molecular clouds hold typical averaged translational temperatures of 10 K, whereas circumstellar shells around carbon stars are heated up to ~4000 K, giving mean translational energies of about 0.1 and 40 kJ mol^{-1}, respectively. Therefore, comparable amounts of both isomers are anticipated to be produced in dark clouds, whereas less c-C_3H than l-C_3H should be formed in the hotter envelope surrounding carbon stars such as IRC+10216. This expected pattern is reflected in the observed number density ratios of c-C_3H versus l-C_3H. Therefore a common HCCH reactant for the formation of interstellar l/c-C_3H radicals via atom-neutral reaction with $C(^3P_j)$ must be included into interstellar reaction networks, taking account of distinct structural isomers.

This work is a step toward a better understanding of reactions of neutral atoms with neutral reactants in the interstellar medium. The direct observation of the C-H exchange channel represents a versatile synthetic route to reactive hydrocarbon radicals in the ISM. Interstellar environments of unsaturated hydrocarbons such as methylacetylene (CH_3CCH), ethinyl (C_2H), vinyl (C_2H_3), ethylene (C_2H_4), and propylene (C_3H_6) which overlap with large concentrations of atomic carbon should be sought. Once these regions have been charted, the search for hitherto unobserved interstellar radicals as reaction products of these atom-neutral reactions is open.

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Essential Yeast Protein with Unexpected Similarity to Subunits of Mammalian Cleavage and Polyadenylation Specificity Factor (CPSF)

Guillaume Chanfreau,* Suzanne M. Noble,* Christine Guthrie†

The 3′ ends of most eukaryotic messenger RNAs are generated by internal cleavage and polyadenylation. In mammals, there is a strict dependence of both reactions on the sequence AAUAAA, which occurs upstream of polyadenylation [poly(A)] sites and which is recognized by CPSF. In contrast, cis-acting signals for yeast 3′-end generation are highly divergent from those of mammals, suggesting that trans-acting factors other than poly(A) polymerase would not be conserved. The essential yeast protein Brr5/Ysh1 shows sequence similarity to subunits of mammalian CPSF and is required for 3′-end processing in vivo and in vitro. These results demonstrate a structural and functional conservation of the yeast and mammalian 3′-end processing machineries despite a lack of conservation of the cis sequences.

The 3′ ends of most eukaryotic mRNAs are generated by a two-step mechanism in which endonucleolytic cleavage of the transcript is closely coupled with poly(A) addition (*1*). The virtually invariant sequence AAUAAA that lies 10 to 30 nucleotides upstream of mammalian poly(A) sites is essential to poly(A) site recognition and 3′-end formation (*1*). CPSF comprises three (*2*) to four (*3*) subunits and likely recognizes the AAUAAA sequence via the 160-kD subunit (*1*). In contrast, the sequences adjacent to poly(A) sites in yeast are highly divergent from those of mammals (*4*). Fractionation of yeast extracts has identified three fractions which, together with poly(A) polymerase, are necessary and sufficient to reconstitute cleavage and polyadenylation in vitro (*5*). Cleavage factor I (CFI) is required for both steps, cleavage factor II (CFII) is required only for cleavage, and polyadenylation factor I (PFI) is required solely for polyadenylation. Although a number of yeast 3′ processing factors have now been cloned and characterized (*6*, *7*), none to date share sequence similarity with known CPSF subunits. In

Department of Biochemistry and Biophysics, UCSF School of Medicine, San Francisco, CA 94143–0448. E-mail: guthrie@cgl.ucsf.edu

*Contributed equally to this work.
†To whom correspondence should be addressed.

