The role of the Brr5/Ysh1 C-terminal domain and its homolog Syc1 in mRNA 3'-end processing in *Saccharomyces cerevisiae*

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ABSTRACT

The cleavage/polyadenylation factor (CPF) of *Saccharomyces cerevisiae* is thought to provide the catalytic activities of the mRNA 3'-end processing machinery, which include endonucleolytic cleavage at the poly(A) site, followed by synthesis of an adenosine polymer onto the new 3'-end by the CPF subunit Pap1. Because of similarity to other nucleases in the metallo- β -lactamase family, the Brr5/Ysh1 subunit has been proposed to be the endonuclease. The C-terminal domain of Brr5 lies outside of β -lactamase homology, and its function has not been elucidated. We show here that this region of Brr5 is necessary for cell viability and mRNA 3'-end processing. It is highly homologous to another CPF subunit, Syc1. Syc1 is not essential, but its removal improves the growth of other processing mutants at restrictive temperatures and restores in vitro processing activity to cleavage/polyadenylation-defective *brr5-1* extract. Our findings suggest that Syc1, by mimicking the essential Brr5 C-terminus, serves as a negative regulator of mRNA 3'-end formation.

Keywords: mRNA 3'-end processing; polyadenylation

INTRODUCTION

Production of functional eukaryotic mRNA requires the addition of a poly(A) tail at the 3'-end by a large complex whose composition is well-conserved from yeast to mammals. This nuclear 3'-end processing machinery recognizes signal sequences on the precursor RNA that specify the poly(A) site, cuts the RNA at this site, and synthesizes a polyadenosine tract onto the new 3'-end. In the yeast *Saccharomyces cerevisiae*, the cleavage/polyadenylation factor (CPF) works in conjunction with a second multi-subunit factor, CF I, to process the 3'-ends of mRNA precursor.

The catalytic subunit responsible for poly(A) addition, the Pap1 poly(A) polymerase, is a subunit of CPF. Recently, comparative protein sequence analysis has suggested that a second CPF component, Brr5/Ysh1 (Chanfreau et al. 1996;

Jenny et al. 1996), might be the nuclease. This protein and its mammalian homolog, CPSF-73, belong to a large family of metallo-hydrolytic enzymes with motifs resembling those forming the active-site fold of β -lactamases (Aravind 1999; Daiyasu et al. 2001; Callebaut et al. 2002). This family contains three proteins with demonstrated nuclease activity: a V(D)J DNA recombination/repair factor called Artemis (Ma et al. 2002), the Snm1 protein involved in DNA cross-link repair (Li et al. 2005), and RNase Z, an endonuclease that trims the 3'-ends of tRNA precursors (Schiffer et al. 2002). A study by Ryan et al. (2004) showed that highly conserved residues in the region of Brr5/Ysh1 with β -lactamase homology are essential for cell growth and that a protein with the properties of CPSF-73 could be cross-linked to the cleavage site of mammalian mRNA precursor. CPSF-73 has recently been implicated in the processing of metazoan histone mRNA transcripts, which are cleaved but not polyadenylated (Dominski et al. 2005; Kolev and Steitz 2005). These findings support the notion that the endonuclease is Brr5/Ysh1 and its homolog CPSF-73, although direct proof of nuclease activity is still lacking.

Affinity purification revealed another interesting feature of the CPF complex related to Brr5/Ysh1—its C-terminus is

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duplicated as a nonessential subunit of CPF called Syc1 (Gavin et al. 2002; He et al. 2003; Nedea et al. 2003). The function of the C-terminus of Brr5/Ysh1 and of Syc1 is not known. In this study, we show that this domain of Brr5/Ysh1 is critical for cell survival, and is needed for both steps of 3'-end processing. Finally, even though Syc1 is not essential, its presence exacerbates the processing and growth defects of several processing mutants, suggesting a role for Syc1 in modulating the efficiency of 3'-end processing.

RESULTS

The C-terminus of Brr5/Ysh1 starting at amino acid 582 exhibits a striking conservation (38% identity) with amino acids 28–188 of Syc1, a 21-kDa protein of 188 amino acids that copurifies with CPF (Gavin et al. 2002; He et al. 2003; Nedea et al. 2003) (Fig. 1A,B). Figure 1A also depicts the metallo- β -lactamase domain of Brr5/Ysh1 in relationship to this C-terminal domain. The first 500 amino acids of Brr5 exhibit the greatest similarity to its mammalian homolog, CPSF-73, and contain the region common to proteins in the β -CASP subfamily whose characterized members act on nucleic acids (Callebaut et al. 2002).

