

Molecular Architecture of the Human Pre-mRNA 3' Processing Complex

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SUMMARY

Pre-mRNA 3' end formation is an essential step in eukaryotic gene expression. Over half of human genes produce alternatively polyadenylated mRNAs, suggesting that regulated polyadenylation is an important mechanism for posttranscriptional gene control. Although a number of mammalian mRNA 3' processing factors have been identified, the full protein composition of the 3' processing machinery has not been determined, and its structure is unknown. Here we report the purification and subsequent proteomic and structural characterization of human mRNA 3' processing complexes. Remarkably, the purified 3' processing complex contains ~85 proteins, including known and new core 3' processing factors and over 50 proteins that may mediate crosstalk with other processes. Electron microscopic analyses show that the core 3' processing complex has a distinct “kidney” shape and is ~250 Å in length. Together, our data has revealed the complexity and molecular architecture of the pre-mRNA 3' processing complex.

INTRODUCTION

Polyadenylation is a nearly universal step in eukaryotic gene expression. Poly(A) tails have profound influence on the stability, export, and translation efficiency of mRNAs (Colgan and Manley, 1997; Zhao et al., 1999). In mammals, biochemical studies have shown that pre-mRNA 3' processing requires four multisubunit protein complexes—CPSF, CstF, CF I, and CF II—in addition to the single subunit poly(A) polymerase (PAP) (Takagaki et al., 1989; reviewed by Colgan and Manley, 1997; Mandel et al., 2008; Zhao et al., 1999). With the aid of RNA polymerase (RNAP) II (McCracken et al., 1997; Hirose and Manley, 1998), these and other factors assemble onto the nascent pre-mRNA to form a macromolecular complex in which the 3' processing reactions take place. Despite significant divergence in the *cis*-

elements required for 3' processing between yeast and mammalian mRNAs, most 3' processing factors are conserved. Interestingly, although 20 polyadenylation factors have been identified in yeast, only 15 have been found in mammals, suggesting either that the yeast machinery is more complex than its mammalian counterpart or that more mammalian 3' processing factors remain to be discovered.

Most of our knowledge of 3' processing has been based on biochemical studies of individual factors. In comparison, the molecular architecture and dynamics of the 3' processing complex remain poorly understood. First, it is not clear what proteins, in addition to the known 3' processing factors, constitute the functional 3' processing complex, and in what stoichiometry. Second, indirect evidence suggests that the 3' processing complex is dynamic and structural and/or compositional rearrangements may occur during the reactions. For example, although all 3' processing factors are required for cleavage, CPSF and PAP are enough to reconstitute specific polyadenylation on precleaved RNAs (Colgan and Manley, 1997; Zhao et al., 1999). Based on these observations, it is possible that CstF, CF I, and CF II dissociate from the 3' processing complex after cleavage. Finally, structural information is critical for understanding the detailed mechanisms of 3' processing. Currently, crystal structures are available for several individual 3' processing factors (Deo et al., 1999; Bard et al., 2000; Martin et al., 2000; Meinhart and Cramer, 2004; Mandel et al., 2006; Perez-Canadillas, 2006; Bai et al., 2007; Legrand et al., 2007; Noble et al., 2007; Qu et al., 2007; Coseno et al., 2008; Grant et al., 2008; Meinke et al., 2008), but the structure of the functional 3' processing complex is unknown. Studies of 3' processing complexes have been hampered by the lack of a method for purifying such complexes in their intact and functional form.

Accumulating evidence suggests that all steps of gene expression are highly coordinated (Hirose and Manley, 2000; Maniatis and Reed, 2002; Proudfoot et al., 2002; Bentley, 2005). For example, 3' processing is necessary both for transcription termination and for the export of mRNAs, although the exact mechanisms are not fully understood. The coupling of different steps in gene expression is often mediated by physical interactions among factors involved in seemingly distinct processes. It has been shown that the 3' processing factor

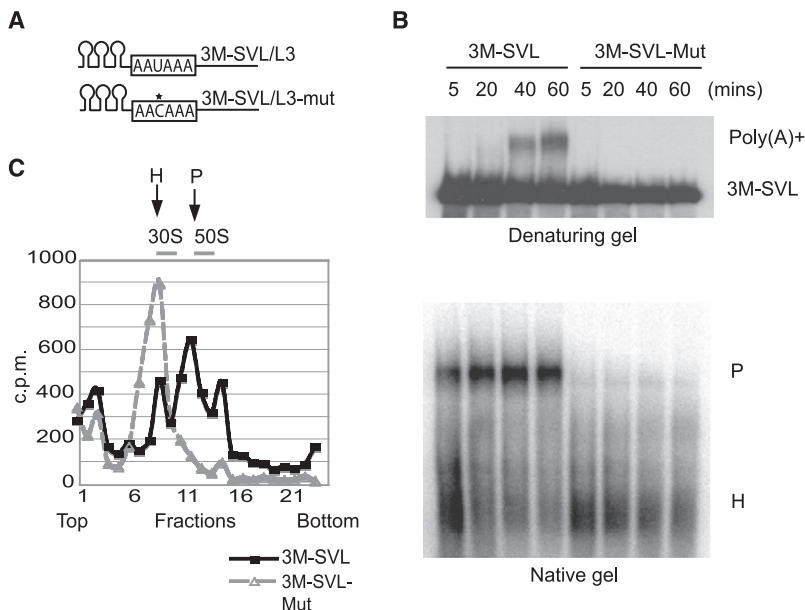


Figure 1. Characterization of RNA Substrates

(A) A schematic drawing of the RNA substrates. 3MS2 tags (hairpins) and the AAUAAA element in wild-type substrates and AACAAA in mutant substrates (boxes) are shown. The site of the single nucleotide change is marked with an asterisk.