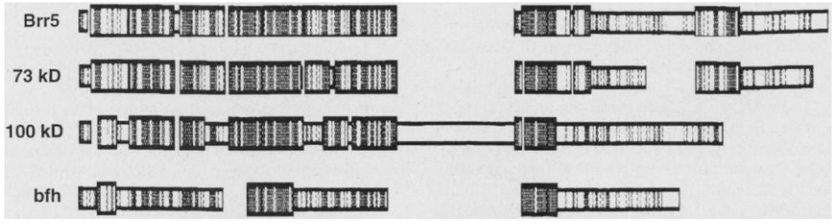

Fig. 1. Brr5/Ysh1 shows sequence similarities to CPSF subunits and a cyanobacterial sequence. Shown is a schematic alignment between Brr5/Ysh1, the 73-kD [provided by Jenny *et al.*; (*13*)] and 100-kD subunits of bovine CPSF, and the bfh sequence from the cyanobacterium *Synechocystis*. The alignment was made with MACAW, and the segment overlap search method was used with a cutoff score of 48. Shading indicates regions of identity and boxes, blocks of strongest similarity, between two or more sequences. Brr5/Ysh1 also exhibit significant sequence relatedness to two other predicted yeast proteins: L9354.1 (GenBank U53878; 859 amino acids long; 20% identical) and a predicted protein from Chromosome XV (code PLF188 of MIPS; 188 amino acids; 43% identical to the COOH-terminal third of Brr5/Ysh1). Accession number for Brr5/Ysh1: PIR S51413; Accession number for bfh: PID g1001329.

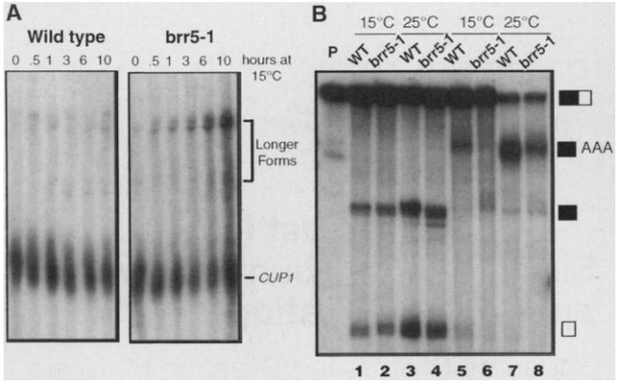

Fig. 2. Cold-sensitivity of 3′-end processing in the *brr5-1* mutant. (**A**) Accumulation of elongated forms of *CUP1* mRNAs in *brr5-1* cells at the nonpermissive temperature. *brr5-1* cells transformed either with a pSE360 plasmid (*10*) carrying *BRR5/YSH1* (WT) or with the vector pSE360 alone (*brr5-1*) were grown to midlog phase at 30°C in synthetic complete medium lacking uracil. The cultures were then shifted to 15°C, and 20-ml samples were removed at the indicated times for total RNA preparation. RNA blot analysis was performed as described (*10*), with a uniformly ^{32}P-labeled Xba I–Kpn I fragment of pWF1 (*14*). (**B**) Cold sensitivity of polyadenylation in *brr5-1* mutant extracts. P, uncleaved *CYC1* precursor. Black box, 5′ cleavage fragment; Grey box, 3′ cleavage product; Black box followed by 3As, polyadenylated 5′ fragment. Extracts from a wild-type or *brr5-1* cells were prepared as described (*5*), except that the cells were lysed by grinding under liquid nitrogen (*23*). ^{32}P-labeled *CYC1* transcript was incubated for 30 min at the indicated temperature as described (*6*). In lanes 1 to 4, ATP was replaced by CTP, and magnesium acetate was replaced by EDTA. This allows the study of the cleavage reaction uncoupled to polyadenylation by inhibiting subsequent polyadenylation of the 5′ cleavage product and degradation of the 3′ product (*6*).