The C-terminal region of Brr5 is essential for cell viability

The C-terminus of Brr5 contains two potential motifs suggestive of function—a leucine zipper, which mediates protein/protein interactions, and a P-loop motif (Saraste et al. 1990) of GX_4-8GKS/T , found in proteins that bind ATP or GTP (Fig. 1B). While deletion of the last 30 amino acids of Brr5 ($\Delta C30$) is lethal, smaller truncations of 19 or 10 amino acids (Fig. 1C, $\Delta C19$ and $\Delta C10$) do not affect cell viability or cause cold- or heat-sensitive growth (data not shown). Thus, if this region is a leucine zipper, it does not mediate an interaction critical for Brr5 function. Overexpression of Syc1, or deletion of the *SYC1* gene, cannot rescue the lethality of $\Delta C30$ Brr5 (data not shown). However, consistent with the high degree of conservation, the C-terminal sequence of Brr5 can be replaced with homologous Syc1 sequence without affecting cell viability or growth rate (Fig. 1C).

Several studies have shown that ATP is important for the cleavage step in the processing of yeast mRNA precursor (Butler and Platt 1988; Chen and Moore 1992; Zhao et al. 1997). To investigate whether the P-loop motif in the Brr5 C-terminus might be involved in the ATP requirement, we made the amino acid replacements K740D or K740H. These substitutions have no effect on cell growth, but a different replacement, T737V, is lethal (Fig. 1C). This amino acid is conserved between Brr5 and Syc1, and while it immediately precedes the GKS of the putative P-loop, it is not part of the P-loop consensus. The inviability of the T737V and brr5- $\Delta C30$ mutants shows that this region of Brr5 is important for activity, while the neutral effect of the point mutations

FIGURE 1. Mutagenesis reveals the critical nature of the Brr5 C-terminal domain. (A) Schematic of the region of Brr5 that places it in the metallo- β -lactamase family and the region homologous to the CPF Syc1 subunit. (B) Alignment of Syc1 amino acids 28–188 with the Brr5 C-terminus beginning at amino acid 582. The putative P-loop motif is boxed, and amino acids T737 and K740 within this motif that were targeted for mutagenesis are marked by an asterisk. A possible leucine-rich motif is marked by shaded amino acids. Identical amino acids are noted, and similar amino acids are indicated by a +. (C) Mutational analysis of the Brr5 C-terminus. The indicated mutations were made in *BRR5* and tested for their ability to rescue lethality caused by deletion of the chromosomal *BRR5* gene. Growth after forcing loss of a *BRR5* covering plasmid on 5'FOA-containing media is shown on the right. (WT) Indicates growth at 16°C, 25°C, and 37°C that is identical to that of the isogenic wild-type strain; (Lethal) No growth.

at K740 indicates that it does not function to bind ATP or GTP. Brr5 has a second match to the P-loop motif in the N-terminus at positions 31–33, but mutations of G31A or K32R in this putative motif also have no effects on cell viability (data not shown).

The C-terminal region of Brr5 is critical for both steps of processing

Because of the lethality of mutations in the C-terminus of Brr5, it is difficult to ascertain the function of this domain in mRNA 3'-end processing. To investigate the behavior of Brr5 lacking the C-terminus, we created a strain in which a

degron tag (Dohmen et al. 1994) was fused to the N-terminus of the chromosomal copy of the *BRR5* gene. Consistent with *BRR5* being an essential gene, the Degron-*brr5* cells die if maintained at 37°C (Fig. 2A). When these cells are grown at 24°C, the Degron-*brr5* is expressed at a level comparable to *Brr5* in the parental strain, as determined from Western blot analysis using a *Brr5*-specific antibody (Fig. 2B, lanes 2,4). When cells are shifted to growth at 37°C, the Degron-*brr5* protein is degraded by 90 min, while the level of *Brr5* in the wild-type parental strain remains the same (Fig. 2B, lanes 3,5). The levels of several other subunits of CPF (Cft1/Yhh1, Cft2/Ydh1, Pta1, Fip1, and Yth1) were examined and did not change upon Degron-*brr5* depletion (data not shown).

We next transformed the Degron-*brr5* strain with a plasmid expressing either *BRR5* or the *brr5*- Δ C30 mutant, and confirmed that the *brr5*- Δ C30 mutant could not support growth on its own (Fig. 2A). By Western blot, the wild-type and *brr5*- Δ C30 proteins are stably expressed in this strain at both 24°C and 37°C (Fig. 2B, lanes 6–9). The activity of

processing extracts made from these different strains after depletion of the Degron-*Brr5* protein was examined by a cleavage assay using precursor containing the *GAL7* poly(A) site and flanking sequences and in the presence of dATP to block poly(A) addition (Fig. 2C), or a poly(A) addition assay using ATP and substrate that terminates at the poly(A) site (Fig. 2D). For these experiments, cells were grown at 24°C, and the cultures were split, with half continuing to grow at 24°C and the other half shifted to 37°C. After 90 min, cultures were harvested for extract preparation. Equal amounts of protein were used in each assay. There was little difference in the processing activity of extracts from the wild-type parental cells grown at 24°C or 37°C (Fig. 2B,C, lanes 2,3), indicating that the activity of extracts from the same cells cultured at the two different temperatures could be compared.

