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The role of the yeast cleavage and polyadenylation factor subunit Ydh1p/Cft2p in pre-mRNA 3'-end formation

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ABSTRACT

Cleavage and polyadenylation factor (CPF) is a multi-protein complex that functions in pre-mRNA 3'-end formation and in the RNA polymerase II (RNAP II) transcription cycle. Ydh1p/Cft2p is an essential component of CPF but its precise role in 3'-end processing remained unclear. We found that mutations in *YDH1* inhibited both the cleavage and the polyadenylation steps of the 3'-end formation reaction *in vitro*. Recently, we demonstrated that an important function of CPF lies in the recognition of poly(A) site sequences and RNA binding analyses suggesting that Ydh1p/Cft2p interacts with the poly(A) site region. Here we show that mutant *ydh1* strains are deficient in the recognition of the *ACT1* cleavage site *in vivo*. The C-terminal domain (CTD) of RNAP II plays a major role in coupling 3'-end processing and transcription. We provide evidence that Ydh1p/Cft2p interacts with the CTD of RNAP II, several other subunits of CPF and with Pcf11p, a component of CF IA. We propose that Ydh1p/Cft2p contributes to the formation of important interaction surfaces that mediate the dynamic association of CPF with RNAP II, the recognition of poly(A) site sequences and the assembly of the polyadenylation machinery on the RNA substrate.

INTRODUCTION

All eukaryotic mRNA precursors (pre-mRNA) are extensively modified before they can serve as templates for protein synthesis. Pre-mRNA 3'-end processing is initiated by endonucleolytic cleavage at the poly(A) site. Subsequently, the upstream cleavage product is polyadenylated whereas the downstream fragment is rapidly degraded (for review see 1). The yeast 3'-end processing reaction can be reconstituted *in vitro* with the cleavage and polyadenylation factor IA (CF IA), cleavage and polyadenylation factor IB (CF IB), cleavage and polyadenylation factor (CPF) and the poly(A) binding protein (Pab1p) (2,3). Interactions between their subunits

allow bridging of the different factors and ensure their coordinated action on the substrate. So far the CPF components Fip1p and Pfs2p have been shown to bridge CPF with CF IA by interacting with Rna14p, a subunit of CF IA (3,4). A table of the factors involved in 3'-end processing and their subunits is provided as supplementary material.

The polyadenylation signals that guide the processing machinery in yeast are redundant and more degenerate compared to the well-conserved sequences in higher eukaryotes. Still, conserved elements can be found, which are the efficiency element (EE), the positioning element (PE), the poly(A) site and U-rich sequences. The EE is located at variable distances upstream of the cleavage site (5). The PE is often found ~20 nt upstream of the cleavage site and consists of an A-rich sequence (6). U-rich regions, located directly upstream and downstream of the cleavage site, and the poly(A) site itself act in concert to produce multiple recognition sites (5,7–9).

RNase H protection mapping experiments with a CYC1 pre-mRNA suggested that CPF is involved in the recognition of the poly(A) site by specific interactions of its subunits Yhh1p/Cft1p, Ydh1p/Cft2p and Yth1p with sequences surrounding the poly(A) site (9–11). Nab4p/Hrp1p (CF IB) binds to the EE (12,13) and, through interaction with Rna14p, possibly enables CF IA to bind to the PE (14). However, it was also reported that Nab4p/Hrp1p (15) as well as the PE and EE (9) are not essential for cleavage *in vitro*, underscoring the importance of the poly(A) site region itself.

The yeast and mammalian 3'-end processing factors are highly homologous (reviewed in 16). In mammals cleavage and polyadenylation specificity factor (CPSF) contributes to poly(A) site selection by binding to the well conserved AAUAAA element upstream of the cleavage site (17); CPSF-160 and possibly additional CPSF subunits are thought to mediate specific interactions to the RNA (18). The binding of purified CPSF is weak, but is strongly enhanced by a cooperative interaction with cleavage stimulation factor (CstF) bound to the downstream elements (19–21).

Transcription by RNA polymerase II (RNAP II) and pre-mRNA processing reactions are coupled events (reviewed in 22–25). The C-terminal domain (CTD) of RNAP II plays a central role in linking the processing reactions to transcription. Current models suggest that the CTD is hypo-phosphorylated

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during transcription initiation and that escape of RNAP II into the elongation phase is accompanied by phosphorylation of the CTD. It has been proposed that the change in charge upon phosphorylation enables proteins involved in pre-mRNA processing to bind to the CTD (26–28). This includes proteins involved in capping (29–32), splicing (33) and 3'-end formation (10,28,34–37). It is assumed that the assembled proteins subsequently travel with the elongating RNAP II during transcription and act on the nascent RNA transcript.

In mammals the CTD was suggested to play a direct role in 3'-end cleavage *in vivo* (34) and *in vitro* (38,39). Experiments in yeast provided evidence that transcription in the absence of the CTD was accompanied by a reduction of cleavage efficiency and the resulting mRNAs had shorter poly(A) tails (28,40). Correct transcription termination requires a functional poly(A) signal in all organisms (41,42, reviewed in 43), and yeast strains carrying mutations in CPF and CF IA were shown to be deficient in correct transcription termination, indicating the coupling between 3'-end processing and transcription termination (10,35,44–47). Furthermore, correct transcriptional termination was found to require the transcription factors Sub1p (48) and Res2p (49) and chromatin remodeling factors (50). The Nrd1p complex was shown to be required for correct termination at snoRNA genes (51).

Ydh1p/Cft2p (which will be referred to as Ydh1p in the remainder of this paper) is the 105 kDa subunit of CPF. It shares 24.4% identity and 43% similarity with the mammalian CPSF-100 protein. It is also significantly related to Ysh1p and to CPSF-73 (52). Ydh1p is essential for cell viability (53) and was shown to bind RNA (54). RNase H protection experiments suggested that the protein interacts with the poly(A) site region (9). Here, we show that Ydh1p is required for cleavage and polyadenylation *in vitro* and for poly(A) site recognition *in vivo*. Furthermore, we provide evidence that Ydh1p interacts with several subunits of CPF, with Pcf11p, a subunit of CF IA, and with the CTD of RNAP II. The results suggest that Ydh1p is part of an interaction surface that mediates important contacts with CF IA, the CTD of RNAP II and the pre-mRNA substrate.

