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16. The disparate effects of *brr5-1* on cleavage in vivo and in vitro are reminiscent of previous observations of a poly(A) polymerase mutant, which affects only polyadenylation in vitro [D. Patel and J. S. Butler, *Mol. Cell. Biol.* **12**, 3297 (1992)], but also affects cleavage site choice in vivo [E. Mandart and R. Parker, *ibid.* **15**, 6579 (1995)]. These observations suggest that the cleavage and polyadenylation steps may be more strictly coupled in vivo than in vitro.
17. Three copies of the hemagglutinin epitope were inserted before the stop codon of the *BRR5/YSH1* ORF. The corresponding replicative plasmid carrying a *URA3* marker and the epitope-tagged version of *BRR5/YSH1* was transformed into the diploid strain heterozygous for the *BRR5/YSH1* disruption. The transformants were sporulated, and the resulting ascospores were dissected. Ascospores prototrophic for uracil and leucine were viable, showing that the epitope-tagged version of the gene is able to complement the gene disruption.
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Sequence Similarity Between the 73-Kilodalton Protein of Mammalian CPSF and a Subunit of Yeast Polyadenylation Factor I

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The 3′ ends of most eukaryotic messenger RNAs are generated by endonucleolytic cleavage and polyadenylation. In mammals, the cleavage and polyadenylation specificity factor (CPSF) plays a central role in both steps of the processing reaction. Here, the cloning of the 73-kilodalton subunit of CPSF is reported. Sequence analyses revealed that a yeast protein (Ysh1) was highly similar to the 73-kD polypeptide. Ysh1 constitutes a new subunit of polyadenylation factor I (PFI), which has a role in yeast pre-mRNA 3′-end formation. This finding was unexpected because in contrast to CPSF, PFI is only required for the polyadenylation reaction. These results contribute to the understanding of how 3′-end processing factors may have evolved.

Almost all eukaryotic pre-mRNAs are cleaved endonucleolytically and are subsequently polyadenylated. In mammals, this process depends on the AAUAAA polyadenylation signal located 10 to 30 nucleotides (nt) upstream of the cleavage site and on a U- or a G- and U-rich "downstream element" (*1*). The polyadenylation signal is highly conserved and is recognized by the cleavage and polyadenylation specificity factor (CPSF), the only protein in addition to poly(A) polymerase (PAP) required for both cleavage and polyadenylation. Apart from conferring specificity to both steps of the reaction, CPSF, together with the poly(A) binding protein II (PAB II), also increases the processivity of PAP during tail elongation (*2*). Three additional components are required only for the cleavage of precursor RNA; these are cleavage factors CFI_m and $CFII_m$ and cleavage stimulation factor CstF (*3*).

Although the basic mammalian pre-mRNA 3′-processing reaction is similar in yeast, the sequence requirements of the RNA substrate are different. A "positioning element" is present 16 to 27 nt upstream of the cleavage site, the efficiency of which is modulated by an "efficiency element" further upstream (*4*). Both of these elements are A- and U-rich. Biochemically, four chromatographic fractions have been identified that are required for the yeast 3′-end processing reaction in vitro. Cleavage factor I (CFI_y) is required for cleavage and polyadenylation, whereas cleavage factor II ($CFII_y$) is only necessary for cleavage. The polyadenylation reaction is performed by CFI_y, polyadenylation factor I (PFI), and PAP (*5*).

A low, but significant sequence similarity has been reported between the 77- and 64-kD proteins of mammalian CstF and the Rna14 and Rna15 subunits of yeast CFI_y, respectively (*6*). So far, PAP has been shown to be the only 3′-processing component that is highly conserved between yeast and mammals.

Mammalian CPSF consists of four subunits with apparent molecular masses of 160, 100, 73, and 30 kD (*7*, *8*). The 160- and 30-kD polypeptides are in close contact with the AAUAAA polyadenylation signal (*1*, *7*). So far, only the 160- and 100-kD subunits have been cloned (*7*, *8*). An affinity-purified antiserum to gel-purified 73-kD subunit was used to screen a cDNA expression library. This library was rescreened with an NH_2-terminal DNA probe to obtain full-length clones (*9*). The assembled sequence of the cDNAs was 2351 bp in length, corresponding to a single band of 2.4 kb detected on Northern (RNA) blots (*10*). It contains an open reading frame of 684 amino acids (aa) (Fig. 1) that is preceded by an in-frame stop codon 51 nt upstream. The protein has a predicted molecular mass of 77.5 kD. Three tryptic peptide fragments sequenced independently (*11*) were found in the open reading frame (Fig. 1).