Extract prepared from Degron-*brr5* cells grown at 24°C is consistently defective for processing in vitro compared to the parental wild-type strain (Fig. 2C,D, lanes 2,4). This observation suggests that even though there is no difference

FIGURE 2. The effects of *brr5* mutants on cell growth and mRNA 3'-end processing. (A) The Degron-*brr5* strain, or the Degron-*brr5* strain with plasmids expressing wild-type *Brr5* or *brr5*- Δ C30 mutant proteins, were grown at 24°C or shifted to growth at 37°C. The lethality of degron-mediated *Brr5* depletion can be prevented by expression of wild-type *Brr5* but not *brr5*- Δ C30. (B) The presence of wild-type *Brr5*, Degron-*brr5*, or the *brr5*- Δ C30 mutant protein was detected by Western blot with *Brr5*-specific antibody. Extract was prepared from the indicated cells grown at 24°C or shifted to growth at 37°C for 90 min to remove the Degron-*Brr5* protein. The positions of the different forms of *Brr5* are indicated on the right. (C) The extracts described in B were examined for cleavage activity using reactions containing radioactive *GAL7* precursor and 2'-dATP to block the poly(A) addition step, as described in Materials and Methods. Reaction products were resolved on a 6% polyacrylamide gel containing 8 M urea and visualized by PhosphorImager analysis. (D) Activity of the same extracts used in C in a poly(A) addition assay containing ATP and *GAL7* substrate that ends at the poly(A) site. Products were analyzed as described for C. The positions of unreacted substrate and products are indicated on the right side of C and D. (Note that the downstream cleavage product is rapidly degraded and not detectable on these gels.) The efficiency of processing for each strain at 37°C relative to that at 24°C (using the amount of processing at 24°C as 100% for each pair) is shown under each pair of lanes and represents the average of three separate experiments.

in growth at this temperature or in the expression level of the Degron-*brr5* compared to wild-type cells, the degron tag has compromised Brr5 function. Removal of Degron-*brr5* at 37°C further impairs cleavage and poly(A) addition to about one-quarter of that seen in the same cells at 24°C (Fig. 2C,D, lanes 4,5). While we do not know the reason for the residual activity at 37°C, it is most likely due to a small amount of Degron-*brr5* or partial degradation products remaining in the cells after a shift to 37°C for 90 min (Fig. 2B, lanes 4,5).

Activity in extract from the Degron-Brr5 strain is rescued to wild-type levels at both temperatures by expression of Brr5 from a plasmid-borne gene (Fig. 2C,D, lanes 6,7). These results confirm previous studies implicating Brr5 as a protein needed for both steps of mRNA 3'-end formation (Chanfreau et al. 1996; Jenny et al. 1996; Zhao et al. 1999b). When the *brr5*- Δ C30 mutant is expressed in the Degron-*brr5* strain, there is no rescue of activity in extract from cells grown at 24°C, as there was with expression of wild-type Brr5 (Fig. 2B,C, cf. lanes 6 and 8). Furthermore, depletion of the Degron-*brr5* protein in cells expressing *brr5*- Δ C30 caused a marked decline in the efficiency of cleavage and poly(A) addition at 37°C to a level comparable to that seen with Degron-*brr5* alone (Fig. 2B,C, cf. lanes 8 and 9). In extracts prepared from the Degron-*brr5* cells, the efficiency of both the cleavage and poly(A) addition reactions at 37°C was fourfold less than it was at 24°C. In Degron-*brr5* cells supplemented with *brr5*- Δ C30, there was a fivefold decrease for cleavage and a 3.7-fold decrease for poly(A) addition. From these data, we conclude that *brr5*- Δ C30 is nonfunctional and that the Brr5 C-terminus is critical for both steps of processing.

To determine if the *brr5*- Δ C30 mutant was incorporated into CPF, we immunoprecipitated Brr5 proteins from wild or mutant extract (after depletion of Degron-Brr5) using Brr5-specific antibody and examined the precipitate by Western blot for the CPF subunits Cft1, Pta1, Pap1, Yth1, and Ssu72. Similar amounts of protein were pulled down with both wild-type and *brr5*- Δ C30 mutant proteins (data not shown), suggesting that the processing defect of *brr5*- Δ C30 is not due to loss of the protein from CPF.

The C-terminus of Brr5 interacts with Pta1

When we conducted a two-hybrid screen for proteins that interacted with full-length Pta1 as bait, we identified four strongly scoring clones containing the C-terminus of Brr5 (data not shown). The shortest fusion protein that gave a robust two-hybrid signal contained only amino acids 546–779 of Brr5, which is only slightly larger than the region of Brr5 homologous to Syc1. In agreement with this result, GST-Pta1 interacts well in a pull-down assay with both Brr5 and its C-terminal homolog Syc1, but not with the Rna15 subunit of CF I (Fig. 3). Thus, the function of this domain of Brr5 may be closely connected to its interaction with Pta1.