MATERIALS AND METHODS

Yeast strains

For random mutagenesis of *YDH1*, mutagenic PCR was carried out with a low concentration of ATP (3). The primers used for PCR were: Ydh1-N (5'-CCCTTACGGATTGAAGT-CATT-3') and Ydh1-C (5'-TTGAACCTTTTATTGTGCTG-3'). Plasmid pBD63 (*YDH1-LEU2-CEN*) was obtained by subcloning of the *YDH1* gene from pIA115 (*YDH1-URA3-CEN*) (53) into the BamHI and SacI restriction sites of pRS415. The plasmid was digested with the restriction enzymes NsiI and SpeI. The fragment containing plasmid sequences and sequences flanking the *YDH1* gene was co-transformed together with the mutagenized PCR product into the yeast strain YPP106 (53). Transformants were selected on minimal medium lacking leucine and replica-plated onto 5-FOA plates in order to shuffle out the pIA115 plasmid. The colonies were then replica-plated onto YPD-plates and incubated at 25, 33 or 37°C, respectively. Plasmids of candidate colonies that showed a ts phenotype were isolated

by standard procedures. The conditional growth phenotype was then verified by retransformation of the isolated plasmids into YPP106, 5-FOA treatment and growth tests at elevated temperatures.

Genotypes of yeast strains used in this study were: YPP106: MAT α ; *ura3-1*; *ade2-1*; *leu2-3,112*; *his3-11,15*; *trp1* Δ ; *ydh1::TRP1* [pIA115; *CEN4-URA3-YDH1*] (53); YAK1: MAT α ; *ura3-1*; *ade2-1*; *leu2-3,112*; *his3-11,15*; *trp1* Δ ; *ydh1::TRP1* [pAK21 *CEN-LEU2-ydh1-1*]; YAK2: MAT α ; *ura3-1*; *ade2-1*; *leu2-3,112*; *his3-11,15*; *trp1* Δ ; *ydh1::TRP1* [pAK22 *CEN-LEU2-ydh1-2*]; YAK3: MAT α ; *ura3-1*; *ade2-1*; *leu2-3,112*; *his3-11,15*; *trp1* Δ ; *ydh1::TRP1* [pAK23 *CEN-LEU2-ydh1-3*]; Y190: *ura3-52*; *trp1-901*; *ade2-101*; *leu2-3,112* *his3-200r*; *gal4D*; *gal 80D*; *URA3:GAL1-lacZ*; *LYS2::GAL1-HIS3*; *cyhr*; Clontech.

Plasmids and primers

The plasmids encoding the C-terminally truncated Ydh1p fragments were obtained by digestion of plasmid pBD71 (9) with restriction enzymes AccI (pBD91; encoding the recombinant protein Δ C338), BamHI/XbaI (pBD92; encoding Δ C182), BamHI/SpeI (pAK5; encoding Δ C137), BamHI/AflII (pAK6; encoding Δ C555), BamHI/AgeI (pAK7; encoding Δ C613). GST-Ysh1p was encoded by pBD38, which was obtained by subcloning of Ysh1p into p20 (GST expression vector) using the restriction enzymes NdeI and BamHI. GST-Tev-Pta1p was encoded by pBD51, which was obtained by PCR amplification of Pta1p followed by digestion with NdeI and BamHI and ligation into p26 (GST-Tev expression vector). GST-Yhh1p-H₆ was encoded by pBD75 (10).

The plasmid encoding the protein used to produce antibody directed against Ydh1p was constructed by digestion of pQE-9 (His6 expression vector Qiagen) with BamHI/HindIII. The insert was constructed by PCR, amplifying the sequence between primer Ydh5' (5'-ATCGCGGATCCCATGACTTAT-AAATACAATTG-3') and Ydh3' (5'-AGCCCAAGCTTATT-TACTCAATTTCGTTTGGT-3') of *YDH1*. For details about pBD-CTD, pACT2-*YHH1*, pACT2-*YSH1* and pACT2-*PCF11* see (10).

Expression of recombinant proteins in *Escherichia coli*

BL21 *E. coli* cells carrying the respective plasmid were grown at 25°C in 2 \times YT until they reached an OD₆₀₀ of ~1. Following induction by 0.5 mM IPTG, incubation was continued for 6 h. The proteins were purified at 4°C on glutathione-Sepharose 4B as recommended (Pharmacia) and the protein was eluted with GST-elution buffer [75 mM KCl, 50 mM Tris-HCl pH 7.9, 10% glycerol, 10 mM glutathione (reduced), 0.01% NP-40, 1 mM DTT].

Protein-protein interactions

In vitro translations were performed with the TNT-coupled transcription-translation system (Promega). GST fusion protein (100 ng) was incubated with *in vitro* translated [³⁵S]methionine-labeled proteins for 1 h. The mixture was bound in a total volume of 860 μ l to 20 μ l glutathione sepharose (Pharmacia), which was previously equilibrated in 1 ml PBS, 0.01% NP-40 and 100 μ g BSA. The matrix was washed three times with IPP150 (150 mM KCl, 20 mM Tris-HCl pH 8.0, 0.01% NP-40) and the proteins were eluted by addition of protein loading buffer and incubation at 95°C.

Bound proteins were separated by SDS-PAGE and visualized by autoradiography. Phosphorylation of GST-CTD was performed as described previously (38) and the assay of the CTD interaction was carried out as described (10). The two-hybrid tests were carried out as described (55).

Extract preparation and *in vitro* cleavage and polyadenylation assays

Extracts competent for *in vitro* processing were prepared following the procedure previously described (3). The cleavage and polyadenylation assays were carried out as described (56). To restrict the assay to cleavage only, EDTA replaced MgAc and CTP replaced ATP. For each reaction 30–40 μ g total protein was used and, in the case of the reactions carried out at 34°C, pre-incubated at this temperature for 5 min. The RNA substrates were prepared by run-off transcription following the previously described procedure (9).

RNA analyses

Northern analyses and RNase H experiments were carried out as described (10). In addition we employed the oligonucleotides anti-U24 (5'-TCAGAGATCTTGGTGATAAT-3') and anti-snR13 (5'-GGCAAAAGCCAAACAGCAACTCGA-GCCAAATGCACTCATATTCATCATAT-3'), which were labeled with [γ -³²P]ATP by T4 polynucleotide kinase.

The reverse transcription analysis was performed as described (45) with primers downstream of snoRNA genes as previously described (51).