To prove that the cloned cDNAs code for the 73-kD subunit of CPSF, we expressed a COOH-terminal fragment starting at position 173 in *Escherichia coli* (*12*). Three monoclonal antibodies to the 73-kD subunit of bovine CPSF also recognized this recombinant protein (*12*). Polyclonal antibodies to the recombinant polypeptide (*12*) recognize the 73-kD protein in purified CPSF (Fig. 2, lane 1) and in purified CPSF that had been immunoprecipitated with a monoclonal antibody to the 100-kD subunit of CPSF (Fig. 2, lane 2). This antibody specifically coprecipitates all four subunits of CPSF (*7*). No signal was detected when CPSF was immunoprecipitated with a control antibody (Fig. 2, lane 3), when no antibody was added to the precipitation (Fig. 2, lane 4), nor when preimmune serum was used (Fig. 2, lane 5).

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A search of the DNA and protein databases (September 1996) revealed striking sequence homologies. As shown in Fig. 1, the 73- and 100-kD subunits of CPSF are significantly related (23% identity, 49% similarity). A number of ESTs from both human and *Caenorhabditis elegans* probably code for homolog of the 73-kD protein (*10*). More surprisingly, three open reading frames with unknown function from the archaebacterium *Methanococcus jannaschii* (*13*) are between 30% and 34% identical to the mammalian protein (see below).

A putative homolog of the 73-kD CPSF subunit is present on chromosome XII of *Saccharomyces cerevisiae* (*14*). The protein is 53% identical (73% similar) to the bovine 73-kD subunit of CPSF over the first 500 aa (Fig. 1). It codes for a protein of unknown function of 779 aa with a predicted molecular mass of 87 kD. Disruption of the open reading frame is lethal, demonstrating that this gene is essential for viability (*10*). We named it *YSH1* for yeast 73-kD homolog 1. In a screen for splicing mutants Noble and Guthrie identified a gene called *BRR5* (*15*), which is identical to *YSH1* (*16*). In addition, a putative open reading frame of 188 aa on yeast chromosome XV (systematic code YOR179c) is 43% identical to the 225 COOH-terminal amino acids of Ysh1/Brr5 (that is, the part that is not homologous between the 73-kD subunit of CPSF and Ysh1/Brr5).

We next examined whether the *YSH1*-encoded protein is involved in 3′-end processing. Chen and Moore have described four chromatographic fractions required for yeast 3′-end processing in vitro based on their separation on a Mono Q–anion exchange column (*5*). To test whether Ysh1 protein elutes from a Mono Q column at the same position as one of these factors, we generated an antiserum to the COOH-terminus of Ysh1 expressed in *E. coli* (*17*). This antibody detected Ysh1 only in the fractions also containing Fip1 (*10*), a subunit of PFI (*18*). A *fip1-1* mutant extract is impaired in polyadenylation but can still specifically cleave an RNA substrate; polyadenylation activity can be restored by addition of PFI fractions (*18*). To check biochemically whether Ysh1 is a subunit of PFI, we used antibody to Ysh1 (anti-Ysh1) to immunodeplete Mono S fractions containing purified PFI (*19*) and tested the supernatant for specific polyadenylation of a *CYC1* precleaved RNA, that is, a substrate already ending at the natural cleavage site, by complementation of a *fip1-1* mutant extract (*18*). Lanes 3 and 4 of Fig. 3A show the PFI activity of the Mono S fraction used for the depletion. Treatment of this fraction with the anti-Ysh1 antiserum completely depleted PFI activity (lanes 7 and 8), whereas the control depletion with preimmune serum had no effect (lanes 5 and 6). The depletion with the antibody to Ysh1 was specific because the polyadenylation reaction was restored by the addition of highly purified PFI [Mini Q fraction; (*19*)] to the depleted reaction (lane 9). In addition, immunoprecipitations with antibodies to epitope-tagged Fip1 quantitatively coprecipitated Ysh1, as assessed by immunoblotting (*10*). These results demonstrate that the yeast homolog of the 73-kD subunit of bovine CPSF is a subunit of PFI.