(B) Comparison of the wild-type and mutant RNA substrates in polyadenylation and complex formation assays. A time course of polyadenylation reactions was analyzed on a denaturing 6% gel (top) or on a 1.5% native agarose gel (bottom) and visualized by phosphorimaging. (C) RNA profiles (measured by radioactivity) on glycerol gradients. The peaks corresponding to the 30S/H and 50S/P complexes and the peak positions of *E. coli* 30S and 50S ribosomes from a parallel gradient are marked.

nucleotide substitution completely abolished cleavage (Figure S1 available online) and polyadenylation (Figure 1B, upper panel). Native gel analyses showed that 3' processing complexes (P complexes) assembled efficiently on the wild-type RNAs, whereas the mutant RNAs were

found almost exclusively in faster-migrating heterogeneous complexes (H complex, Figure 1B, lower panel). Complexes assembled on the wild-type and mutant substrates were further analyzed by glycerol gradient sedimentation (Figure 1C). The RNA distribution profile along the gradient showed that the mutant RNAs were concentrated in a peak of ~30S that corresponds to the H complex. Although a small portion of wild-type RNAs was also present in the same peak, the majority was found in an ~50S peak that corresponds to the 3' processing complexes (see below).

To purify assembled 3' processing complexes, RNA substrates were first bound to the adaptor protein MBP-MS2 (MBP is maltose-binding protein) and then incubated with HeLa nuclear extract (NE) under polyadenylation conditions (with ATP) to allow assembly of the complexes. Reaction mixtures were then fractionated by glycerol gradient sedimentation as described above. The 30S and 50S fractions were then used for affinity purification with amylose beads. Analysis of the RNAs in the purified complexes showed that the mutant RNA was exclusively found in the 30S/H complexes (Figure 2A, bottom panel). Although some wild-type RNA was also detected in this peak, the majority of it was found in the 50S/P complexes (Figure 2A, top panel), consistent with the glycerol gradient profile (Figure 1C). Silver staining of the eluted complexes revealed that a large number of proteins were specifically purified with wild-type substrate in the 50S/P complexes (Figure 2B). The protein profile of the purified 50S/P complex was distinct from that of the purified 30S/H complex (Figure 2B, compare the left and right panel), suggesting that P complexes were effectively separated from the H complexes. Western blotting showed that all 3' processing factors tested, including CPSF73, CstF64, and symplekin, were specifically detected in the P complexes, but not in the H complexes or with mutant RNA substrates (Figure 2C). In contrast, hnRNP A1, a protein commonly found in H complexes, was highly enriched in the H complex assembled on the mutant substrate. We conclude

CPSF is associated with the transcription machinery as early as in the preinitiation complex through a direct interaction with TFIID (Dantoni et al., 1997), and both CPSF and CstF can remain associated with RNAP II throughout the coding region (Venkataraman et al., 2005; Glover-Cutter et al., 2008). In addition, interactions between CPSF and a component of the U2 snRNP, SF3b, are important for coupling between splicing and 3' processing (Kyburz et al., 2006). A comprehensive characterization of the crosstalk between 3' processing and other cellular processes will be important for better understanding gene regulation on a systems level.

In this study, we purified the human 3' processing complex and determined its protein composition. Remarkably, the purified complex contains ~85 proteins. In addition to known polyadenylation factors, we identified new essential 3' processing factors and over 50 proteins that may mediate coupling with other cellular processes. We also visualized the core 3' processing complex using electron microscopy for the first time and describe its basic features.

RESULTS AND DISCUSSION

Purification of Functional Human Pre-mRNA 3' Processing Complex

To purify the human pre-mRNA 3' processing complex, we adopted an RNA-tagging strategy used previously to purify spliceosomal complexes (Jurica et al., 2002; Zhou et al., 2002; Deckert et al., 2006). Briefly, SV40 late (SVL) and adenovirus L3 pre-mRNAs, two commonly used substrates for in vitro 3' processing analyses (Takagaki et al., 1988), were fused at their 5' ends to 3 copies of the hairpin that specifically binds to the bacteriophage coat protein MS2 (3M-SVL and 3M-L3, Figure 1A). As controls, we used mutant RNA substrates with single point mutations (U to C) in the highly conserved AAUAAA sequence (3M-SVL-mut and 3M-L3-mut, Figure 1A). In vitro 3' processing assays showed that, as expected, this single

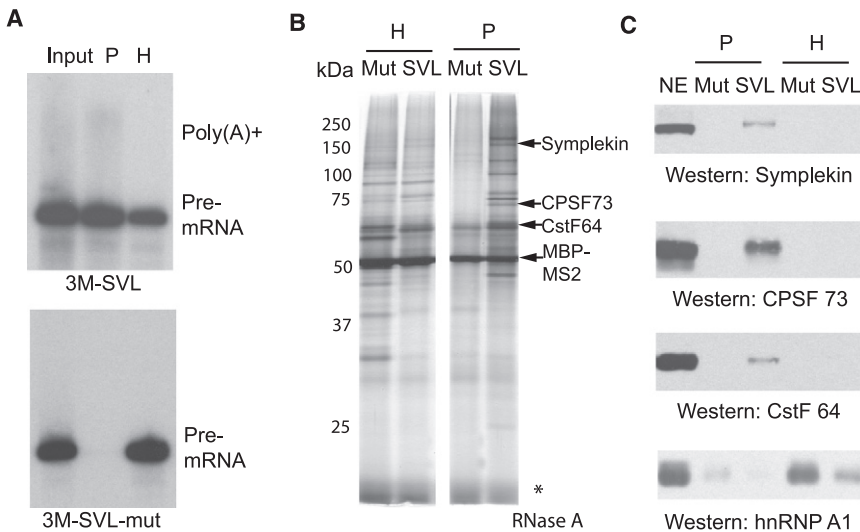


Figure 2. Purification of 3' Processing Complexes

(A) RNAs from input and purified 30S/H and 50S/P complexes were purified, resolved, and visualized as described above.

(B and C) Proteins from the purified 30S/H and 50S/P complexes were resolved by SDS-PAGE and analyzed by silver staining (MBP-MS2 and several western-confirmed bands are labeled) (B) and western blotting (C).

that we have successfully purified 3' processing complexes. It is important to point out that under the conditions used there was a significant time window (~20 min) during which the 3' processing machinery was fully assembled (Figure 1B, lower panel, from 20 min time point to 40 min), but no significant 3' processing had occurred (Figure 1B, top panel). Since we purified the 3' processing complexes from this time window, the vast majority of the purified complexes were in the precleavage stage.