Protein extraction for western blotting

The cells were grown at 25°C and shifted to 37°C; during incubation their growth was kept in the log phase. At each time point, 40 ml of the culture (OD₆₀₀ = 0.4) was harvested. The following procedure was carried out on ice. The cells were resuspended in IPP150 and an equal volume of glass beads was added. The cells were opened by rigorous vortexing. Five millilitres of IPP150 and protease inhibitors were added and the mixture was centrifuged for 1 h at 8000 *g* r.p.m. Four millilitres of the supernatant was thereafter centrifuged for 2 h at 200 000 *g* and the protein was concentrated by centrifugation in a centricon YM10 (Millipore). The protein concentration was determined by Bradford analysis and equal amounts of total proteins were separated by SDS-PAGE.

RESULTS

Ydh1p is required for cleavage and polyadenylation *in vitro*

In order to investigate the role of Ydh1p in 3'-end processing we generated temperature sensitive *ydh1* alleles (see Materials and Methods). Figure 1A shows growth curves of *ydh1* mutant and isogenic wild-type cells following shift from 25 to 37°C. The *ydh1-1* strain displayed growth arrest at 37°C after 3 h, whereas the strains *ydh1-2* and *ydh1-3* ceased growth after ~5 h. A drop-test revealed that the mutant strains did not form colonies at the restrictive temperature (Fig. 1B).

Next, we analysed whether mutations in *YDH1* affect cleavage and polyadenylation *in vitro* and tested extracts from wild-type, *ydh1-1*, *ydh1-2* and *ydh1-3* cells for their ability to

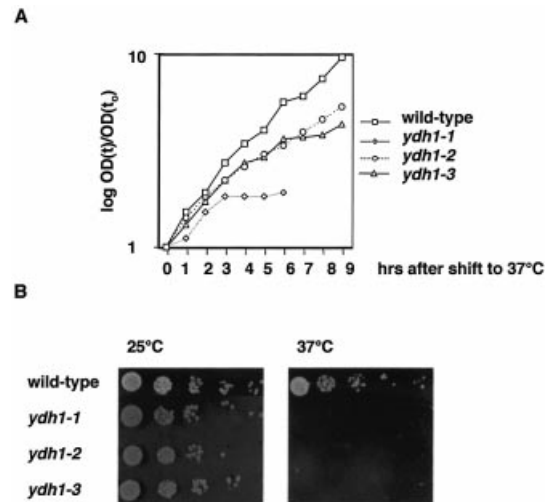


Figure 1. *YDH1* mutant strains display a temperature sensitive phenotype. (A) Growth curves of wild-type and mutant *ydh1* strains at 37°C. (B) 10-fold serial dilution of cultures spotted on YPD plates followed by incubation at the indicated temperature for 2 days.

cleave a synthetic *CYC1* pre-mRNA (Fig. 2A). Extracts from wild-type (lanes 2 and 6), *ydh1-2* (lanes 4 and 8) and *ydh1-3* cells (lanes 5 and 9) accurately cleaved the substrate RNA at 30 and 34°C. Notably, *ydh1-3* extract (lane 9) showed a reduced efficiency of cleavage at 34°C compared to wild-type (lane 6). In contrast, extract of *ydh1-1* cells was deficient in cleavage at both temperatures (lanes 3 and 7).

To show that the deficiency in cleaving the RNA substrate is due to inactive Ydh1p or CPF, respectively, we carried out reconstitution assays either with extract which is mutant in a CF IA subunit (*rna15-1*) or with purified factors at 30°C (Fig. 2B). The cleavage activity of *ydh1-1* extract was restored upon addition of *rna15-1* extract (lane 4) or purified CPF (lane 6), but not by addition of purified CF IA (lane 7). As expected, the *rna15-1* extract on its own lacked cleavage activity (lane 5) (56) and purified CPF and CF IA alone were not able to cleave the substrate (lanes 8 and 9); cleavage occurred at specific and at cryptic sites when both factors were added to the reaction (lane 10); site-specific cleavage was obtained by including CF IB in the reaction (lane 11) (15).

Next we analysed whether mutations in *YDH1* affect the polyadenylation reaction of pre-cleaved *CYC1* substrate (*CYC1*-Pre) *in vitro* (Fig. 2C). No polyadenylation activity could be observed in *ydh1-1* extract at 30 and 34°C (lanes 3 and 7). In contrast, the *ydh1-2* and *ydh1-3* extracts specifically and efficiently polyadenylated the substrate at both temperatures (lanes 4, 5, 8 and 9), comparable to wild-type (lanes 2 and 6). Specific polyadenylation activity was restored in *ydh1-1* extract by addition of purified CPF (Fig. 2D, lane 4) but not by addition of purified CF IA (lane 5). As shown before, CPF by itself unspecifically polyadenylated the substrate (lane 6) (3), whereas CF IA alone displayed no polyadenylation activity (lane 7). Specific polyadenylation activity was restored upon combination of CPF, CF IA, CF IB and Pab1p (lane 8).

Notably, addition of 100–250 ng recombinantly expressed Ydh1p failed to reconstitute the cleavage and polyadenylation activities in *ydh1-1* extract, possibly because the recombinant