By analogy with *fip1-1*, conditional alleles of *YSH1* should allow the cleavage reaction to occur, but should be unable to polyadenylate the 5′-cleavage product. To address this question, we constructed a strain (LM111) in which the Ysh1 protein could be conditionally depleted. Depletion relied on a destabilizing element in combination with a regulatable promoter (*20*). A plasmid (pGUR-YSH1) expressing a fusion of the ubiquitin sequence to Ysh1 was con-

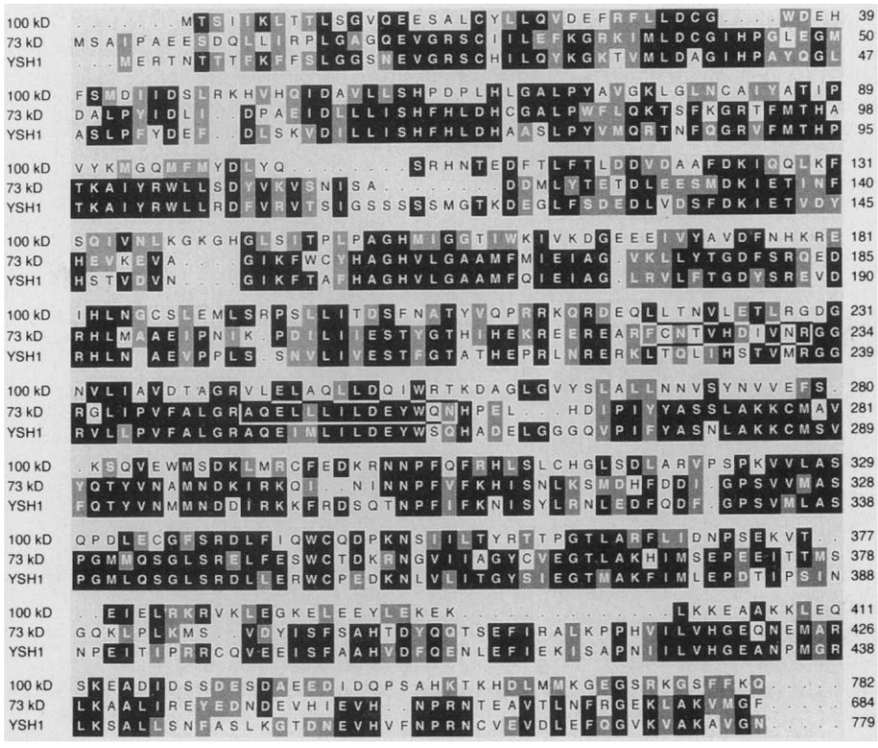

Fig. 1. Amino acid sequence comparison of the 100-kD and 73-kD subunits of CPSF and YSH1; only the conserved regions are shown. Two tryptic peptide sequences derived from the 73-kD subunit of CPSF are boxed. Amino acids identical or conserved between either two sequences are highlighted in black and grey, respectively. The EMBL accession number for the 73-kD coding sequence is X95906. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

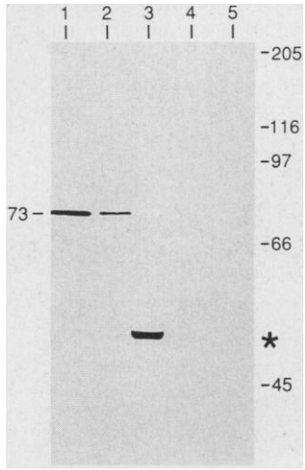

Fig. 2. Antibodies to the cloned cDNA expressed in *E. coli* recognize the 73-kD subunit of CPSF on an immunoblot. (Lane 1) Purified CPSF [Superose 6 fraction (*7*)]. (Lanes 2 and 5) CPSF immunoprecipitated with monoclonal antibody J1/27 to the 100-kD subunit of CPSF (*7*). (Lane 3) Immunoprecipitation of CPSF with an unrelated control antibody. (Lane 4) No CPSF added to the immunoprecipitation. Lanes 1 to 4 were probed with the antiserum to the histidine-tagged fusion protein, lane 5 with preimmune serum (at a dilution of 1:10000 each). The asterisk marks the position of the immunoglobulin G (IgG) heavy chains used for the immunoprecipitation, which cross-react with the secondary antibody (*25*).