We next wished to determine whether the purified 3' processing complexes were functional. To this end, we assembled 3' processing complexes on the 3M-SVL RNA as above. Following purification, the complexes were tested for cleavage activity (in the presence of 3' dATP, a polyadenylation inhibitor). No significant cleavage was observed with the purified complexes alone (Figure 3, lane 2), possibly indicating that one or more factors were missing or limiting. Indeed, proteomic analyses (see below) indicated that the CF II component Pcf11 was detected at substoichiometric levels and the other CF II subunit, Clp1, was completely missing. We therefore tested whether supplementing the purified complexes with CF components could allow cleavage to take place. To this end, we added partially purified cleavage factor complex (CF) that contains both CF I and II (Takagaki et al., 1989) to the purified 3' processing complexes, and indeed, cleavage products were now detected (Figure 3, lanes 3–5). Therefore, we conclude that the purified 3' processing complexes were functional in this complementation assay.

It is notable that other highly purified RNA processing complexes, such as the spliceosome, are also not active on their own but can be activated in complementation experiments analogous to the one described above (Jurica et al., 2002; Zhou et al., 2002; Deckert et al., 2006). Due to the dynamic nature of such complexes, certain factors may be missing at any given time point.

Proteomic Analyses of the Purified 3' Processing Complexes

For proteomic analyses, we purified 3' processing complexes assembled on the two aforementioned substrates, 3M-SVL

and 3M-L3. For comparison, we also purified the H complexes assembled on 3M-SVL-mut RNAs. The protein composition of each complex was determined using the Multidimensional Protein Identification Technology (MudPIT; see Link et al., 1999 and the Experimental Procedures).

Table 1 lists proteins found in either the SVL and L3 complexes, but only those found in both complexes were considered to be components of the 3' processing complex.

The purified 3' processing complexes contained ~85 proteins, including nearly all previously known 3' processing factors. The only exception was Clp1, a component of the CF II complex (de Vries et al., 2000), which plays an unknown role in 3' processing. Pcf11, another subunit of CF II, also seemed to be present at low levels, as only a small number of unique peptides from this protein were detected. These observations indicate that the association between CF II and the core 3' processing complex may be weak and/or transient. Interestingly, instead of the canonical PAP (Lingner et al., 1991; Raabe et al., 1991), the

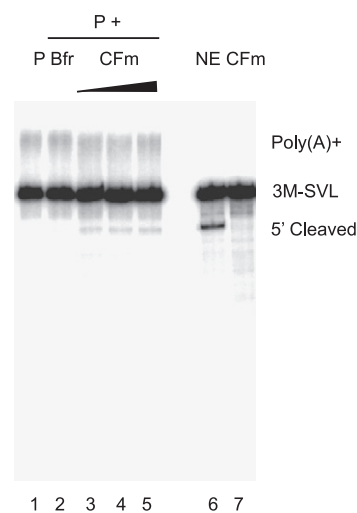


Figure 3. Purified 3' Processing Complexes Are Functional in a Complementation Assay

Purified 3M-SVL P complexes alone (P, lane 1), supplemented with buffer D (Bfr, lane 2) or increasing amounts of CF fraction (CF, lanes 3–5), were incubated under cleavage conditions. Standard cleavage assays with NE and CF are shown as controls.

related neo-PAP/PAPOLG (Kyriakopoulou et al., 2001; Topalian et al., 2001) was the sole PAP detected. The reason for this is unclear but is consistent with many studies indicating that PAP is not tightly associated with other processing factors (e.g., Takagaki et al., 1988) and may reflect a dynamic association between PAP and the core 3' processing complex and/or functional redundancy between the two poly(A) polymerases.

The complexes described above were assembled under polyadenylation conditions (with ATP). For comparison, we also purified 3' processing complexes assembled under cleavage conditions (in the presence of the polyadenylation inhibitor 3'-dATP and without ATP) and analyzed them by mass spectrometry (Figure S2 and Table S1). Again, most RNAs within the purified complexes were unprocessed (Figure S2), indicating the majority of the purified complexes were in the precleavage stage. Comparison between Table 1 and Table S1 shows that the protein compositions of the 3' processing complexes assembled under the two conditions were highly similar. For example, under both conditions, all subunits of CPSF, CstF, and CF I were identified, while CF II components were either detected only by a small number of unique peptides or entirely missing. In addition, many other factors, including WDR33, PP1, Rbbp6, and CstF64 tau (see below), were identified in both analyses. These observations suggest that the assembly conditions have very little effect on the general protein composition of the precleavage 3' processing complexes.

Characterization of New 3' Processing Factors

Three proteins identified in our study not previously implicated in mRNA 3' processing in mammals (WDR33, Rbbp6, and PP1) are known or putative homologs of yeast 3' processing factors. WDR33/WDC146 is a WD40 repeat-containing protein and is the putative mammalian homolog of the yeast 3' processing factor Pfs2 (Ohnacker et al., 2000). To characterize its potential functions in 3' processing, we first tested whether WDR33 interacts with the CPSF complex since Pfs2 binds strongly to Ysh1, the yeast homolog of CPSF73 (Ohnacker et al., 2000). To this end, we established a stable cell line expressing Flag-tagged CPSF73 and purified CPSF73 and associated proteins by immunoprecipitation (IP) (Figure 4A). We analyzed the CPSF73-containing complexes by mass spectrometry, and the identified proteins are listed in Table S2 next to those found in the 3' processing complexes. All the known subunits of the CPSF complex (CPSF-160, -100, -73 and -30, and Fip1) were identified. Although not detected in CPSF previously purified through multiple chromatographic steps (Bienroth et al., 1991; Murthy and Manley, 1992), symplekin was detected at close to stoichiometric levels, consistent with previous studies that it associates with CPSF as well as CstF (Takagaki and Manley, 2000). Strikingly, WDR33 was also present in the CPSF complex, as indicated by the large number of unique peptides detected (Figure S3 and Table S2) and confirmed by western blotting (Figure 4B). The WDR33 band partially overlapped the CPSF160 band on SDS-PAGE (Figure 4A), perhaps explaining in part why WDR33 previously escaped detection. Gel filtration analysis of the purified CPSF complex showed that WDR33 coeluted with CPSF (Figure 4C).

We next immunodepleted WDR33 from NE to determine whether WDR33 is required for 3' processing. Quantitative western analyses showed that ~95% of WDR33 was removed (Figure 4D). About two-thirds of CPSF73 was also codepleted, indicating that the majority of CPSF73 is associated with WDR33. CstF64 levels were reduced by about a third while the level of the phosphatase PP1 was not significantly affected. When WDR33-depleted NE was used in 3' processing assays, both cleavage (Figure 4E) and polyadenylation (Figure S4) were essentially abolished. Add-back of the immunopurified CPSF complex restored cleavage (Figure 4E). Together, these data indicate that WDR33, despite the fact that it had not been previously identified, is a bona fide component of the CPSF complex and suggest that it plays an essential role in mammalian 3' processing.