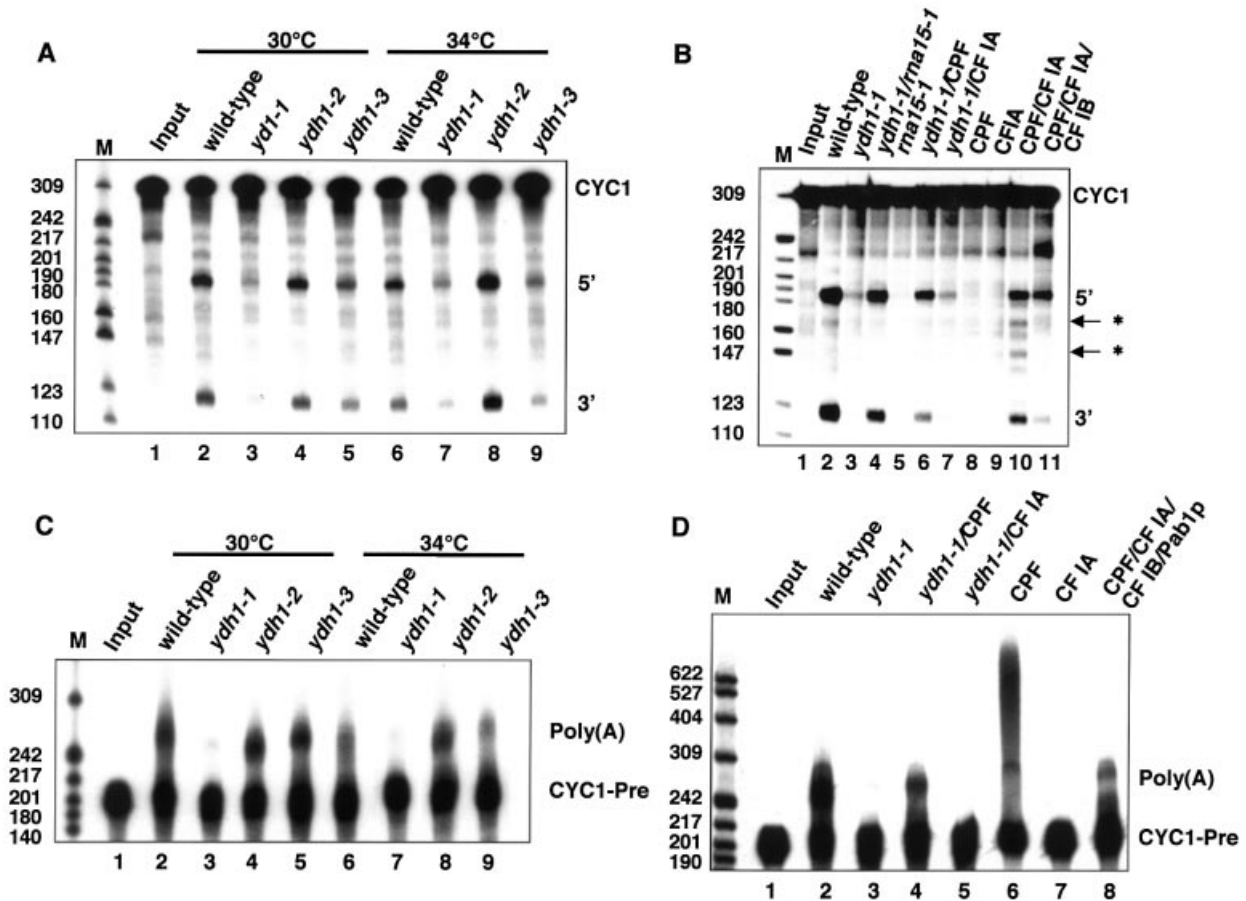


Figure 2. Ydh1p is required for cleavage and polyadenylation *in vitro*. *In vitro* analysis of cleavage (A and B) and polyadenylation activities (C and D) of *ydh1* mutant extracts. Input lanes (1) represent mock-treated reactions. The migration positions of the *CYC1* and *CYC1*-precleaved (*CYC1*-Pre) RNA substrate, specific (5' and 3') and cryptic (asterisks) cleavage products and the polyadenylation products [Poly(A)] are indicated on the right of each panel. The position and size (in number of nucleotides) of the marker bands are indicated on the left. (A) Extracts prepared from yeast strains (as indicated above the lanes) were monitored for their ability to cleave internally ^{32}P -labelled *CYC1* RNA substrate at 30 and 34°C. (B) Reconstitution of cleavage activity in *ydh1-1* extract at 30°C. As indicated above the lanes, *ydh1-1* extract was combined with equal amounts of *ma15-1* extract (lane 4), purified CPF (lane 6) or CF IA (lane 7). (C) Specific polyadenylation was analysed at 30 and 34°C with internally ^{32}P -labelled pre-cleaved *CYC1* RNA substrate that ends at the natural poly(A) site. (D) Reconstitution of specific polyadenylation activity in *ydh1-1* extract at 30°C. *ydh1-1* extract was combined with purified CPF (lane 4) or CF IA (lane 5).

protein was not able to replace its mutant counterpart in the CPF complex (results not shown).

The above results showed that the *ydh1-1* extract was strongly reduced in cleavage and polyadenylation activities *in vitro* and that both activities could be reconstituted by addition of purified CPF. Furthermore, the cleavage activity of the *ydh1-3* mutant was lower at 34°C compared to 30°C. The results indicate that Ydh1p is involved in both steps of the 3'-end processing reaction.

mRNAs are unstable in *ydh1* mutant strains at restrictive temperature

mRNAs without poly(A) tails are rapidly degraded in living cells. Yeast strains with a 3'-end processing deficiency are therefore expected to under-accumulate mRNAs after shift to the restrictive temperature. For this reason, we performed northern blot analyses on total RNA extracted from strains grown at 25°C and after shift to 37°C (Fig. 3A). The amount of 18S rRNA served as control for the loading of the RNA (panel

VI). *ADH1*, *ACT1*, *CYH2* and *CYC1* mRNA levels diminished in the *ydh1-1* mutant cells after 2 h shift to 37°C (panels I–IV, lanes 3–6). The level of the stable *PGK1* mRNA ($t_{1/2} = 45$ min) dropped significantly only after the cells were shifted to 37°C for 6 h (panel V, lanes 3–6). In comparison the *ADH1*, *ACT1*, *CYH2* and *CYC1* mRNA levels of the *ydh1-2* mutant strain only dropped slightly after 4 h, and the *PGK1* mRNA level remained stable even after shift to 37°C for 6 h (panels I–V, lanes 7–10). The mutant strain *ydh1-3* under-accumulated *ACT1* mRNA after only 2 h at 37°C; *ADH1*, *CYH2* and *CYC1* mRNA levels were reduced after 4 h (panels I–IV, lanes 11–14). The *PGK1* mRNA level showed a slight decrease after 4 h and more so after 6 h (panel V, lanes 11–14). The RNA polymerase II transcribed U24, U14 and snR13 snoRNAs were stable in all mutants tested, even after shift to 37°C for 6 h (panels VII–IX), indicating that snoRNA processing was not affected in *ydh1* mutant strains. These results showed that *ydh1* mutant strains under-accumulated mRNAs at restrictive temperature. The *ydh1* mutant phenotypes are possibly due to

