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6. Our calculations used the CACSD(T) (single- and double-excitation coupled cluster with a perturbative estimate of triple excitations) level [K. Raghavachari, G. W. Trucks, J. A. Pople, M. Head-Gordon, Chem. Phys. Lett. 157, 479 (1989)] based on restricted Hartree-Fock (UHF) wave functions. Only the pure spherical harmonic components of d, f, and g functions were included. The ACES II program package was used [J. F. Stanton, J. Gauss, J. D. Delos, R. K. Hodel, R. J. Bartlett, Int. J. Quantum Chem. Symp. 26, 879 (1992)]. Structures of local minima were fully optimized using a triple zeta polarization (TZP) basis set [A. Schäfer, H. Horn, R. Ahlrichs, J. Chem. Phys. 97, 2571 (1992)], and stationary points were characterized by vibrational frequencies within the harmonic approximation. Vibrational frequencies were computed numerically with analytical CIPSD(T) gradients [J. D. Watts, J. Gauss, R. J. Bartlett, Chem. Phys. Lett. 200, 1 (1992)]. Energy differences and reaction energies were obtained by single-point calculations with a quadruple zeta double polarization [QZ2P; H:7s2p1d1f (4s2p2d1f); C: (11s7p2d1f) [6s5p3d2f[1]; A. Schäfer, H. Horn, R. Ahlrichs, J. Chem. Phys. 97, 2571 (1992)] and a correlation consistent polarized valence quadruple zeta [cc-pVQZ; H:6s3p2d1f (4s2p2d1f); C:12s9p6d7f5p5d4s2d2f1g] (T. H. Dunning, J. Chem. Phys. 90, 1007 [1988]) basis. Zero-point energies were included as computed at the CACSD(T)/TZP level; the zero-point vibrational energy of c-C3H was taken from J. F. Stanton [Chem. Phys. Lett. 237, 20 (1995)].


11. Y. T. Lee, D. J. McDonald, P. R. LeBreton, D. R. Herschbach, Rev. Sci. Instrum. 40, 1402 (1969); Y. T. Lee, Science 236, 793 (1987). The peak velocities of the carbon beam were determined to 1180, 2463, and 3196 ms−1 and those of the acetylene beam to 866 ms−1.


15. In this framework, the C atom orbits the HCH molecule prior to reaction. The HCH molecules are treated as point masses, and therefore sterical effects do not play a role in this simple model [R. D. Levine and B. R. Bernstein, Molecular Reaction Dynamics and Chemical Reactivity (Oxford University Press, Oxford, 1987)]. Therefore, any deviation from this theory indicates that the actual structure of the HCH molecule plays a significant role.


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

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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 machinery 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 3′ ends of both sequences AAUAAA, that is, 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

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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 br5-1 mutant. (A) Accumulation of elongated forms of CUP1 mRNAs in br5-1 cells at the nonpermissive temperature. br5-1 cells transformed either with a pSE360 plasmid (WT) or with the vector pSE360 alone (br5-1) 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 32P-labeled Xba I-Kpn I fragment of pWF1 (14). (B) Cold sensitivity of polyadenylation in br5-1 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 br5-1 cells were prepared as described (5), except that the cells were lysed by grinding under liquid nitrogen (23). 32P-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 br5-1 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 br5-1 mutation influences this reaction in vivo. Both br5-1 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 br5-1 strain at the restrictive temperature suggests that the br5-1 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 br5-1 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 Br5/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 Br5/Ysh1 functions in both steps of the 3' processing reaction. Alternatively, Br5/Ysh1 may associate with a factor required for cleavage in vitro, and this factor is coimmunoprecipitated with epitope-tagged Br5/Ysh1. For example, immunodepletion of Fip1 from extracts inhibits not only polyadenylation, which one would expect for a component of FPI, 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 Br5/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 Br5/Ysh1 protein cofractionated with Fip1, a component of FPI (7). Further evidence for the association of Br5/Ysh1 with FPI was provided by the observation that antibodies to Fip1 (7) could coimmunoprecipitate Br5/Ysh1 (18). In addition, CFII activity, defined by cleavage reconstitution assays, cofractionated with Br5 and FPI (Fig. 4A). An association between CFII and FPI would offer an attractive explanation for the decreased cleavage activity observed on immunodepletion of Br5/Ysh1 (Fig. 3A). Consistent with this possibility, fractions containing FPI and CFII and enriched in Br5/Ysh1 can complement the cold sensitivity of polyadenylation in the br5-1 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, CFII, FPI, and the poly(A) polymerase function together as a holozyme 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. Br5/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 Br5/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, br5-1 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 br5-1 and immunodepleted extracts, but have detected no defects in vitro (18). In vivo, however, the kinetics of the polyadenylation defect in br5-1 parallel those of splicing defects [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.