Pfs2 was initially suggested to be the yeast equivalent of mammalian CstF50, another WD40 repeat protein with which it shares limited similarity (Ohnacker et al., 2000). This was consistent with the fact that the yeast CstF equivalent, CF IA, lacks a CstF50 homolog (Zhao et al., 1999). However, our data now indicate that the human 3' processing machinery contains two WD40 proteins, one in CPSF and another in CstF. It is noteworthy that the plant PFS2/WDR33 homolog, FY, has been implicated as playing an important role during floral transition (Simpson et al., 2003).

Rbbp6/PACT is the putative homolog of the yeast 3' processing factor Mpe1 (Vo et al., 2001). Interestingly, Rbbp6 was originally identified as a p53- and Rb-binding protein, playing important roles in apoptosis, cell-cycle, and p53 regulation (Sakai et al., 1995; Simons et al., 1997). Rbbp6 is significantly larger than Mpe1 and contains additional domains, including an arginine/serine-rich (RS) domain that is found in many splicing factors and a RING-finger-related domain (Pugh et al., 2006). Although we have not directly tested its role in processing, it is likely that Rbbp6, like its yeast counterpart Mpe1, functions in 3' processing. It is possible that Rbbp6 may link mRNA 3' end formation to the Rb/p53 pathways and tumorigenesis.

Our results show that the serine/threonine phosphatase PP1 and its regulator PNUTS are components of the human 3' processing complex (Table 1). The PP1 homolog in yeast, Glc7, is a known 3' processing factor, and its phosphatase activity is specifically required for polyadenylation, but not for cleavage (He and Moore, 2005). To test if PP1 is involved in mammalian 3' processing, we depleted PP1 and the related PP2A family phosphatases using microcystin (MC)-conjugated beads (Figure 5A). MC is a specific small-molecule inhibitor of the PP1/2A phosphatases, and we have shown previously that MC-conjugated beads can be used to efficiently deplete these phosphatases from NE (Shi et al., 2006). When mock- or MC-treated NE were used in standard in vitro 3' processing assays, similar levels of cleaved products were observed (Figure 5B). Polyadenylation, however, was significantly reduced in MC-treated NE, and add-back of recombinant PP1 restored polyadenylation (Figure 5C), suggesting that PP1 is specifically required for polyadenylation. Therefore, dephosphorylation by PP1 is an evolutionarily conserved step in 3' processing.

We also found CstF64 tau in our purified 3' processing complex. CstF64 tau is highly homologous to CstF64 and

Table 1. Protein Composition of the Human Pre-mRNA 3' Processing Complex

Protein Name	Accession No.	Yeast homolog	Motifs/Notes on Functions	Cal. Mass	No. of peptides	
					L3	SVL
Known Polyadenylation Factors						
CPSF complex						
CPSF160 (CPSF1)	NP_037423	CFT1	SFT1	160822	80	72
CPSF100 (CPSF2)	NP_059133.1	CFT2	β-CASP	88487	59	52
CPSF73 (CPSF3)	NP_057291	YSH1	β-CASP	77486	42	22
CPSF30 (CPSF4)	NP_006684	YTH1	Zinc finger	30124	18	13
hFip1	NP_112179	FIP1		66526	23	19
CstF complex						
CstF77 (CSTF3)	NP_001317	RNA14	HAT	82922	60	54
CstF64 (CSTF2)	NP_001316	RNA15	RRM	60959	31	22
CstF50 (CSTF1)	NP_001315		WD repeats	48358	21	18
CF Im complex						
CF Im 68 (CPSF6)	NP_008938		RRM	59209	15	10
CF Im 59	NP_079087		RRM	52050	15	12
Cf Im 25 (CPSF5)	NP_008937			26227	26	21
CF IIm complex						
Pcf11	NP_056969.2	PCF11	CID	173050	5	3
Other Known Polyadenylation Factors						
Symplekin	NP_004810	PTA1		126500	51	39
Symplekin variant	BAE06092.1			118793	48	38
PAPOLG	NP_075045	PAP	RRM	82803	2	3
PABPC1	NP_002559.2	PAB1	RRM	70671	27	33
PABPC4	NP_003810.1		RRM	70783	20	23
PABPN1	NP_004634		RRM	31749	14	12
(Putative) Homologs of Yeast Polyadenylation Factors						
CstF64 tau (CSTF2T)	NP_056050.1	SPAC644.16 (<i>S. pombe</i>)	RRM	64436	21	16
WDR33	NP_060853	PFS2	WD repeats	145921	49	45
RBBP6	NP_008841	MPE1	RS, DWNN	201563	4	3
PP1 alpha	NP_002699.1	GLC7	Phosphatase	37512	2	7
PP1 beta	NP_002700	GLC7	Phosphatase	37187	2	5
DNA-Damage-Response Factors						
DNA-PK	NP_008835		Kinase	469093	21	15
Ku 70	NP_001460	YKU70	DNA helicase	69843	4	7
Ku 86/XRCC5	NP_066964	YKU86	DNA helicase	82705	6	13
PARP-1*	NP_001609			113135	5	
RNAP II and Associated Factors						
Pol II large subunit Rpb1	NP_000928.1	RPB1	Polymerase	217204	2	2
Pol II B*	NP_000929.1	RPB2		133896		2
Rpb5 (Pol II E)*	NP_002686.2	RPB5		24611	2	
Rpb11*	AF468111_1	RPB11		17208		2
Transcription factors						
TF II, I	NP_127492.1			112416	8	2
TAF15	AAH46099.1		RRM, zf-RanBP	52061	6	5
Integrator complex						
INTS2	NP_065799.1			134346	2	2
INTS3	NP_075391.3			118013	4	9
INTS4	NP_291025.3			108171	2	3

(Continued on next page)

