFERENCES

1. Maniatis, T., and Reed, R. (2002) Nature 416, 499–506
2. Neugebauer, K. M. (2002) J. Cell Sci. 115, 3865–3871
3. Pandit, S., Wang, D., and Fu, X. D. (2008) Curr. Opin. Cell Biol. 20, 260–265
4. Kornblith, A. R., de la Mata, M., Fededa, J. P., Munoz, M. J., and Nogues, G. (2004) RNA 10, 1489–1498
5. Proudfoot, N. J., Forger, A., and Dye, M. J. (2002) Cell 108, 501–512
6. Bentley, D. L. (2005) Curr. Opin. Cell Biol. 17, 251–256
7. Goldstrohn, A. C., Greenleaf, A. L., and Garcia-Blanco, M. A. (2001) Gene (Amst.) 277, 31–47
8. Hirose, Y., and Manley, J. L. (2000) Genes Dev. 14, 1415–1429
9. Corden, J. L. (1990) Trends Biochem. Sci. 15, 383–387
10. Allison, L. A., Moyle, M., Shales, M., and Ingles, C. J. (1985) Cell 42, 599–610
11. Egloff, S., and Murphy, S. (2008) Trends Genet. 24, 280–288
12. Phatnani, H. P., and Greenleaf, A. L. (2006) Genes Dev. 20, 2922–2936
13. Chapman, R. D., Heidemann, M., Hintermair, C., and Eick, D. (2008) Trends Genet. 24, 289–296
14. Buratowski, S. (2003) Nat. Struct. Mol. Biol. 10, 679–680
15. Komarnitsky, P., Cho, E. J., and Buratowski, S. (2000) Genes Dev. 14, 2452–2460
16. Schroeder, S. C., Schwer, B., Shuman, S., and Bentley, D. (2000) Genes Dev. 14, 2435–2440
17. Saunders, A., Core, L. J., and Lis, J. T. (2006) Nat. Rev. Mol. Cell Biol. 7, 557–567
18. Prelich, G. (2002) Eukaryot. Cell 1, 153–162
19. Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) J. Biol. Chem. 271, 27176–27183
20. Marshall, N. F., and Price, D. H. (1995) J. Biol. Chem. 270, 12332–12338
21. McCracken, S., Rosonina, E., Fong, N., Sikes, M., Beyer, A., O’Hare, K., Shuman, S., and Bentley, D. (1998) Cold Spring Harbor Symp. Quant. Biol. 63, 301–309
22. McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. (1997) Nature 385, 357–361
23. Bird, G., Zorio, D. A., and Bentley, D. L. (2004) Mol. Cell. Biol. 24, 8963–8969
24. Cho, E. J., Takagi, T., Moore, C. R., and Buratowski, S. (1997) Genes Dev. 11, 3319–3326
25. Palancade, B., and Bensaude, O. (2003) Eur. J. Biochem. 270, 3859–3870
26. Pei, Y., Hausmann, S., Ho, C. K., Schwer, B., and Shuman, S. (2001) J. Biol. Chem. 276, 28075–28082
27. Rodriguez, C. R., Cho, E. J., Keogh, M. C., Moore, C. L., Greenleaf, A. L., and Buratowski, S. (2000) Mol. Cell. Biol. 20, 104–112
28. Fabrega, C., Shen, V., Shuman, S., and Lima, C. D. (2003) Mol. Cell 11, 1549–1561
29. Ho, C. K., and Shuman, S. (1999) Mol. Cell 3, 405–411
30. Misteli, T., and Spector, D. L. (1999) Mol. Cell 3, 697–705
31. Rosonina, E., Ip, J. Y., Calarco, J. A., Bakowski, M. A., Emili, A., McCracken, S., Tucker, P., Ingles, C. J., and Blencowe, B. J. (2005) Mol. Cell. Biol. 25, 6734–6746
32. Emili, A., Shales, M., McCracken, S., Xie, W., Tucker, P. W., Kobayashi, R., Blencowe, B. J., and Ingles, C. J. (2002) RNA 8, 1102–1111
33. Morris, D. P., and Greenleaf, A. L. (2000) J. Biol. Chem. 275, 39935–39943
34. Carty, S. M., Goldstrohn, A. C., Sune, C., Garcia-Blanco, M. A., and Greenleaf, A. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9015–9020
35. de la Mata, M., and Kornblith, A. R. (2006) Nat. Struct. Mol. Biol. 13, 973–980
36. Zeng, C., and Berget, S. M. (2000) Mol. Cell. Biol. 20, 8290–8301
37. Natalicio, B. J., and Garcia-Blanco, M. A. (2005) Methods 37, 314–322
38. Siosid, S. S., Sollier-Webb, B., and Cleveland, D. W. (1987) Mol. Cell. Biol. 7, 3602–3612
39. Edery, I., Chu, L. L., Sonenberg, N., and Pelletier, J. (1995) Mol. Cell. Biol. 15, 3363–3371
40. Kaneko, S., Chu, C., Shatkin, A. J., and Manley, J. L. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 17620–17625
41. Wagner, E. J., Curtis, M. L., Robson, N. D., Baranik, A. P., Eis, P. S., and Garcia-Blanco, M. A. (2003) RNA 9, 1552–1561
42. Sepehri, S., and Hernandez, N. (1997) Genome Res. 7, 1006–1019
43. McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Broders, G., Sidirovski, D., Hessel, A., Foster, S., Shuman, S., and Bentley, D. L. (1997) Genes Dev. 11, 3306–3318
44. Wagner, E. J., and Garcia-Blanco, M. A. (2002) Mol. Cell 10, 943–949
45. Borodulina, O. R., and Kramarov, D. A. (2008) RNA 14, 1865–1873
46. Niwa, M., and Berget, S. M. (1991) Genes Dev. 5, 2086–2095
47. Ghosh, S., and Garcia-Blanco, M. A. (2000) RNA 6, 1325–1334
48. Woychik, N. A., and Hampsey, M. (2002) Cell 108, 453–463
49. Chao, E. J., Podolny, V., and Buratowski, S. (2008) Mol. Cell. Biol. 28, 1883–1891
50. Ujvari, A., and Luse, D. S. (2006) Nat. Struct. Mol. Biol. 13, 49–54
51. Mitsuwa, H., Kanda, E., and Ishihama, A. (2003) Nucleic Acids Res. 31, 4696–4701