FIGURE 3. GST pull-down assays. Brr5, Syc1, or Rna15 was radioactively labeled by in vitro translation and then incubated with GST or GST-Pta1, and complexes were isolated and analyzed as described in Materials and Methods. GST-Pta1 interacts with Brr5 and Syc1, but not with the Rna15 subunit of CF I. Ten percent of the in vitro translated input is shown.

Deletion of *SYC1* affects the viability of many mRNA 3'-end processing mutants

The strong homology to the essential Brr5 C-terminus and the association of Syc1 with CPF suggests that Syc1 is involved in either the processing event itself or in coordinating 3'-end processing with other steps in mRNA synthesis. However, deletion of the *SYC1* gene caused no defect in growth of *S. cerevisiae* on rich media at 25°C, 30°C, or 37°C (Nedea et al. 2003; data not shown). In addition, yeast lacking *SYC1* could grow as well as wild-type cells on other carbon sources such as galactose, glycerol, or ethanol, and did not show any increased sensitivity to ultraviolet light or treatment with methyl-methanesulfonate (data not shown).

To probe for a possible function for Syc1, we paired *sys1* Δ with mutations in other genes needed for both cleavage and polyadenylation (*BRR5*, *PTA1*, *PFS2*, *RNA14*, and *RNA15*), or for only the polyadenylation step (*PAP1* and *FIP1*). This analysis revealed numerous genetic interactions. For example, *sys1* Δ rescues the thermosensitive (ts) but not the cold-sensitive (cs) phenotype of *brr5-1* (Fig. 4A). The deletion of *SYC1* also suppresses the lethality of the *pta1-2* mutant at 37°C (Fig. 4B), but is synthetic lethal with *pta1-3* at 24°C (Fig. 4C). The following interactions with other CPF mutants were also observed (Fig. 4D): (1) *sys1* Δ enhances the ts and cs growth defect of *pfs2-1*; (2) *sys1* Δ enhances the cs growth defect of *fip1-206*, but does not affect the ts growth; and (3) *sys1* Δ has no effect on the growth of *pap1-1*. Suppression of growth defects were also observed with mutations in CF I components—*sys1* Δ rescues *rna14-3* at 34°C and *rna15-2* at 33°C, but not at 37°C (Fig. 4E; data not shown).

Deletion of the *SYC1* gene alleviates the in vitro processing defect of the *brr5-1* mutant

The *brr5-1* mutant strain was isolated by Chanfreau et al. (1996) and shown to be cold-sensitive. We have found that it is also thermosensitive and grows very slowly at 37°C (Fig. 4A). Extract from the *brr5-1* mutant is deficient in

FIGURE 4. Genetic interactions of *syc1Δ* with mutations in cleavage/polyadenylation factors. (A) Deletion of *SYC1* suppresses the ts growth of the *brr5-1* mutant. A haploid strain containing the *brr5-1* and *syc1Δ* alleles was transformed with vector or plasmid bearing the *SYC1* gene, and incubated at the indicated temperatures. (B) Deletion of *SYC1* suppresses the ts growth defect of the *pta1-2* mutant. (C) Deletion of *SYC1* is synthetic lethal with the *pta1-3* mutant. The *pta1-3/syc1Δ* and the *PTA1/syc1Δ* strains were transformed with a plasmid containing the *PTA1* and *URA3* genes, and grown at 24°C on complete media lacking uracil, or in the presence of 5-FOA to remove the plasmid. (D) The *syc1Δ* allele affects the growth of strains containing the *pfs2-1* and *fip1-206* mutations, but not that of *pap1-1*. The growth of the *pfs2-1*, *fip1-206*, and *pap1-1* mutants is shown at different temperatures in the presence or absence of *SYC1*. (E) The growth of the *rna14-3* and *rna15-2* mutants is shown at different temperatures in the presence or absence of *SYC1*. Deletion of *SYC1* allows *rna14-3* to grow at 34°C, and improves the growth of *rna15-2* at 32°C.

poly(A) addition at 15°C (Chanfreau et al. 1996) and defective for cleavage and poly(A) addition at 30°C (Zhao et al. 1999b). By DNA sequencing, we have mapped the mutation in *brr5-1* to a single amino acid change (A407T) in the β -CASP B-motif, indicating that this region of Brr5 has a role in both steps of mRNA 3'-end formation.

The genetic interactions of *syc1Δ* with several 3'-end processing mutants suggest that even though Syc1 is not essential, it might negatively influence the efficiency of the cleavage/polyadenylation complex. If this hypothesis is correct, *syc1Δ* might rescue the thermosensitive growth defect of *brr5-1* by relieving the mRNA 3'-end-processing defect of this mutant. As seen in Figure 5, A and B (lanes 1,2), deletion of *SYC1* by itself has no effect on mRNA cleavage and poly(A) addition in vitro. In agreement with Zhao et al. (1999b), extract from the *brr5-1* strain is not very active in either step of 3'-end processing (Fig. 5A,B, lane 3). However, when this mutant is paired with *syc1Δ*, cleavage activity is restored (Fig. 5A, lane 4). Poly(A) addition is partially rescued, with substrate processed at

an efficiency almost reaching wild-type levels, but receiving shorter tails than normal (Fig. 5B, lane 4). This observation suggests that poly(A) addition is either less processive in the *brr5-1/syc1Δ* extract, or is terminating prematurely.