Table 1. Continued

Protein Name	Accession No.	Yeast homolog	Motifs/Notes on Functions	Cal. Mass	No. of peptides	
					L3	SVL
Known Polyadenylation Factors						
INTS6	NP_036273.1			100390	2	3
INTS7	NP_056249.1			106834	2	2
INTS8*	NP_060334.2			113088		3
INTS9*	NP_060720.1		YSH1 homology	73815		4
INTS10*	NP_060612.2			82236		2
PAF complex						
Cdc73	AAH14351.2	CDC73		46675	2	2
FACT complex						
FACT complex large subunit	NP_009123	SPT16		119914	4	4
Splicing Factors						
p54nrb	NP_031389.3			54232	11	3
PSF	NP_005057.1			76150	10	4
PUF60	NP_510965.1		RRMs	59876	3	9
UAP56	NP_004631.1	SUB2	RNA Helicase	48991	12	9
SF1	NP_004621.2	BBP		68330	5	5
SF3A, subunit 1	NP_005868.1	PRP21	Surp	88886	9	15
SF3A, subunit 2	NP_009096.2	PRP11		49256	2	4
SF3A60	CAA57388	PRP9		58777	3	18
SF3B, subunit 1	NP_036565.2	HSH155		145830	16	34
SF3B, subunit 2	NP_006833.2	CUS1		100228	2	21
SF3B, subunit 4	NP_005841.1	HSH49		44386	3	5
SF3B, 14 kDa*	NP_057131.1	SNU17		14585		5
Prpf38b	NP_060531.1			64441	2	8
SRm300	NP_057417.2		RS	299676	13	15
U1 70K	NP_003080.2	SNP1	RRM	51557	7	7
U2AF65	NP_009210.1	MUD2	RRM	53501	6	11
U2AF35	NP_006749.1		RRM	27872	2	3
PRP19	NP_055317.1	PRP19	RING, WD	55181	5	3
Exosome						
SKIV2L2 (hMTR4)	NP_056175.2	MTR4	DEXD	117933	29	28
Translation Factors						
eEF1-alpha	NP_001393.1	TEF1/2	GTPase	50141	14	12
eEF1-gamma	NP_001395.1	TEF4	GST	50119	5	4
eIF2A	NP_114414.2	YGR054W		64990	4	4
eIF 4A	NP_001407.1	TIF1	Helicase	46154	12	6
eIF3 gamma	NP_003747.1		JAB/MPN	39930	5	6
eIF3S2	NP_003748.1	TIF34	WD	36502	2	2
eIF3S5	NP_003745.1		PCI	37564	3	2
eIF3S6	NP_001559.1			52221	2	4
eIF3S9 eta	NP_003742.2		RRM	92482	7	7
eIF3A	NP_003741.1	RPG1		166569	16	9
eIF4G1	NP_886553.2	TIF4631		175460	2	2
RACK1/GNB2L1/lung cancer oncogene 7	NP_006089.1	ASC1	WD	35077	5	5
Factors with Known Motifs						
RNA binding motif protein 7	NP_057174.1		RRM	30503	4	4

Table 1. Continued

Protein Name	Accession No.	Yeast homolog	Motifs/Notes on Functions	Cal. Mass	No. of peptides	
					L3	SVL
Known Polyadenylation Factors						
RNA binding motif protein 9/ Ataxin-binding protein/Fox-1	NP_055124		RRM	39515	2	7
RNA binding motif protein 25	NP_067062.1		RRM	100186	4	16
RNA binding motif protein 39, isoform a	NP_909122.1		RRM	59380	2	4
RNA binding motif protein 39, isoform b	NP_004893.1		RRM	58657	2	4
DEAH box polypeptide 3	NP_001347.3	DBP1	RNA helicase	7244	13	9
DEAH box polypeptide 5	NP_004387.1	DBP2	RNA helicase	69148	4	5
DEAH box polypeptide 9	NP_001348.2		RNA helicase	140958	32	3
DEAH box polypeptide 15	NP_001349.2	PRP43	RNA helicase	90933	3	17
DEAH box polypeptide 36	NP_065916.1		RNA helicase	114776	12	27
DEAH box polypeptide 39	NP_005795.2		RNA helicase	49130	8	5
DEAD box polypeptide 6	NP_004388.1	DHH1	RNA helicase	82432	3	2
DEAD box polypeptide 17 (p82)	NP_006377.2		RNA helicase	80273	6	4
Gemin3 (DEAD box polypeptide 20)	NP_009135.3		RNA helicase	92213	4	2
DEAD box polypeptide 23	NP_004809.2	PRP28 (<i>S. Pombe</i>)	RNA helicase	95583	4	5
ZCCHC8	NP_060082		Zinc finger	79375	15	16
EBNA2 coactivator, p100/SND1	NP_055205.2		Staphylococcal nuclease domain, Tudor	101997	12	2
Interferon-induced protein with tetratricopeptide repeats 1 isoform 2	NP_001539.3		TPR	55360	9	7
Interferon-induced protein with tetratricopeptide repeats 3	NP_001540		TPR	55985	8	5
Other Factors						
G3BP/Ras-GTPase-activating protein SH3-domain-binding protein	NP_005745.1		Endoribonuclease	52164	5	2
JUP protein/ gamma-catenin	NP_002221.1			81745	5	3
PNUTS (PPP1R10)	NP_002705.2		PP1 regulator	99058	6	9

Components of multi-subunit complexes that are present in only one purified complex are listed and are indicated with asterisks (*). Proteins that are abundant in H complexes were designated H complex components and not listed here.

reportedly found only in testis and not expressed in HeLa cells (Wallace et al., 1999). To determine whether CstF64 tau is a component of the CstF complex, we established a HEK293 cell line stably expressing Flag-tagged CstF77 and purified the CstF complex by IP (Figure S5A). Results of mass spectrometry analyses of purified CstF are listed in Table S2, and CstF64 tau was indeed detected (Figure S5B and Table S2). We suspect that CstF64 tau may be a general component of the CstF complex. An intriguing possibility is that CstF complexes may contain either CstF64 or CstF64 tau, and their functions may be partially redundant. This is consistent with earlier observations that an ~90% reduction in CstF64 levels had no significant effect on cell growth (Takagaki and Manley, 1998) and that CstF may function as a dimer in 3' processing (Bai et al., 2007).