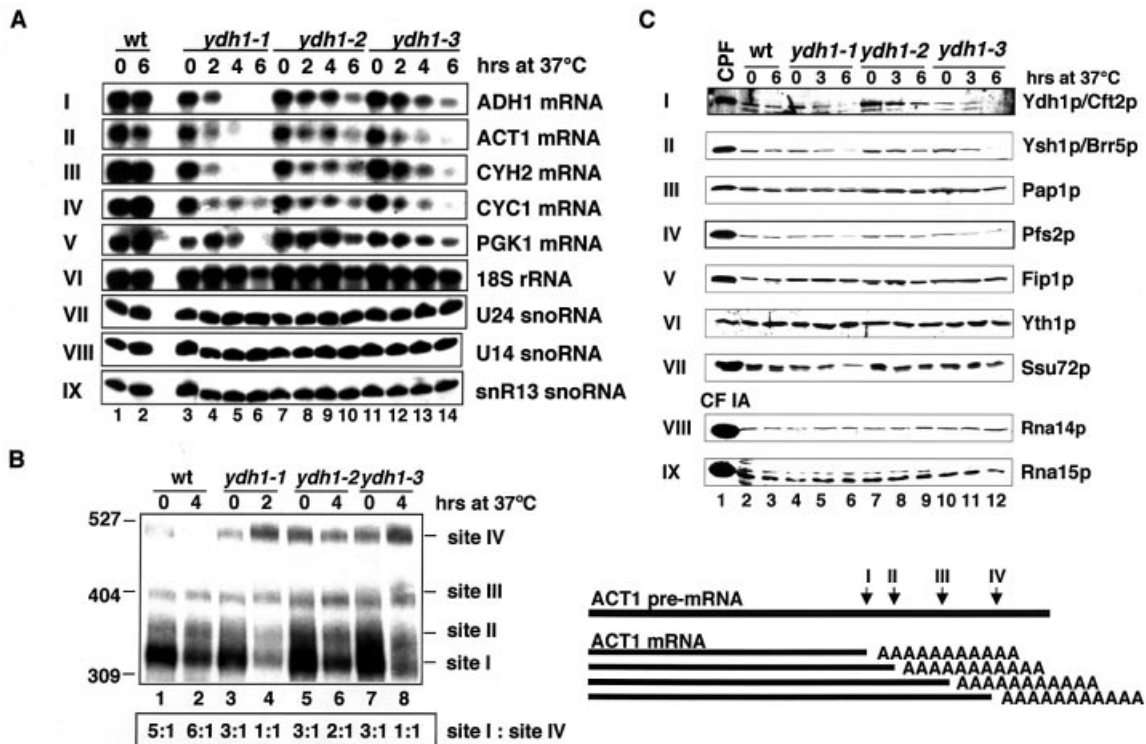


Figure 3. mRNAs are unstable in *ydh1* mutant strains at restrictive temperature and the mutant strains are deficient in the recognition of *ACT1* poly(A) site *in vivo*. (A) Northern analysis of total RNA extracted from wild-type and mutant *ydh1* cells grown at 23°C, or after shift to 37°C for 2, 4 and 6 h as indicated above each lane. The RNAs were separated on formaldehyde/1.2% agarose gels (panels I–VI) or 8.3 M urea/8% polyacrylamide gels (panels VII–IX). The filters were developed with random-primed labeled probe (panels I–V) or end-labeled oligonucleotides (panels VI–IX) directed against the RNA species indicated at the right of each panel. (B) Analysis of *ACT1* poly(A) site usage in wild-type and mutant *ydh1* cells. The schematic on the right shows the relative positions of the different poly(A) sites. Total RNA extracted from wild-type and mutant *ydh1* cells after growth at 23°C, or following shift to 37°C as indicated above each lane, was treated with an oligonucleotide complementary to the 3' region of the *ACT1*-mRNA (*ACT1*-RnaseH) and RNaseH. The RNAs were separated on an 8 M urea/6% polyacrylamide gel and the filters were incubated with random primed labeled probe directed against the 3'-end of the *ACT1* mRNA. RNA levels were quantified by PhosphorImager scanning (Molecular Dynamics); the ratios of poly(A) site I:site IV usage for each lane are indicated at the bottom. (C) Western analysis of wild-type and *ydh1* mutant extracts prepared from cells grown at 23°C, or after shift to 37°C for 3 and 6 h. Equal amounts of total protein were loaded in each lane. Lane 1 shows purified CPF (panels I–VII) and CF IA (panels VIII and IX). The filters were treated with antibodies directed against the proteins indicated at the right.

a deficiency of the strains in poly(A) site recognition (see below) which might impair the efficiency in 3'-end processing.

Ydh1p is required for poly(A) site selection of *ACT1* pre-mRNA

The previous observation that Ydh1p binds RNA around the poly(A) site (9), raised the possibility that the protein is required for poly(A) site recognition. To test this, we analysed poly(A) site usage of *ACT1* pre-mRNA in wild-type and mutant cells (57). The *ACT1* 3' untranslated region (UTR) contains at least four polyadenylation sites, of which the most proximal one (site I) is used with highest frequency in wild-type cells (Fig. 3B; lanes 1 and 2). All tested *ydh1* mutants used site I three times more often than site IV at 25°C (lanes 3, 5 and 7). At restrictive temperature, however, processing shifted from site I towards site IV in a ratio of 1:1 in *ydh1-1* and *ydh1-3* cells (lanes 4 and 8); the *ydh1-2* mutant showed a ratio of 2:1 (lane 6). These results indicated that mutations in *ydh1-1*, *ydh1-2* and *ydh1-3* impair poly(A) site recognition *in vivo*. To verify that the *ACT1* mRNAs were polyadenylated we incubated the reactions with RNase H and the oligos *ACT1*-RNaseH and dT to digest the poly(A) tails. This resulted

in a slight downward shift and sharpening of the RNA bands. This showed that the *ACT1* mRNAs were polyadenylated (results not shown).

Western blot analyses were carried out to test whether the observed phenotypes were the result of an under-accumulation of Ydh1p or other subunits of the 3'-end processing machinery (Fig. 3C). As control, purified CPF or CF IA was analysed in parallel (lane 1). In wild-type and *ydh1-2* cells Ydh1p was stable even after 6 h at 37°C (panel I, lanes 2, 3 and 7–9), whereas it was reduced in *ydh1-1* and *ydh1-3* cells after 3 h at 37°C (lanes 5, 6, 11 and 12). Ysh1p/Brr5p, Pfs2p and Ssu72p (panels II, IV and VII) under-accumulated in *ydh1-1* and *ydh1-3* cells after 6 h at 37°C (lanes 6 and 12). The levels of Pap1p, Fip1p and Yth1p were constant even after 6 h at 37°C (panels III, V and VI). In addition, mutations in *YDH1* did not affect the levels of the CF IA subunits Rna14p and Rna15p (panels VIII and IX).