Many studies have now shown that efficient termination of RNAP II transcription downstream of a poly(A) site requires a functional mRNA 3'-end-processing machinery. This coupling is reflected in the finding that some, but not all yeast mutants that are defective in cleavage also cause defects in transcription termination (Birse et al. 1998; Dichtl et al. 2002a,b; He et al. 2003; Sadowski et al. 2003). As shown in Figure 5A, the *brr5-1* mutant is compromised for cleavage activity. To test whether this defect is also associated with difficulty in efficient transcription termination, we used a transcription run-on (TRO) assay developed by Steinmetz and Brow (2003), in which the level of transcription extending through two G-less cassettes of different sizes is measured directly after T1 RNase treatment and denaturing gel electrophoresis (Fig. 5C).

FIGURE 5. Effects of *syc1Δ* on mRNA 3'-end processing activity in vitro. Processing extracts were prepared from the *BRR5/SYC1* (wild-type), *BRR5/syc1Δ*, *brr5-1/SYC1*, and *brr5-1/syc1Δ* strains grown at 24°C and analyzed for activity as described in Figure 2. The percentage of processed RNA is indicated under each lane. The Input lanes contain unreacted RNA substrate. (A) Cleavage assay. (B) Poly(A) addition. (C) Diagram of tandem G-less cassette constructs used for transcription run-on (TRO) assays, as described by Steinmetz and Brow (2003). An inert spacer sequence (black rectangle) from a region downstream of the *CYC1* poly(A) site, beginning at nucleotide 587 relative to the first nucleotide of the *CYC1* open reading frame, is inserted between the two G-less cassettes. The G-less cassettes (light gray) and the *CYC1* polyadenylation signal (dark gray) are also indicated. (D) Transcription termination downstream of the *CYC1* poly(A) site, as assayed by TRO analysis, is not affected by the *brr5-1* or *syc1Δ* alleles, alone or in combination. Wild-type (*BRR5/SYC1*), *brr5-1/syc1Δ*, *BRR5/syc1Δ*, and *brr5-1/SYC1* cells were transformed with the *CYC1* ds (lanes 1,3,5,7) or the *CYC1* pA (lanes 2,4,6,8) reporter plasmids were grown at 25°C and then shifted to 37°C for 90 min before harvesting for the TRO procedure. Loss of the 132-nt fragment with the *CYC1* pA construct indicates efficient transcription termination.

Inserting elements that direct 3'-end formation between the two G-less cassettes causes transcription termination to occur before the downstream cassette is transcribed. With this assay, the efficiency of transcription termination can be determined by comparing the amount of radioactivity incorporated into the 262-nt upstream product and the 132-nt downstream product (Fig. 5D). The 132-nt fragment does not accumulate when a wild-type strain or strains with the *brr5-1* and *syc1Δ* alleles are incubated at 37°C for 90 min and assayed for transcription through the *CYC1* poly(A) site (Fig. 5E, lanes 2,4,6,8). This result indicates that *brr5-1* and *syc1Δ* mutations do not cause a termination defect.

DISCUSSION

In this study, we investigated the function of the C-terminal domain of the Brr5 subunit of the yeast cleavage/polyadenylation factor, CPF, and its homolog Syc1 in the 3'-end processing of eukaryotic mRNA. We have found that the C-terminus of Brr5 is essential for both cleavage and poly(A) addition of mRNA precursor. However, the function of this domain does not require the putative leucine-zipper or P-loop motifs present in this part of Brr5. Moreover, the Syc1 protein sequence can substitute for the Brr5 C-terminus when it is part of Brr5 but not when expressed separately.

Putative P-loops in Brr5 are not important for function

The original studies demonstrating mRNA 3'-end processing in vitro found that ATP was needed for the cleavage steps, as well as for poly(A) synthesis (Zhao et al. 1999a). This requirement was maintained even upon purification of the processing factors. In these studies, creatine phosphate was included in the reactions for the purpose of regenerating ATP. Manley and colleagues have shown that creatine phosphate was sufficient, and moreover, could be replaced in the cleavage reaction by the C-terminal domain (CTD) of RNA polymerase II (RNAP II) in the mammalian system (Hirose and Manley 1998; Ryan et al. 2002). These findings correlated well with numerous other studies showing that the efficiency and specificity of mRNA 3'-end formation in vivo were stimulated by the CTD (McCracken et al. 1997; Fong and Bentley 2001; Licatalosi et al. 2002; Ahn et al. 2004; Bird et al. 2004). This collection of work supported a model in which physical interaction with the CTD of elongating RNAP II helps recruit 3'-end processing factors to the nascent RNA.