Accumulating evidence suggests that all steps of gene expression are highly coordinated, and coupling of different steps is often mediated by physical interactions among factors involved in seemingly distinct processes (Hirose and Manley,

2000; Maniatis and Reed, 2002; Proudfoot et al., 2002; Bentley, 2005). Consistent with this theme, we identified in our purified 3' processing complex a large number of proteins that have known or putative functions in transcription and splicing, both of which are known to be connected to 3' processing. In fact, splicing factors detected in our complexes, such as SF3b, U2AF, and U1-70K, have been shown to associate with specific 3' processing factors and mediate crosstalk between splicing and polyadenylation (Gunderson et al., 1998; Vagner et al., 2000; Kyburz et al., 2006). We detected RNAP II in the purified 3' processing complex, consistent with earlier findings that RNAP II is necessary for efficient 3' cleavage in vitro (Hirose and Manley, 1998). In addition, we identified the PAF complex, a RNAP II-associated transcription elongation factor that was recently shown to function in 3' end formation of polyadenylated mRNAs in yeast (Penheiter et al., 2005). Most subunits of another RNAP II-associated complex, the Integrator, were also found in the 3' processing complexes. The Integrator complex was recently shown to

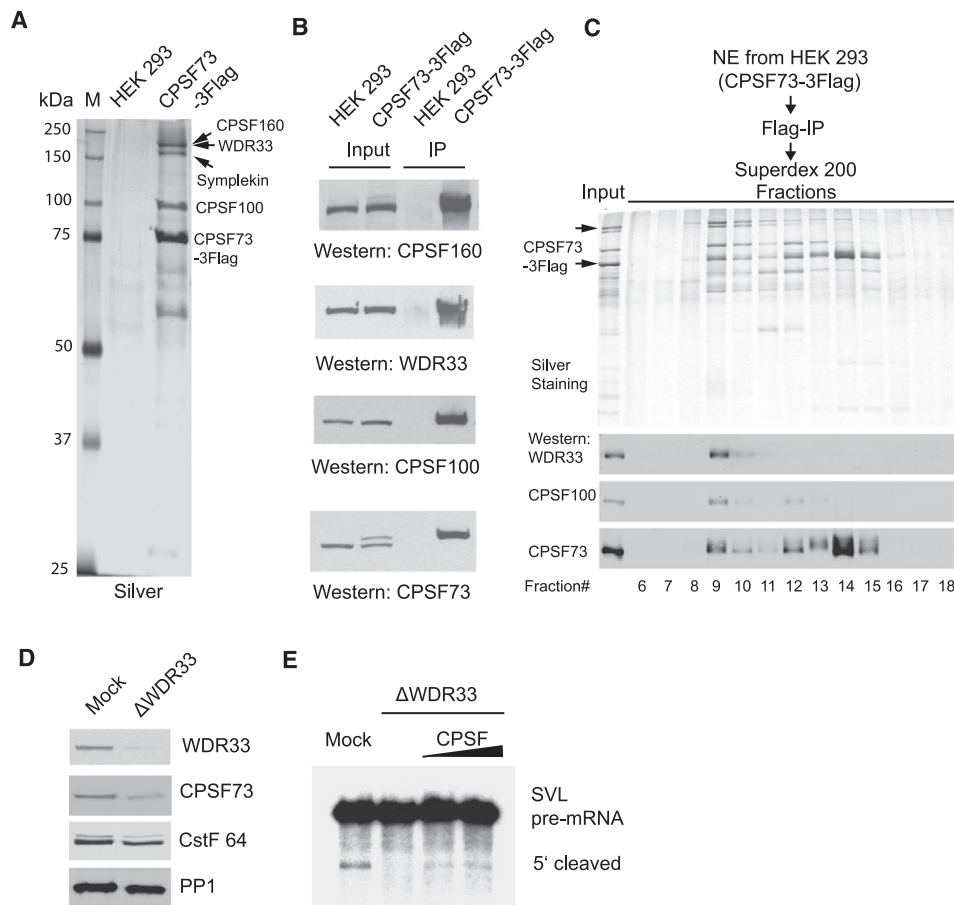


Figure 4. WDR33 Is a Bona Fide Component of the CPSF Complex

(A and B) Characterization of the immunopurified CPSF73 complex. Proteins from Flag-IP of NE from HEK293 or the CPSF73-3Flag cell line were resolved by SDS-PAGE and stained with silver (A) or analyzed by western blotting using specific antibodies (B). Protein bands in (A) whose identities were confirmed by western are labeled.

(C) Immunopurified CPSF complexes were analyzed by gel filtration using a Superdex-200 column. Proteins from input and each fraction were analyzed by SDS-PAGE and silver staining (top panel) and western (bottom panel). WDR33 is marked by an arrow.

(D) Western analyses of the mock-treated (mock) or WDR33-depleted (Δ WDR33) NE. Intensities of individual bands were quantified using the Odyssey Infrared scanner (Li-Cor).

(E) WDR33 is essential for 3' processing. Mock, Δ WDR33 NE alone, or Δ WDR33 supplemented with immunopurified CPSF were used in standard cleavage assays. RNAs were resolved and visualized as described above.

function in the 3' processing of snRNAs, and two of its subunits, Ints9 and Ints11, display sequence homology with CPSF 100 and 73, respectively (Baillat et al., 2005). The integrator subunits INTS8, -9, and -10 were missing in the L3 complex. The reason for their absence is unclear but may be due to slightly lower levels of the Integrator complex in the L3 complexes or the association between these subunits, and the rest of the complex might be weak and/or transient. It is currently unclear what, if any, role the Integrator might play in the 3' processing of pre-mRNAs.

Our study also identified a number of factors that may mediate unexpected connections between 3' processing and other cellular processes. For example, we found that the DNA-activated protein kinase complex (DNA-PKcs/Ku70/Ku86), well-studied for its functions in DNA-damage repair, is associated with the 3' processing complex (Table 1). This is potentially

similar to transcription where several DNA repair factors, such as XPB and XPD, are also essential transcription factors as components of the TFII H (Drapkin et al., 1994). These results are also consistent with previous studies showing that 3' processing is connected to DNA-damage response (Kleiman and Manley, 2001; Mirkin et al., 2008). Another intriguing factor associated with the 3' processing complex was the translation elongation factor and GTPase eEF1 alpha. Interestingly, Tef1, the yeast homolog of eEF1 alpha, copurifies with the yeast 3' processing factor CF I (Gross and Moore, 2001). It will be of interest to examine what, if any, roles these and other factors identified in our proteomic analyses play in 3' processing.