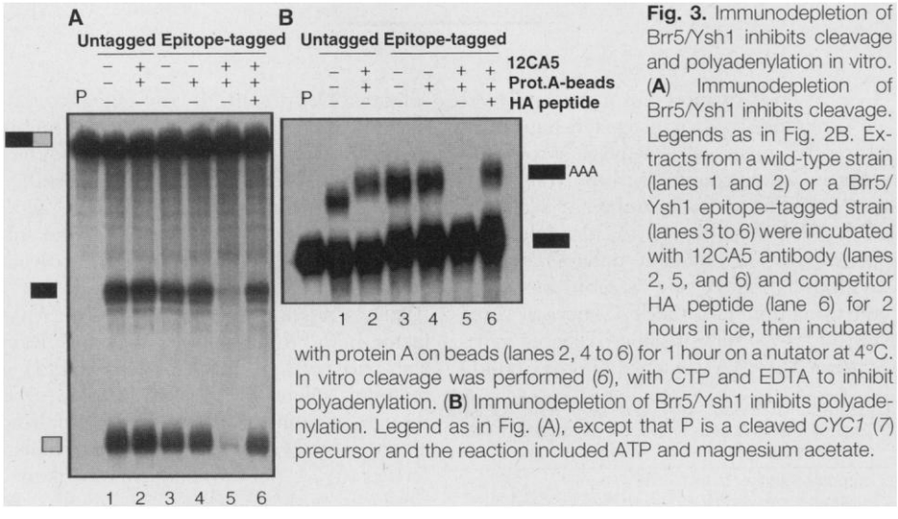

Fig. 3. Immunodepletion of Brr5/Ysh1 inhibits cleavage and polyadenylation in vitro. (**A**) Immunodepletion of Brr5/Ysh1 inhibits cleavage. Legends as in Fig. 2B. Extracts from a wild-type strain (lanes 1 and 2) or a Brr5/Ysh1 epitope–tagged strain (lanes 3 to 6) were incubated with 12CA5 antibody (lanes 2, 5, and 6) and competitor HA peptide (lane 6) for 2 hours in ice, then incubated with protein A on beads (lanes 2, 4 to 6) for 1 hour on a nutator at 4°C. In vitro cleavage was performed (*6*), with CTP and EDTA to inhibit polyadenylation. (**B**) Immunodepletion of Brr5/Ysh1 inhibits polyadenylation. Legend as in Fig. (A), except that P is a cleaved *CYC1* (*7*) precursor and the reaction included ATP and magnesium acetate.

fact, poly(A) polymerase has been the only component with strong conservation between yeast and mammals (*8*, *9*), prompting the hypothesis that the mechanisms of poly(A) site recognition and 3′-end generation may be fundamentally distinct in yeast and mammals.

The *brr5-1* mutant was identified in a screen for cold-sensitive pre-mRNA splicing mutants in *Saccharomyces cerevisiae* (*10*). We isolated the wild-type *BRR5/YSH1* gene and found it to encode a predicted polypeptide of 779 amino acids (*11*). Gene disruption analysis revealed that *BRR5/YSH1* is essential for viability (*11*). Surprisingly, the amino acid sequence showed sequence similarity through its entire length to the 100-kD subunit of bovine CPSF (*12*), with 23% identity and 48% similarity overall (Fig. 1). Even stronger similarity was apparent upon subsequent comparison to the 73-kD subunit of bovine CPSF [whose sequence was provided by Jenny *et al.*; (*13*)], with 53% identity in the first 500 amino acids (Fig. 1). Finally, Brr5/Ysh1 and the CPSF subunits bear sequence similarity to a predicted open reading frame from the cyanobacterium *Synechocystis*, which we name bfh for Brr five homolog (Fig. 1).