However, in yeast, a coupling with RNAP II is not essential for efficient processing (McNeil et al. 1998; Dower and Rosbash 2002). In addition, creatine phosphate alone cannot serve as a cofactor for cleavage in the yeast in vitro system (Chen and Moore 1992), although other nucleotides such as CTP or dATP are effective substitutes for ATP (Butler and Platt 1988). Butler and Platt also showed that

cleavage depends on hydrolysis of the β - γ bond of ATP. Moreover, RNA cross-linking of the Cft2/Ydh1 subunit in the CF II subcomplex of CPF required ATP and a functional poly(A) site (Zhao et al. 1997). Overall, these studies suggested that NTP hydrolysis or NTP binding might be important for the cleavage step. Brr5 contains two possible P-loops in its N- and C-termini, but we have found that mutations in Brr5 that would have destroyed P-loop function in other ATP-binding proteins have no effect on cell viability, ruling out the idea that Brr5 binds or hydrolyzes ATP. Thus, while cleavage in yeast may need ATP as a cofactor, our findings indicate that its usage is unlikely to be mediated by Brr5. The recent discovery that the activity of a CPF-associated phosphatase, Glc7, is critical for execution of the poly(A) addition step (He and Moore 2005) suggests that ATP may be needed for a phosphorylation event.

The role of the Brr5 C-terminus and its homolog, Syc1

While the P-loop motif in the Brr5 C-terminus is not important, the last 30 amino acids and the T737 that lies in the vicinity of the P-loop homology are essential (Fig. 1C). In this report, we show that the C-terminus is critical for both cleavage and poly(A) addition. It is highly homologous to another CPF subunit, Syc1, and these two proteins interact with the CPF subunit Pta1, which is also needed for both steps of processing (Preker et al. 1997; Zhao et al. 1999b). However, we have found that cells lacking Syc1 give extract normal for in vitro mRNA 3'-end processing, a result consistent with the finding that deletion of *SYC1* does not affect cell viability. Nevertheless, the presence of Syc1 exacerbates the growth defects of several processing mutants, and removal of Syc1 by deletion of the gene rescues the cleavage defect of *brr5-1* cells and partially relieves the defect in poly(A) addition. These observations suggest that Syc1 works as a negative regulator of 3'-end processing, perhaps by competing with sites that interact with the Brr5 C-terminus.

There are two possible ways in which Syc1 could execute such a function. First, Syc1, while not essential, may help to ensure the correct timing of different events in mRNA 3'-end formation. Certain mutations in CPF or CF I could create a situation in which Syc1 cannot be as easily displaced from a part of CPF that needs to bind to the C-terminus of Brr5. Removal of Syc1 would now allow correct interaction of Brr5 so that it can function in cleavage and poly(A) addition. One such contact might take place through Pta1, which has been proposed to be a scaffold that helps to organize the 3'-end processing machinery (Takagaki and Manley 2000; Nedeja et al. 2003).

Alternatively, Syc1 might act as a checkpoint to slow processing down when there is a problem. Removal of Syc1 in some cleavage/polyadenylation mutants such as *brr5-1*, *pta1-2*, *rna14-3*, or *rna15-2* could allow enough

transcripts to be processed to restore growth at a previously restrictive temperature. With other mutants such as *pta1-3*, *pfs2-1*, and *fip1-206*, growth defects are more severe in the *syc1* Δ background, with the most extreme case being *pta1-3*, in which *syc1* Δ causes lethality. The *pta1-3* mutant grows much more slowly than *brr5-1* or *pta1-2* at the permissive temperature (He et al. 2003), and perhaps loss of Syc1 cannot be tolerated if the defect in processing is too severe. Similarly, *pfs2-1* and *fip1-206* are the most defective, yet still viable alleles that have been described for these two genes (Ohnacker et al. 2000; Helmling et al. 2001). While it is difficult to fully explain the genetic interactions without a better understanding of the architecture of the 3'-end-processing complex, these results nevertheless highlight the importance of Syc1 to optimal functioning of this machinery.

The Syc1 of *S. cerevisiae* is highly conserved in other *Saccharomyces* species such as *Saccharomyces bayanus*, *Saccharomyces kudriavzevii*, *Saccharomyces milatae*, and *Saccharomyces paradoxus*, but it is not found in metazoans and is present in only one other fungus (*Candida glabrata*) of those whose genomes are available in the public protein databases. A similar situation exists for the C-terminus of Brr5, which is homologous to Syc1. It does not resemble the C-terminus of the metazoan orthologs, but similar domains are found in the Brr5 protein from *Ashbya gossypii*, *Kluyveromyces lactis*, *Candida glabrata*, *Candida albicans*, *Debaryomyces hansenii*, and *Schizosaccharomyces pombe*. Perhaps the fine-tuning of mRNA 3'-end processing afforded by Syc1 and the Brr5 C-terminus supports a more efficient response to a rapidly changing environment, and has led to the persistence of this domain in the genomes of these single-cell eukaryotes.