A comparison between our proteomic analyses of the 3' processing complexes and previously studies of the spliceosome (Jurica et al., 2002; Zhou et al., 2002; Deckert et al., 2006) revealed both similarities and differences. One common

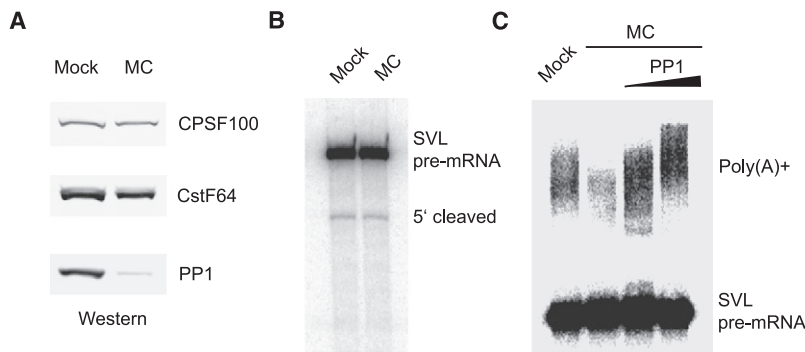


Figure 5. PP1 Is Required for Polyadenylation, but Not for Cleavage

(A) Western analyses of mock-treated (Mock) or microcystin-treated (MC) NE.

(B) Depletion of PP1/2A phosphatases has no effect on cleavage. Mock and MC NE were used in standard in vitro cleavage assays with SVL as the substrate. Pre-mRNA and the 5' cleaved products are marked.

(C) PP1 is required for polyadenylation. Mock, MC NE alone, or MC NE supplemented with recombinant PP1 were used in standard polyadenylation assays with SVL as the substrate. Pre-mRNA and poly(A)⁺ RNAs are marked.

there is that all of these studies strongly support a link between splicing and 3' processing. An interesting difference is that although the spliceosome includes a number of factors that have been implicated in transcription (e.g., TREX and TAT-SF1) (Zhou et al., 2002), the 3' processing complex contains RNAP II and RNAP II-associated factors, such as the Integrator and PAF (Table 1). Therefore, splicing and 3' processing are both connected to transcription but probably by different factors.

Structural Analyses of 3' Processing Complexes by EM

We next wished to begin to analyze the structure of the 3' processing complex. To this end, the isolated complexes were processed following the GraFix method (Kastner et al., 2008). Briefly, the 3' processing complexes purified as described above were subject to a second glycerol gradient sedimentation during which the complexes are centrifuged into increasing concentration of the fixation reagent glutaraldehyde. This step serves to further purify and gently fix the complexes to preserve their integrity and has been successfully used, for example, to process spliceosomes for electron microscopic (EM) analyses (Deckert et al., 2006; Behzadnia et al., 2007). During this second glycerol gradient sedimentation, the 3' processing complexes were found again in an ~50S peak (data not shown), indicating that structural integrity of the complex was preserved throughout the purification. The peak fraction was negatively stained with uranyl formate using the carbon-sandwich method (Radermacher et al., 1987) and analyzed by EM.

A typical raw image of the 3' processing complex shows monodisperse particles of similar sizes (Figure 6A). We also obtained tilted images of the particles to confirm that they were fully sandwiched between carbon membranes and that the staining was homogeneous (Figure S6). Images of representative particles show a distinct “kidney” shape, slightly elongated and bent (Figures 6B). There appears to be a central cavity surrounded by two or more peripheral densities. The maximum dimension of the complex is ~250 Å, which is consistent with its ~50S sedimentation coefficient.

3,671 molecular images were collected for image processing using both SPIDER (Frank, 1996) and EMAN (Ludtke et al., 1999). After reference-free alignment and classification, 50 (using SPIDER, shown in Figure S7) and 47 (using EMAN, shown in Figure S8) two-dimensional class averages were obtained. Although most of the class averages have defined edges and consistent sizes, they seem to lack strong internal features. This could potentially be due to heterogeneity among particles caused by the dynamic nature of this complex and/or the presence of substoichiometric factors. Another possible explanation is that classification of negative stain images focuses on the shape of the boundary so that small changes in orientation will not affect the class designation yet can result in changes of internal features seen in projection. As a result, internal features of class averages can be blurred. Nonetheless, our results have provided a first view of the polyadenylation complex and revealed its general structural features.

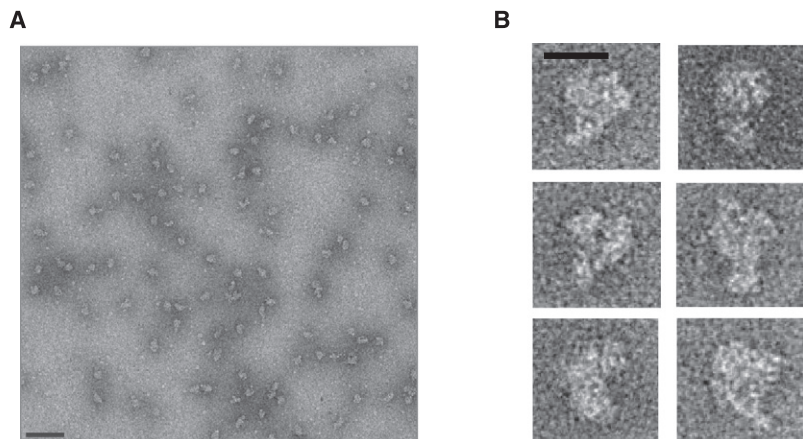


Figure 6. EM Analyses of the Purified 3' Processing Complex

(A) A typical CCD micrograph of the negatively stained purified 3' processing complexes. Bar, 100 nm.

(B) A gallery of representative particles. Bar, 20 nm.

Given the seemingly simple nature of the polyadenylation reaction, it is remarkable that it involves such a large complex. The size of the 3' processing complex is close to that of the bacterial ribosome large subunit (Radermacher et al., 1987) and the spliceosomal A complex (Behzadnia et al., 2007). The major components of our purified complexes, such as CPSF, symplekin, CstF, and CF I, likely constitute the "core" of the 3' processing complex seen in the class averages, as their collective molecular weight (~1.1 MDa) is already close to that of the bacterial ribosome large subunit (1.5 MDa) (Radermacher et al., 1987). These core factors, at least one of which, CstF, may be present as a dimer (Bai et al., 2007), likely correspond to some of the observed major densities. For the rest of the factors identified in the proteomics (total molecular weight ~7 MDa), the majority are present at substoichiometric levels and likely contribute to the heterogeneity observed among particles. The structure described here, we believe, corresponds to the core polyadenylation machinery.