These results indicate that the defects observed in the *ydh1* mutant strains might be caused by a destabilization of the Ydh1p protein. Since the destabilization at restrictive temperature of Ysh1p/Brr5p, Pfs2p and Ssu72p was detectable at later time-points compared to the appearance of phenotypes

[see northern blot and *ACT1* poly(A) site selection], we consider it unlikely that lower levels of factors other than Ydh1p are responsible for the deficiencies of the *ydh1* mutant cells.

Ydh1p interacts with the C-terminal domain of RNA polymerase II

The CTD of RNAP II plays an important role in coupling transcription and 3'-end formation (reviewed in 22,23). We were interested to know whether Ydh1p contributes to this process by physically interacting with the CTD. To test this, we carried out *GAL4*-based yeast two-hybrid tests and assayed for the activation of expression of the *HIS3* and *lacZ* reporter genes. Strain Y190 was co-transformed with plasmids carrying the *GAL4* DNA binding domain fused to the CTD (pBD-CTD) and the *GAL4* activation domain fused to test genes. As positive controls we analysed the known CTD interactors *YHH1/BRR5* (pACT2-YHH1) and *PCF11* (pACT2-PCF11) (10,28,47) and pACT2-YSH1 as negative control. As shown in Figure 4A the plasmids carrying *YDH1*, *YHH1* and *PCF11* in combination with the plasmid pBD-CTD enabled cells to grow on medium lacking histidine under stringent conditions (in the presence of 35 mM 3'-aminotriazole). In contrast, no growth was observed with pACT2-YSH1 or when empty pBD and pACT2 plasmids were tested. These results were confirmed by an X-Gal filter lift assay, which monitors β -galactosidase expression (results not shown). Thus, *YDH1* showed a two-hybrid interaction with the CTD of RNAP II.

To confirm that Ydh1p interacts with the CTD, and to test whether this interaction is influenced by the phosphorylation state of the CTD we performed GST pull-down experiments with *in vitro* translated radioactively labeled proteins and recombinant GST-CTD fusion protein. Ydh1p interacted with the CTD (Fig. 4B, lane 3) and this interaction was enhanced upon phosphorylation of the CTD (lane 4); no signal was observed with GST alone (lane 2). Ysh1p/Cft1p interacted with neither form of the GST-CTD protein. These results suggested that Ydh1p binds the CTD and that the interaction is enhanced upon phosphorylation of the CTD.

Correct transcription termination by RNAP II requires a functional poly(A) site on the nascent RNA and the CTD plays an important role in coupling transcription and pre-mRNA processing (reviewed in 43). The observations that Ydh1p is involved in poly(A) site recognition and that it interacted with the CTD raised the possibility that Ydh1p might also be involved in transcriptional termination. Therefore, we carried out transcriptional run-on analysis with the well-characterized *GAL1/10* controlled *CYC1* gene *in vivo* (44). We did not observe a significantly increased RNAP II density downstream of the *CYC1* terminator in *ydh1-1* and *ydh1-3* cells grown at 25°C or following shift to 37°C compared to wild-type (results not shown). Similarly, we did not detect the accumulation of read-through products in *ydh1* strains in reverse transcription experiments with primers annealing downstream of the cleavage site of different snoRNA species (snR39b, snR45, snR3, snR50, snR71) (51). This suggested that Ydh1p is not generally required for termination of RNAP II during transcription of pre-mRNAs and snoRNAs.

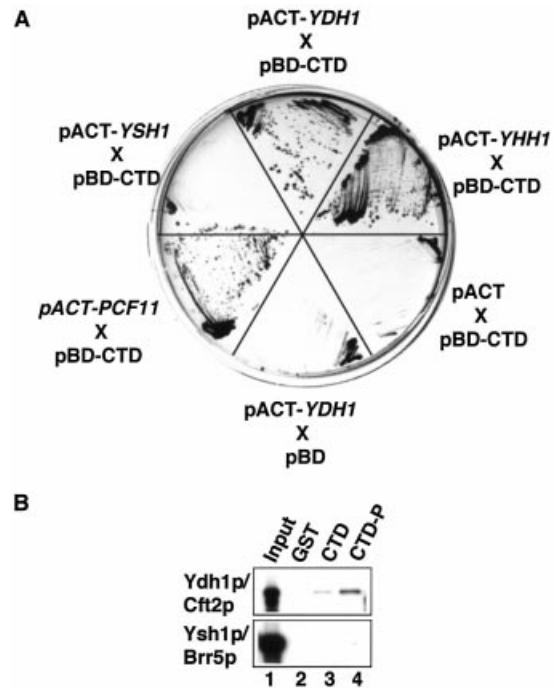


Figure 4. Ydh1p interacts with the CTD of RNAP II. (A) *GAL4*-based two-hybrid analysis. Y190 cells were co-transformed with a plasmid encoding the *GAL4* DNA binding domain fused to the CTD (pBD-CTD) and plasmids (pACT2) encoding the *GAL4* activation domain fused to the genes indicated. Transformants were tested for expression of the *HIS3* reporter gene on medium lacking histidine, and containing 35 mM 3-amino-1,2,4-triazole. As control, pBD CTD was co-transformed with the empty pACT2 plasmid and pACT2-YDH1 with the empty pBD plasmid. (B) Pull-down experiments with 1 μ g GST (lane 2), GST-CTD (lane 3), phosphorylated GST-CTD (lane 4) and *in vitro* translated, [³⁵S]methionine-labeled proteins (indicated on the left). Lane 1 shows 10% of the *in vitro* translated reactions used in the assay. Bound proteins were separated by SDS-PAGE and visualized by autoradiography.