structed such that cleavage after the ubiquitin moiety by a cellular deubiquitinylating enzyme results in a protein with the open reading frame that starts with an arginine (R). According to the N-end rule (*20*), such a protein is extremely unstable. Under the control of a UAS-GAL-CYC1 hybrid promoter the ubiquitin-R-Ysh1 fusion protein can rescue a chromosomal deletion of *YSH1* on 2% galactose-containing medium, but not on medium supplemented with 5% glucose, that is, when transcription is repressed.

3′-End processing extracts were prepared from LM111 cells before and after a shift to glucose-containing medium for various lengths of time (*21*). Immunoblot analysis confirmed that the amount of Ysh1 protein decreases in the extracts following shift (*10*). Likewise, the amount of Fip1 decreases, indicating that depletion of Ysh1 leads to degradation of PFI. In contrast, the amount of CFI_y, as assessed by immunoblot analysis with antibodies to Rna15, remained constant over the time of the experiment (*10*). In Fig. 3B, cleavage of a *CYC1* pre-mRNA was assayed separately from polyadenylation. Cleavage activity was only modestly affected in extracts prepared from LM111 cells after a shift to repressive conditions (lanes 8 to 10), as compared to extracts from cells grown in galactose, wild-type cells, or *fip1-1* mutant cells (lanes 7, 3, and 6, respectively). In contrast, CFI_y mutant extracts [*rna14-1* and *rna15-1* (*5*)] are unable to cleave at all (lanes 4 and 5).

In coupled cleavage-polyadenylation assays (Fig. 3C) extracts from LM111 grown in galactose cleave and polyadenylate with similar efficiency as wild-type extracts (compare lanes 7 and 3). However, after shift to glucose-containing medium for the times indicated on the top of lanes 8 to 10 the extracts progressively lost the ability to process the substrate. A similar decrease in polyadenylation activity was observed when a precleaved *CYC1* RNA was used (*10*). Activity could be restored by addition of purified PFI to extracts prepared from cells shifted to glucose for 12 hours, confirming that Ysh1 is a subunit of PFI (lane 11). In contrast to *fip1-1* extracts, Ysh1-depleted extracts do not accumulate the upstream cleavage product. Even though we did not detect any Ysh1 in column fractions that restored cleavage activity in in vitro reconstitution assays [CFI_y, $CFII_y$; (*5*, *10*)], we cannot rule out the possibility that depletion of Ysh1 (and thus PFI) may affect cleavage activity in the coupled cleavage and polyadenylation assay. It is possible that disintegration of PFI eventually destabilizes $CFII_y$ as well. However, this effect is much less pronounced under conditions where polyadenylation is prevented (Fig. 3B).

Neither the 73-kD subunit of CPSF nor bovine-yeast hybrid genes could replace a Ysh1 null mutation in vivo. The COOH-terminally truncated forms of Ysh1 could not replace the wild-type gene either, an indication that the nonhomologous part of the protein is essential (*10*).

We previously cloned the 160- and 100-kD subunits of bovine CPSF (*7*). Here, we describe the cloning of a third component of this multimeric factor, the 73-kD subunit. This protein shows several interesting features. First, a low, but significant homology between the 73- and the 100-kD components of CPSF (Fig. 1) suggests that these subunits have evolved from a common ancestral gene and have related functions in CPSF. Second, we identified Ysh1/Brr5 as a new component of yeast PFI based on the high conservation of the amino acid sequence between these two proteins. This is the most conserved component (so far) of the 3′-end processing machinery between these two highly divergent organisms. Besides Ysh1/Brr5 and the 73-kD protein, only the poly(A) polymerases have a comparable degree of sequence homology [47% identity between the yeast and bovine enzymes; (*22*)]. It is likely that key functions in the 3′-end formation of pre-mRNAs are exerted by proteins that have been highly conserved throughout evolution, such as the poly(A) polymerases, the 73-kD subunit of CPSF, and the Ysh1/Brr5 proteins.