To test the involvement of Brr5/Ysh1 in yeast 3′-end formation, we first investigated whether the *brr5-1* mutation influences this reaction in vivo. Both *brr5-1* and isogenic wild-type cells were shifted to the restrictive temperature (15°C), and mRNAs from the *CUP1* gene were detected by RNA blotting (Fig. 2A) [*CUP1* was previously shown to be sensitive to defects in 3′ processing; (*14*)]. The accumulation of longer forms of *CUP1* transcripts in the *brr5-1* strain at the restrictive temperature suggests that the *brr5-1* mutation disrupts 3′-end processing and that the 3′ processing machinery is unable to efficiently recognize or cleave the proper 3′ processing signals (*15*). To confirm and extend this result in vitro, we prepared 3′ processing extracts from wild-type and *brr5-1* strains, with which radioactive precursors containing *CYC1* cleavage-polyadenylation signals were incubated at restrictive and permissive temperatures (Fig. 2B). Mutant extracts showed a defect in the polyadenylation step at the restrictive temperature (Fig. 2B, lanes 5 and 6). This defect was partially relieved at the permissive temperature (compare lanes 7 and 8), while cleavage was not affected (*16*). In an independent test of the role of Brr5/Ysh1 in vitro, we immunodepleted extracts containing an epitope-tagged version of the Brr5/Ysh1 protein (*17*). Both cleavage and polyadenylation were inhibited (Fig. 3, lane 5). This inhibition was specific for the depletion of Brr5/Ysh1 because

mock depletion with protein A–Sepharose beads alone (Fig. 3, lane 4) or immunodepletion of an extract from a strain containing untagged Brr5/Ysh1 (lane 2) resulted in no significant defect. Moreover, the inhibition of cleavage and polyadenylation was efficiently blocked by the addition of peptide competitor of the epitope tag (lane 6). These results could be explained by a model in which Brr5/Ysh1 functions in both steps of the 3′ processing reaction. Alternatively, Brr5/Ysh1 may associate with a factor required for cleavage in vitro, and this factor is coimmunodepleted with epitope-tagged Brr5/Ysh1. For example, immunodepletion of Fip1 from extracts inhibits not only polyadenylation, which one would expect for a component of PFI, but also decreases the efficiency of cleavage (*7*); the last-mentioned effect can be explained by the association of Fip1 with Rna14 (*7*), which is required for cleavage (*6*).

To determine whether Brr5/Ysh1 associates with other 3′ processing factors, we fractionated extracts by Mono Q chromatography [Fig. 4A; (*5*)]. Analysis of the fractions by immunoblotting revealed that most of the Brr5/Ysh1 protein cofractionated with Fip1, a component of PFI (*7*). Further evidence for the association of Brr5/Ysh1 with PFI was provided by the observation that antibodies to Fip1 (*7*) could coimmunoprecipitate Brr5/Ysh1 (*18*). In addition, CFII activity, defined by cleavage reconstitution assays, cofractionated with Brr5 and PFI (Fig. 4A). An association between CFII and PFI would offer an attractive explanation for the decreased cleavage activity observed on immunodepletion of Brr5/Ysh1 (Fig. 3A). Consistent with this possibility, fractions containing PFI and CFII and enriched in Brr5/Ysh1 can complement the cold sensitivity of polyadenylation in the *brr5-1* mutant extract (Fig. 4B), as well as the polyadenylation (Fig. 4B) and cleavage (Fig. 4C) defects of the immunodepleted extract. It is possible that, in vivo, CFI, CFII, PFI, and the poly(A) polymerase function together as a holoenzyme and that some aspects of this association can be maintained in vitro. There are precedents for this possibility in mammalian polyadenylation (*19*) and RNA polymerase II transcription (*20*), where multiple factors originally defined in vitro as independent activities have been shown to compose a preassembled complex.