Ydh1p interacts with other subunits of CPF and with Pcf11p, a subunit of CF IA

As Ydh1p is part of a multi-protein complex we were interested to examine with which of the other CPF subunits the protein interacts. Therefore, we expressed a N-terminally GST-tagged and C-terminally His-tagged version of the Ydh1 protein (G-Ydh1-H) in *E.coli* and carried out GST pull-down experiments with radioactively labeled *in vitro* translated proteins. As shown in Figure 5A, G-Ydh1-H interacted with itself and with the CPF subunits Yhh1p/Cft1, Ysh1p/Brr5p, Pta1p, Pfs2p, Ssu72p, YDL094cp (panels I–VII) and the CF IA subunit Pcf11p (panel X). G-Ydh1-H did not pull-down the CPF subunits Fip1p and Yth1p (panels VII and IX), nor the CF IA subunits Rna14p, Clp1p and Rna15p (panels XI–XIII), Nab4p/Hrp1 (CF IB) or Pab1p (panels XIV and XV). Since we identified a large number of potential Ydh1p interaction partners, we decided to investigate whether these interactions were specific and therefore assignable to distinct regions of Ydh1p. For this purpose the pull-downs were repeated with C-terminally truncated Ydh1p proteins (Fig. 5B). Figure 5A shows that the very C-terminus was required for interaction of Ydh1p with itself (panel II) as most of the interaction was lost upon deletion of the C-terminal 137 amino acids. The

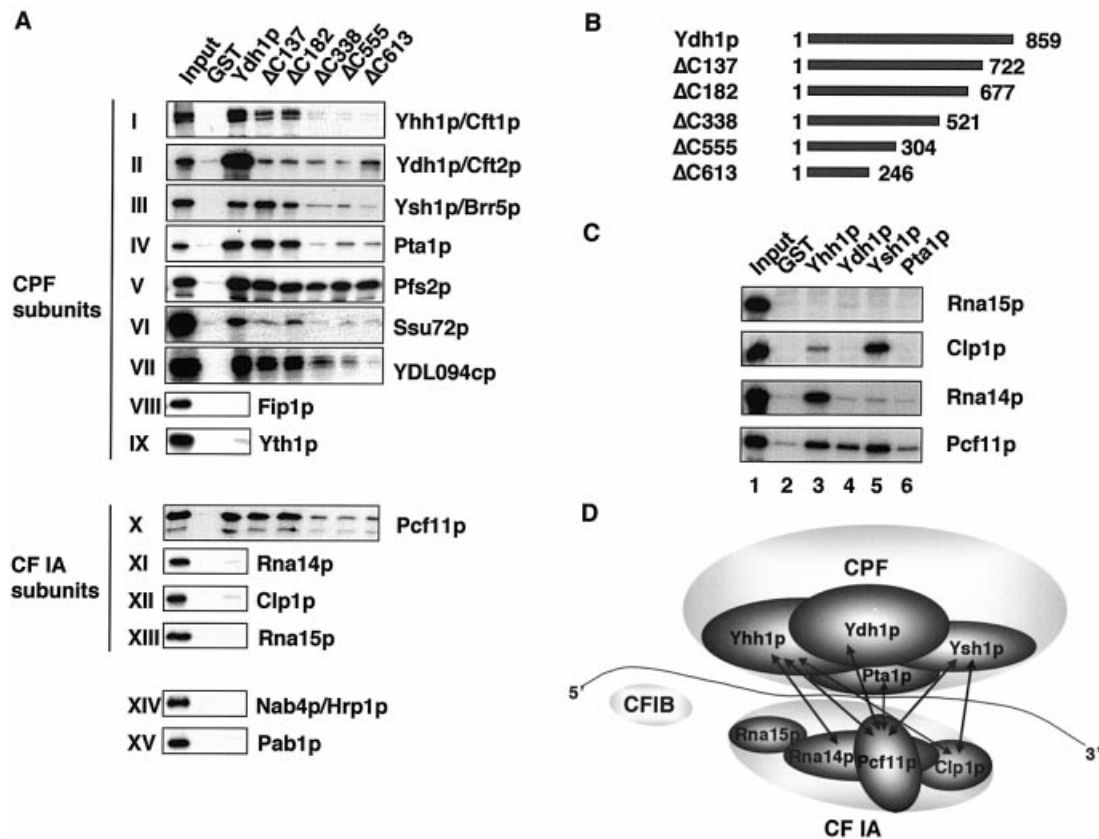


Figure 5. Ydh1p interacts with other subunits of CPF and with Pcf11p, a subunit of CF IA. (A) GST pull-down experiments with 0.5 μ g GST, 100 ng G-Ydh1-H (Ydh1p) or 100 ng of the different C-terminally truncated, GST-tagged Ydh1 fragments (as indicated above each lane) and *in vitro* translated [35 S]methionine-labeled proteins (indicated on the right). The first lane shows 10% of the *in vitro* translated protein used in the binding reactions. (B) Schematic drawing of the full length and C-terminal truncations of Ydh1p. The numbers indicate the length of the constructs in amino acids. (C) GST pull-down experiment with GST-tagged recombinant proteins (indicated above each lane) and *in vitro* translated, [35 S]methionine-labeled proteins (indicated on the right). (D) Model of protein-protein interaction surface (not all CPF subunits and protein interactions are shown).

N-terminal 246 amino acids were sufficient for interaction with Pfs2p (panel V) and central protein sequences most likely mediated interactions with Yhh1p/Cft1p, Ysh1p/Brr5p, Pta1p, Ssu72, pYDL094c and Pcf11p (panels I, III, IV, VI, VII and X). These results suggested that the Ydh1p subunit might contribute to the stability and structural order of CPF and bridge the two factors CPF and CF IA.

To obtain a better understanding of how CPF interacts with CF IA we decided to assay for further interactions between the two factors. Figure 5C shows a GST pull-down with *in vitro* translated protein and GST-tagged recombinant proteins. Of the analysed CPF subunits we found that only Yhh1p/Cft1p interacted strongly with Rna14p. Yhh1p/Cft1p also bound to Pcf11p, and more weakly to Clp1p (lane 3). Ydh1p pulled-down only Pcf11p (lane 4). Ysh1p/Brr5p interacted strongly with Clp1p and Pcf11p (lane 5). A weak signal was observed for the interaction between Pta1p and Pcf11p (lane 6). These results suggested that Ydh1p, Yhh1p/Cft1p, Ysh1p/Brr5p and possibly also Pta1p are involved in forming a protein-protein interaction surface between CPF and CF IA. Our combined results indicate that Ydh1p plays an important role in determining the RNA binding specificity of CPF and in the assembly of the 3'-end formation machinery and its tethering to RNAP II.

DISCUSSION

The yeast and mammalian 3'-end formation machineries display a surprisingly complex subunit composition. At present, up to 20 polypeptides are known that constitute the factors that catalyse the yeast 3'-end formation reaction *in vitro* (45,58). The ongoing characterization of the components suggests distinct and specialized tasks of the individual polypeptides in both steps of the 3'-end processing reaction as well as in the coupling of 3'-end formation to transcription. In this work we characterized the role of the yeast CPF subunit Ydh1p in these processes.