In the same vein, it can be predicted that functional homologs of the other mammalian 3′-processing components exist in yeast. Indeed, recent work on the composition of purified PFI indicates that besides Fip1 this factor contains several additional polypeptides, including homologs of the 160-, 100-, and 30-kD subunits of CPSF [U28374, Z73287, and (*23*)]. In mammals, CPSF confers specificity to the cleavage and the polyadenylation reactions. It is conceivable that PFI, like CPSF, acts as an enhancer of poly(A) polymerase processivity during poly(A) tail elongation. The Fip1 subunit of PFI tethers PAP1

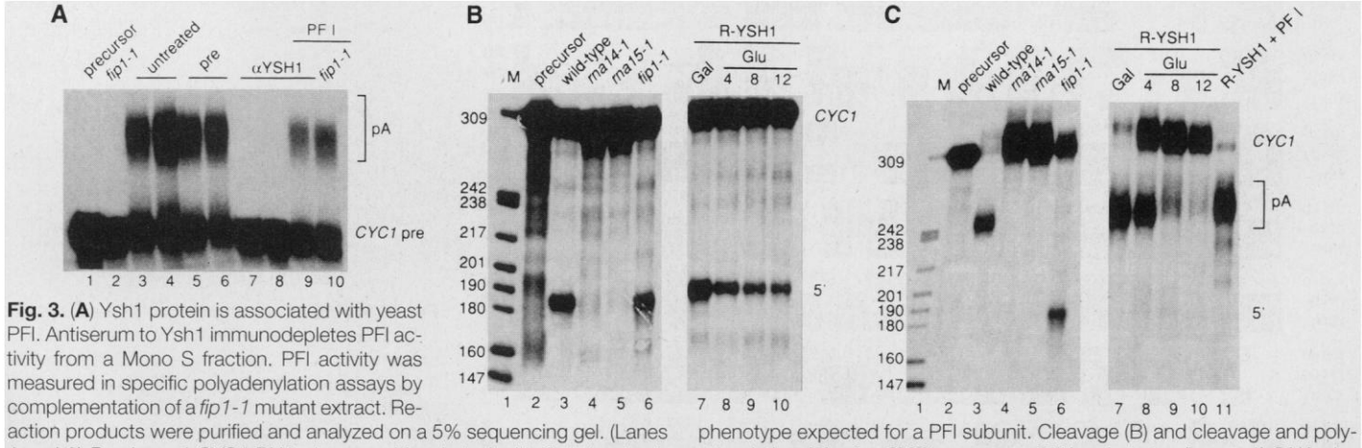

Fig. 3. (**A**) Ysh1 protein is associated with yeast PFI. Antiserum to Ysh1 immunodepletes PFI activity from a Mono S fraction. PFI activity was measured in specific polyadenylation assays by complementation of a *fip1-1* mutant extract. Reaction products were purified and analyzed on a 5% sequencing gel. (Lanes 1 and 2) Precleaved *CYC1* RNA alone, and incubated with *fip1-1* extract; (lanes 3 and 4) 1 and 2 μl, respectively, of untreated input fraction; (lanes 5 and 6) 2 and 4 μl, respectively, of PFI fraction, depleted with preimmune serum (pre); (lanes 7 and 8) 2 and 4 μl, respectively, of PFI fraction, depleted with antibodies to YSH1 (αYSH1). After depletion, polyadenylation could be restored by the addition of 2 μl of pure PFI (lane 9). The same PFI fraction also restored polyadenylation activity to the *fip1-1* extract (lane 10). The positions of the precursor (*CYC1* pre) and the polyadenylated RNA products are indicated on the right. (**B** and **C**) In vivo depletion of Ysh1 leads to the phenotype expected for a PFI subunit. Cleavage (B) and cleavage and polyadenylation (C) of a *CYC1* precursor RNA substrate. (Lanes 1) DNA size standard indicated in nucleotides. (Lanes 2) Precursor RNA. (Lanes 3) Activity of a wild-type yeast extract. (Lanes 4, 5, and 6) Processing activities of *rna14-1*, *rna15-1*, and *fip1-1* mutant extracts, respectively. (Lanes 7) Activity of LM111 (*20*) grown in medium containing galactose. (Lanes 8 to 10) 3′-Processing activity of the same strain after shift to medium containing glucose for the time indicated at top of the lanes (hours). [Lane 11 in (C)] The deficient polyadenylation activity of LM111 shifted to glucose for 12 hours is restored upon addition of pure PFI.