We have now identified a yeast factor that is essential to 3′-end processing in vivo and in vitro. Brr5/Ysh1 displays sequence similarity throughout its length to two components of mammalian CPSF, suggesting that, despite striking differences in the cis requirements for 3′-end formation of mRNAs, the factors necessary for poly(A) site recognition and polyadenylation are conserved among eukaryotes. The existence of a prokaryotic protein bearing sequence similarity with Brr5/Ysh1 also raises the possibility that a CPSF-like activity may be responsible for the polyadenylation of 3′ ends of some eubacterial mRNAs (*21*). Originally, *brr5-1* was identified in a screen for pre-mRNA splicing mutants (*10*). The coupling of splicing and 3′-end formation and the communication between splicing and 3′-end processing components have been well-documented in mammals (*22*) but not in yeast. We have assessed splicing in both *brr5-1* and immunodepleted extracts, but have detected no defects in vitro (*18*). In vivo, however, the kinetics of the polyadenylation defect in *brr5-1* parallel those of the splicing defect [assayed by primer extension of *U3* and *RP51A* transcripts; (*10*)]. Thus, we cannot distinguish whether the splicing defect is indirect, or, more interestingly, whether a coupling between splicing and polyadenylation that occurs in vivo cannot be reproduced under standard in vitro conditions.

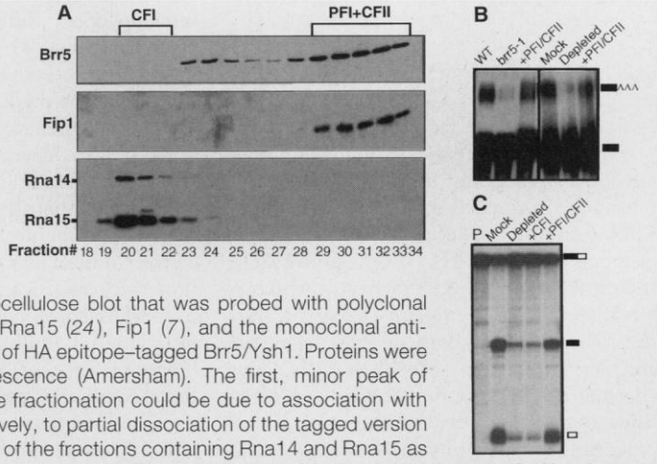

Fig. 4. Association of Brr5/Ysh1 with PFI and CFII. (**A**) Cofractionation of Brr5/Ysh1 with Fip1. The 3′ processing extracts from the Brr5/Ysh1 epitope–tagged strain were fractionated by Mono Q chromatography and a KCl gradient (*5*), and proteins were resolved by SDS-PAGE and transferred to a nitrocellulose blot that was probed with polyclonal antibodies to Rna14 and Rna15 (*24*), Fip1 (*7*), and the monoclonal antibody 12CA5 for detection of HA epitope–tagged Brr5/Ysh1. Proteins were detected by chemiluminescence (Amersham). The first, minor peak of Brr5/Ysh1 observed in the fractionation could be due to association with other proteins or, alternatively, to partial dissociation of the tagged version of the protein. The identity of the fractions containing Rna14 and Rna15 as CFI was confirmed by assays for cleavage activity (*18*). The CFII fractions were identified by cleavage activity, and found to be overlapping with PFI (*18*). Therefore, using the protocol described (*5*), we were unable to resolve the CFII and PFI activities. (**B**) Complementation of the polyadenylation defect of the *brr5-1* and immunodepleted extracts. Cleaved *CYC1* substrate (*7*) was incubated with wild-type (WT) or mutant extract (*brr5-1*) at 15°C, and in mock-depleted or immunodepleted extract at 30°C, with 4 μl of buffer D (*5*) or with 4 μl of fraction 32 (Fig. 4A) enriched in Brr5/Ysh1 and containing the PFI and CFII activities. Mock-depleted or depleted extracts were incubated at 30°C. (**C**) Complementation of the cleavage defect of the immunodepleted extract. P, Full-length *CYC1* transcript. The RNA was incubated at 30°C with 4 μl of buffer D or 4 μl of fraction 20 (CFI) or 32 (PFI-CFII). The addition of CFI was unable to complement the defect.

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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

Andreas Jenny,* Lionel Minvielle-Sebastia, Pascal J. Preker, Walter Keller†

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).

Department of Cell Biology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland. The first three authors contributed equally to this work.

*Present address: EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.
†E-mail: Keller2@ubaclu.unibas.ch