To analyse if Ydh1p is necessary for cleavage and polyadenylation we produced conditional *ydh1* mutant strains and carried out *in vitro* assays. The experiments revealed a deficiency of the mutant extracts in both steps of the 3'-end processing reaction. The activity could be rescued upon addition of purified CPF. mRNAs without a poly(A) tail are prone to rapid degradation *in vivo*. Northern analysis revealed that the levels of a number of different mRNAs were reduced at restrictive temperature in Ydh1p mutants. The results support a requirement for Ydh1p in both steps of 3'-end processing. However, we do not expect Ydh1p to be directly involved in the catalysis of the 3'-end processing reaction,

because it took several hours at the restrictive temperature before the mRNA levels were reduced. Furthermore, the *ACT1* poly(A) site selection assay revealed that a substantial amount of pre-mRNA appeared to be cleaved and polyadenylated *in vivo* at the restrictive temperature in all mutants.

We reported previously that Ydh1p binds to a *CYC1* pre-mRNA around the poly(A) site; this suggested a role for the protein in poly(A) site selection (9). Here we show that Ydh1p is involved in poly(A) site selection of *ACT1* pre-mRNA *in vivo*, as all analysed *ydd1* mutant strains displayed more frequent use of alternative poly(A) sites compared to the wild-type strain. These results underscore our previously postulated model that Ydh1p contributes to poly(A) site selection (9). In contrast to mammalian polyadenylation signals, the *cis*-acting sequences in yeast are highly degenerate (reviewed in 1,8). We proposed that the RNA binding subunits of CPF act in concert to achieve specific recognition of the correct poly(A) site (9). The preferential use of cleavage sites located downstream of the major site in Ydh1 mutants may be caused by the coupling of transcription elongation and 3'-end processing. Reduced RNA binding efficiency of CPF containing mutant Ydh1p could lead to skipping of the first cleavage site of the nascent pre-mRNA emerging from the elongating RNAP II. Ydh1p (9), Yhh1p/Cft1p (10) and Yth1p (11) were shown to bind preferentially to U-rich elements localized directly upstream and downstream of the poly(A) site, thus colocalizing with the region to which the complete CPF factor binds (9). These interactions with the RNA substrate are thought to be crucial for poly(A) site selection and for cleavage activity. Thus, the observed *in vitro* cleavage deficiency of the *ydd1* mutant cells might result from insufficient poly(A) site selection. It remains to be determined how Ydh1p interacts with RNA. The primary sequence of the protein does not display any clear similarities to known RNA binding domains. Preliminary RNA binding experiments with portions of Ydh1p indicated that the RNA binding activity is distributed throughout the entire length of the protein (results not shown). However, detailed analyses have not been done yet. Interestingly, RNA binding activity has not been demonstrated for the mammalian homolog of Ydh1p, CPSF 100 kDa. Considering the highly conserved *cis*-acting elements in mammalian pre-mRNAs (reviewed in 1), it seems possible that a smaller set of proteins is sufficient for specific poly(A) site recognition, whereas in yeast a cooperative interplay of more RNA binding proteins is required to recognize the more degenerate sequence elements in a specific fashion.

We have shown that Ydh1p interacted with the CTD of RNAP II both in a two-hybrid test and in a GST pull-down assay. This interaction was enhanced upon phosphorylation of the CTD. The only other CPF subunit found to interact with the phosphorylated CTD is Yhh1p/Cft1p (10). We suggest that Ydh1p might be involved in tethering CPF to transcribing RNAP II and thereby contributes to the coupling of 3'-end processing and transcription. However, the *ydd1* mutants did not reveal a defect in transcriptional termination at the *CYC1* terminator. As this was the only pre-mRNA that was tested by transcriptional run-on analysis we cannot exclude that Ydh1p might be involved in transcriptional termination of other genes. Moreover, reverse transcription analysis on snoRNAs in *ydd1* mutant strains did not reveal 3'-extended transcripts, indicating that Ydh1p was not required for snoRNA

termination either. We propose that Ydh1p helps to tether CPF to elongating RNAP II but that it is not needed for general transcription termination. Ydh1p and Yhh1p/Cft1p show a phenotypic similarity in that both proteins bind RNA, are involved in poly(A) site recognition and bind to the CTD (10). However, the observation that mutants in *yhh1* are severely impaired in transcription termination at the *CYC1* gene (10) whereas *ydd1* mutants have no general termination defect, indicates that the functional roles of the individual 3'-end formation factor subunits can be substantially different.

It is not well understood how the subunits of the CPF and CF IA factors assemble to form functional 3'-end processing complexes on the RNA substrate. CPF consists of up to 15 polypeptides, which interact with each other and form a stable factor. We found that Ydh1p interacted with the CPF subunits Yhh1p/Cft1p, Ysh1p/Brr5p, Pta1p, Pfs2p, Ssu72p and pYDL094c. These many interactions indicate that the protein contributes substantially to the assembly and structural order of the CPF factor. Strikingly, Ydh1p also bound strongly to itself in the GST pull-down assays. This may indicate that there is more than one Ydh1p molecule present per 3'-end processing unit. However, silver stain analysis of purified CPF factor suggested an apparent stoichiometric presence of Ydh1p compared to other CPF subunits (45). Interestingly, we found that the CF IA subunit Pcf11p interacted with the CPF components Ydh1p, Yhh1p/Cft1p, Ysh1p/Brr5p, and weakly with Pta1p. In addition, we showed that the CPF subunits Yhh1p/Cft1p and Ysh1p/Brr5p interacted with the CF IA subunits Rna14p and Clp1p, respectively. The latter interactions were also observed between the homologous subunits of the mammalian 3'-end formation machinery. CPSF 160 kDa interacts with the CstF 77 kDa protein (18) and CPSF 73 kDa interacts with hClp1p (59). So far, only Pfs2p and Fip1p were shown to bridge CPF and CF IA (3,4). We propose that Ydh1p, Yhh1p/Cft1p, Ysh1p/Brr5p, Pfs2p, Fip1p, and possibly Pta1p, contribute to a protein-protein interaction surface which acts in the assembly of the 3'-end formation machinery and which appears to be conserved in evolution (Fig. 5D).

Our analysis of Ydh1p suggests that the protein is an important constituent of protein interaction surfaces which act in the association of 3'-end processing factors with RNAP II, the recognition of poly(A) signal sequences and the assembly of an active 3'-end formation complex on the RNA substrate.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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