to CFI_y (*18*). Given that in mammals CPSF interacts with poly(A) polymerase, cleavage factors, and the pre-mRNA, it is possible that the CPSF-related subunits of PFI also participate in specific interactions with CFI_y as well as with the RNA substrate. Thus in yeast, specificity and processivity may be conferred upon poly(A) polymerase by the combined action of CFI_y and PFI. The reason why PFI is not required for the cleavage reaction, in spite of its homology with CPSF, might be because the poly(A) polymerase itself does not participate in cleavage (*5*). However, the fact that neither the signal sequences on the RNA substrates nor the protein factors are interchangeable between yeast and metazoans may reflect subtle but important differences in protein-protein and RNA-protein interactions between the polyadenylation systems of yeast and metazoans.

Do bacteria have CPSF or PFI? Three open reading frames of unknown function in the M. *jannaschii* (*13*) are significantly related to the 73-kD protein and to Ysh1/Brr5. In view of recent surprising findings in the field of bacterial polyadenylation (*26*), this raises the question whether these proteins are involved in pre-mRNA 3′-end formation. However, M. *jannaschii* does not encode homologs of the 160-, 100-, or 30-kD subunits of CPSF nor of Fip1. As far as sequence data are available, this bias also holds true for eubacteria (*10, 16*). It thus remains an open question as to which degree pre-mRNA 3′-end formation is conserved between the different domains of life. In any case, the high degree of sequence conservation suggests that Ysh1/Brr5 and its related proteins have important and essential functions.

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9. A rabbit was injected three times with purified CPSF 73-kD subunit and once with complete CPSF. The serum was affinity-purified and used for screening a calf aorta endothelial cDNA expression library (Stratagene 936705) as described (*24*). Additional clones were obtained with a NH_2-terminal probe (*24*).
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12. A cDNA fragment starting at the Hind III site and containing the authentic stop codon was subcloned into pQE11 (Qiagen) and expressed in *Escherichia coli* (*7*). A rabbit was injected four times with 100 μg of protein (*25*). Monoclonal antibodies were obtained from the fusion described earlier (*7*).
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17. *YSH1* was cloned by PCR and the screening of a genomic library (*10*). A truncated form of *YSH1* starting at the internal Bgl II site and containing the authentic stop codon was subcloned into pQE10 (Qiagen) and expressed in *E. coli* (*7*). A rabbit was injected four times with 100 μg of SDS-polyacrylamide gel eluted fusion protein (*25*).
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Dependence of Yeast Pre-mRNA 3′-End Processing on CFT1: A Sequence Homolog of the Mammalian AAUAAA Binding Factor

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3′-End formation of pre-mRNA in yeast and mammals follows a similar but distinct pathway. In *Saccharomyces cerevisiae*, the cleavage reaction can be reconstituted by two activities called cleavage factor I and II (CFI and CFII). A CFII component, designated CFT1 (cleavage factor two) was identified by its sequence similarity to the AAUAAA-binding subunit of the mammalian cleavage and polyadenylation specificity factor (CPSF), even though the AAUAAA signal sequence appears to play no role in yeast pre-mRNA 3′ processing. Depletion of a yeast whole-cell extract with antibodies to CFT1 protein abolished cleavage and polyadenylation of pre-mRNAs. Addition of CFII restored cleavage activity, but not polyadenylation. Polyadenylation required the further addition of poly(A) polymerase and polyadenylation factor I, suggesting a close but not necessarily direct association of these two factors with the CFT1 protein.

Most eukaryotic pre-mRNAs are processed at their 3′ ends by endonucleolytic cleavage and subsequent addition of a poly(A) tail at the upstream fragment (*1*). Some studies have provided evidence for a possible evolutionary link between yeast and mammalian trans-acting factors involved in 3′-end formation (*2–4*). The poly(A) polymerase (PAP) has so far revealed the highest sequence similarity among the factors identified for cleavage and polyadenylation in yeast and mammals (*5*). The two known subunits of CFI (*6*), the Rna14 and Rna15 proteins (*7*), have been proposed to be the yeast counterparts of the 77-kD and 64-kD subunits of the mammalian cleavage stimulatory factor (CstF) (*2, 3*). Nevertheless,

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