The role of the β -CASP domain of Brr5

Brr5 is a member of the β -CASP subfamily of the metallo- β -lactamases, whose characterized members act on nucleic acids (Callebaut et al. 2002). The proteins in this family do not have a clearly identifiable match to the fifth of the five motifs that are found in other metallo- β -lactamase subfamilies and instead share in common three motifs designated motifs A, B, and C. Ryan et al. (2004) recently reported that mutations in conserved amino acids in two of these motifs (E209Q and H408F in motifs A and B) cause lethality in *S. cerevisiae*, underscoring the critical importance of this region. We also find that mutation of conserved histidines in the β -CASP B (H408A) or C motifs (H430D) is lethal (data not shown). Furthermore, we have mapped the mutation in the *brr5-1* mutant strain to a single amino acid replacement of A407T, another conserved amino acid in the B-motif. Interestingly, the *brr5-1* mutant is both cold-sensitive (Chanfreau et al. 1996) and heat-sensitive for growth (this study). Extract from the *brr5-1* strain is deficient in poly(A) addition at 15°C (Chanfreau

et al. 1996) and defective for cleavage and poly(A) addition at 30°C (Zhao et al. 1999b). Thus, the β -CASP domain may function in both processing steps. Alternatively, if Brr5 is the mRNA 3'-end nuclease, this region could be important in positioning RNA in the active site of the nuclease. In this model, the *brr5-1* mutant could negatively impact on poly(A) addition by interacting nonproductively with RNA substrate in a way that prevented poly(A) polymerase from accessing the 3'-end of precleaved RNA.

Summary

Our results on the function of Brr5 and Syc1 extend our understanding of the organization of the yeast mRNA 3'-end processing machinery and the function of its various parts. Previous research indicates that CFI and CPF are both important in directing the processing machinery to the correct site on mRNA precursor. This is accomplished by recognition of signal sequences that reside upstream of the cleavage site by CF I (Kessler et al. 1997; Gross and Moore 2001), interaction with sequences flanking the poly(A) site by CPF (Barabino et al. 2000; Dichtl and Keller 2001; Dichtl et al. 2002b; Tacahashi et al. 2003), and numerous potential cross-factor interactions between CF I and CPF (Preker et al. 1995; Ohnacker et al. 2000; He et al.

2003; Kyburz et al. 2003; Cheng et al. 2004; Dichtl et al. 2004). Our current study lends support to a model in which the action of Brr5, through its β -CASP domain and its C-terminus, affects both the endonuclease and poly(A) polymerase activities of the processing complex. These activities are regulated by other subunits of CPF such as Syc1 as the reaction proceeds from complex assembly, cleavage, poly(A) addition, termination of polyadenylation, and release from the final product.

MATERIALS AND METHODS

Yeast strains and genetic analysis

The yeast strains and sources used in this study are given in Table 1. We introduced mutations into the *BRR5* coding sequence by PCR-based mutagenesis using Deep Vent DNA polymerase (NEB) and primers containing the desired mutation, a new restriction site that does not affect the amino acid sequences and a unique restriction site for cloning. The wild-type Brr5 sequence, cloned into pET16b as described by Tacahashi et al. (2003), was used as a template, and the relevant part of the wild-type Brr5 in this plasmid was then replaced with the amplified fragment. Mutations were confirmed by digestion with restriction enzymes followed by sequencing. The mutated *brr5* sequence was then transferred into YCP50 (*URA3*) or YCPlac111 (*LEU2*) plasmids for expression in