REFERENCES AND NOTES

9. Sequences similarities have been noted between the 77-kD and the 64-kD subunits of human CstF and yeast Rna14 and Rna15, respectively (Y. Takagaki and J. L. Manley, Nature 372, 471 (1994)). However, these similarities are not very strong or restricted to an RNA-binding domain.
11. A minimal fragment of 2.8 kb complementing the br5-1 mutant was identified by partial restriction enzyme digestion of the original complementing plasmid. Sequencing this insert revealed a single open reading frame (ORF). We confirmed that this ORF corresponded to Br5/Ysh1 (and not to a suppressor) by creating a wild-type strain in which the chromosomal version of the gene was marked by URA3. This strain was crossed to a ura3 br5-1 strain, the diploid was sporulated, and the meiotic progeny were assessed for cold sensitivity (cs) and uracil auxotrophy. Among 18 tetrads, all of the cs spores were ura3 uracil auxotrophs, whereas all cold-resistant spores were uracil prototrophs, indicating linkage of the markers. To determine whether BR5/YSH1 is essential, we prepared a disrupt- ed version by replacing almost the entire coding sequence with the LEU2 gene. This disrupted allele was used to replace one copy of BR5/YSH1 in a wild-type diploid strain by homologous recombin- ation. A diploid heterozygous for BR5/YSH1 disruption was sporulated. The disrupted spores were dissected. Each of the 12 ascii dis- sectected gave rise to only two colonies, both leucine auxotrophs, indicating that BR5/YSH1 is essen- tial for viability.
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), 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 Cfl1 and Cfl1 and cleavage stimulation factor Csf (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 (CFL1) is required for cleavage and polyadenylation, whereas cleavage factor II (CFL2) is only necessary for cleavage. The polyadenylation reaction is performed by CFL1, 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 Csf and the Rna14 and Rna15 subunits of yeast Cfl1, 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 NH2-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 trypic 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 precipitates 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).

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 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

13. BRR5 was independently cloned and characterized by A. Jenny, L. Minvielle-Sebastia, P. Preker, and W. Keller as similar to the 73-kD subunit of CPSF and named YSH1 (Yeast Seventy-three Homolog 1; personal communication). We adopted the name BRR5/YSH1.
15. The total amount of poly(A) RNA was decreased in brr5-1 at the nonpermissive temperature. The poly(A) tail lengths in the brr5-1 strain showed a significant difference with the wild type, even at the nonpermissive temperature.
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 vivo (D. Patel and J. S. Butler, Mot. Cell Biol. 12, 3297 (1992), but also affects cleavage site choice in vivo (E. Mandart and R. Parker, ibid. 15, 6573 (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.
25. We thank W. Keller and F. Lacroute for antibodies and P. Preker for plasmids and helpful advice; A. Gamarnik and R. Andino for help with FPLC; A. Jenny, L. Minvielle-Sebastia, P. Preker, and W. Keller for communication of unpublished results; L. Esperras, C. Pudlov, and H. Roiba for technical assistance; A. Frankel, E. O'Shea, C. Siebel, and J. Staley for critical reading of the manuscript; H. Machani for noting the Synechocystis bfh sequence and major help with the manuscript; and members of the Guthrie laborator for sharing experimental expertise. Supported by NIH grant GM21119 (C.G.), a Human Frontier Science Program long-term postdoctoral fellowship (G.C.), and an American Heart Association Predoctoral Fellowship (S.N.). C. G. is an American Cancer Society Research Professor of Molecular Genetics.
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