In this study, we purified functional human pre-mRNA 3' processing complexes and determined their protein composition. We detected all but one known 3' processing factor and identified several new and potentially essential ones. We identified a number of proteins involved in other cellular processes, expanding the view that 3' processing is integrated with other cellular events. We also visualized the 3' processing complex for the first time and characterized its basic structural features. Together, our study has provided critical insights into the molecular composition and the structure of the 3' processing complex, revealing a molecular architecture that is much more complex than previously expected.

EXPERIMENTAL PROCEDURES

Antibodies

Anti-CPSF160, -100, -73, and WDR33 were kindly provided by Orit Rosenblatt and Bethyl Laboratories; anti-CstF64 6A9 was described previously (Takagaki et al., 1990); anti-symplekin was from BD Biosciences; and anti-hnRNP A1 was from ImmuQuest.

In Vitro 3' Processing Assays

Constructs used in this study were derived from pG3SVL-A and pG3L3-A, which contain the SV40 late site and adenovirus 2 L3 poly(A) site, respectively (Takagaki et al., 1988). 3 MS2-binding sequences were as described previously (Zhou et al., 2002) and were inserted between Acc I and Xba I sites before the SVL and L3 sequences. ³²P-labeled pre-mRNAs were prepared with SP6 RNA polymerase (Promega) from linearized plasmids. Polyadenylation reactions typically contain 8 pmol radiolabeled RNA/ml reaction, 40% NE, 8.8 mM HEPES (pH 7.9), 44 mM KCl, 0.4 mM DTT, 0.7 mM MgCl₂, 1 mM ATP, and 20 mM creatine phosphate. In cleavage reactions, ATP was omitted and 0.2 mM 3' dATP (Sigma), 2.5% PVA, and 40 mM creatine phosphate were added.

Purification of 3' Processing Complexes

Radiolabeled RNA substrates were incubated with 50 molar excess of MBP-MS2 adaptor protein for 30 min on ice. Then, the other ingredients of the polyadenylation reaction were added, and the reactions were incubated in 200 μl aliquots at 30°C for 40 min or otherwise specified time. The reactions were chilled and loaded onto 11 ml 10%–30% glycerol gradients (20 mM HEPES [pH 7.9], 100 mM KCl, 0.1 mM EDTA, and 1 mM DTT). The gradients were centrifuged at 22,000 rpm for 16 hr in a SW41 rotor, and then 500 μl fractions were manually collected from the top to the bottom. Radioactivity of each fraction was measured using a liquid scintillation counter. Peak frac-

tions were pooled and mixed with amylose beads for 1 hr at 4°C. After washing with wash buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT), the complexes were eluted in wash buffer plus 12 mM maltose. For mass spectrometry analyses, the eluted complexes were treated with RNase A and precipitated before analyses. For purification of complexes assembled on mutant substrates and cleavage complexes, reaction mixtures were loaded onto a Sephacryl S-400 size-exclusion column, and RNP-containing fractions were pooled and used for affinity purification with amylose beads (Jurica et al., 2002).

Proteomic Analyses of Purified 3' Processing Complex Using Multidimensional Protein Identification Technology

Precipitated protein preparations were dissolved in digestion buffer, digested by trypsin, and analyzed by LC/LC/MS/MS according to published protocols (Link et al., 1999). MS/MS spectra obtained were analyzed by SEQUEST using a nonredundant NCBI protein database. The SEQUEST outputs were then analyzed by DTASelect (version 2.0) program. The type of digestion method used was specified (-trypstat for tryptic digests) so as to specifically filter for peptides with trypsin specificity. A user-specified false positive rate was used to dynamically set XCorr and DeltaCN thresholds through quadratic discriminant analysis. This data set was then further filtered to remove contaminants (i.e., keratin) through the use of Contrast (version 2.0). A minimum of two peptides and half tryptic status (-p 2-y 1) were set in the Contrast.params file. For analyses of the cleavage complexes, minimum peptide number was set to 1.

Immunopurification of CPSF73- and CstF77-Associated Proteins

The plasmids CPSF73-3Flag-pCMV14 and Flag-CstF77-pCDNA3.1 were transfected into HEK293 cells, and stable transfectants were selected using G418 (Invitrogen). NE was made from these stable cell lines using standard protocol, and IP was performed using M2 beads (Sigma). For functional analyses, eluted proteins were concentrated using Centricon Y-30 (Millipore) and directly used in *in vitro* 3' processing assays.

Immunodepletion of WDR33

100 μl NE was diluted with equal volume of Buffer D, and NP-40 was added to final concentration of 0.1%. The diluted NE was then mixed with either protein G-agarose (mock) or with anti-WDR33-conjugated protein G-agarose (ΔWDR33) for 2 hr at 4°C. The depletion efficiency was measured by quantitative western blotting using the Odyssey infrared scanner (Li-Cor).

Electron Microscopy

A 9 ml polyadenylation reaction was used for purification as described above. Following affinity purification, the complexes were eluted in 300 μl and further treated using the GraFix method (Kastner et al., 2008). Briefly, the eluted complexes were loaded on a 4 ml 10%–30% glycerol and 0%–0.1% glutaraldehyde gradient and centrifuged at 51,000 rpm for 2.5 hr in an SW55Ti rotor. Afterwards, 180 μl fractions were taken manually from the top. Negative staining was performed using the carbon-sandwich method (Radermacher et al., 1987). Images were acquired on a JEOL JEM2100F electron microscope operating at 200 kV. Imaging was performed at a set magnification of 30,000× under low-dose conditions at an underfocus of 2.3 microns, and images were collected on a Tietz 224HD 2Kx2K CCD camera with 24 micron pixel size. The calibrated pixel size was 5.11 Å/pixel. Images were processed using the standard SPIDER protocol (Frank, 1996) or the refine2d.py script from EMAN (Ludtke et al., 1999).

SUPPLEMENTAL DATA

The Supplemental Data include eight figures and two tables and can be found with this article online at [http://www.cell.com/molecular-cell/supplemental/S1097-2765\(09\)00025-2](http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00025-2).

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