TABLE 1. *Saccharomyces cerevisiae* strains used in this study

Name	Genotype	Source
FY23	<i>MATa, ura3-52, leu2Δ1, trp1Δ63</i>	Winston et al. 1995
YAZ212	<i>MATa, ura3-52, leu2Δ1, trp1Δ63, brr5::DHFR^{ts}-BRR5</i>	This study
YAZ216	<i>MATa, ura3-52, leu2Δ1, trp1Δ63, brr5::DHFR^{ts}-BRR5 [pSE360]</i>	This study
YAZ220	<i>MATa, ura3-52, leu2Δ1, trp1Δ63, brr5::DHFR^{ts}-BRR5 [YCP50-ΔC30brr5]</i>	This study
CEN PK2	<i>MATa, leu2-3,112, ura3-52, trp1-289, his3-Δ1</i>	EUROSCARF
YSN399	<i>MATα, ura3-52 his3-Δ200, ade12-100o, leu2-Δ1, lys2-801a, brr5-1</i>	Chanfreau et al. 1996
YSN378	<i>MATa/α, his3Δ/his3Δ, ura3/ura3, lys2/lys2, leu2/leu2, ade2/ade2, brr5::LEU2/BRR5</i>	Chanfreau et al. 1996
YOM7	YSN378 haploid, pSE360	Chanfreau et al. 1996
BY4743	<i>MATa/α, his3Δ1/his3Δ1, ura3Δ0/ura3Δ0, lys2Δ0/LYS2, leu2Δ0/leu2Δ0, met15/MET, brr5::kanMX/BRR5</i>	ATCC 201390
HKY1	Same as CEN PK2 with <i>syc1::kanMX</i>	This study
YAZ142	<i>brr5-1/syc1Δ</i> derived from mating YSN399 and HKY1	This study
MO17	<i>ura3-1, trp1, ade2-1, leu2-3, 112, his3-11, 15, pfs2::TRP1, pFL36-pfs2-1</i>	Ohnacker et al. 2000
YAZ161	<i>pfs2-1/syc1Δ</i> derived from mating MO17 and HKY1	This study
UR3148	<i>MATα, ade1/ade2, lys2, gal1, ura3-52, pap1-1</i>	Patel and Butler 1992
YAZ167	<i>pap1-1/syc1Δ</i> derived from mating UR3148 and HKY1	This study
fip1-206	<i>MATα, leu2-3, 112, ura3-52, trp1, his4, fip1::LEU2, [p314/fip1-206]</i>	Helmling et al. 2001
YAZ165	<i>fip1-206/syc1Δ</i> derived from mating FY1284 and HKY1	This study
FY1284	<i>MATa pta1-2 ura3-52 ade8 his4-917</i>	Madison and Winston 1997
YAZ121	<i>pta1-2/syc1Δ</i> derived from mating YSN399 and HKY1	This study
XH15	<i>MATa ura3-52 leu21 trp163 his3200 pta1::TRP1 [YcPLEU2-pta1-3]</i>	He et al. 2003
YAZ184	<i>pta1-3/syc1Δ [YCP50-PTA1]</i>	This study
W303	<i>ade2,-1 his3-11, 15, trp1-1, ura3-1, leu2-3, 112 can1-100</i>	F. Lacroute
THJY83	W303, but <i>MATα, rna14-3</i>	F. Lacroute
THJY84	W303, but <i>MATα, rna15-2</i>	F. Lacroute
THJY977	<i>MATa, ade2-1, ura3-1, his3-11,15, leu2-3, 112, trp1-1, can1-100, SYC1::HIS3</i>	This study
THJY978	<i>MATα, ade2-1, ura3-1, his3-11,15, leu2-3, 112, trp1-1, can1-100, SYC1::HIS3</i>	This study
THJY1189	<i>MATα, ade2-1, leu2, his3-11,15, rna14-3, SYC1::HIS3</i>	This study
THJY1193	<i>MATα, ade2-1, leu2, his3-11,15, rna15-2, SYC1::HIS3</i>	This study

yeast. Viability and phenotype of Brr5 truncations were determined by spore analysis of YSN378 diploid cells carrying a deletion of one chromosomal copy of *BRR5* (Chanfreau et al. 1996) transformed with a YCP50 plasmid (*URA3*) containing the mutant *brr5* sequence. Point mutations of *BRR5* were analyzed using plasmid shuffle and counterselection on medium containing 5-fluoroorotic acid (5-FOA) to replace the pSE360 (*BRR5*, *URA3*) plasmid (Noble and Guthrie 1996) with YCplac111 containing mutant *brr5*.

The deletion of *SYC1* in CEN-PK2 cells was accomplished by integration of the *kanMX6* module as described previously (Longtine et al. 1998) to give the strain HKY1. The genetic interactions of the *syc1Δ* mutant with various RNA processing mutants were performed at different temperatures as indicated in figure legends using haploids derived by mating the HKY1 strain with those mutants, sporulating and dissecting tetrads, and selection of double mutants using selective medium, cell viability, and temperature sensitivity.

In vivo depletion of Brr5

To create the Degron-*brr5* strain, the *SspI*/*PvuII* fragment of the pPW66R plasmid (Dohmen et al. 1994) containing the CUP1-DHFR sequence was ligated to pUG6 (Guldener et al. 1996) linearized with *PvuII*. A 630-nt PCR fragment of *BRR5* encoding the N-terminus was then inserted into the *KpnI*/*MscI* sites of that plasmid. The resulting plasmid was linearized with *Bsu36I* and transformed into FY23 yeast cells, and recombinants were selected on YPD medium containing 200 μg/mL G418, followed by temperature-sensitivity screening and confirmation that the thermo-sensitive growth could be rescued by plasmid containing *BRR5*. The expression of Degron-*brr5* at permissive temperature (24°C) and its degradation at nonpermissive temperature (37°C) as well as lack of wild-type Brr5 protein was confirmed by Western blot analysis using rabbit polyclonal antibody specific to Brr5.

In vitro processing and transcription run-on assays

Extract preparation and processing assays were performed as described previously (He and Moore 2005). Equal amounts of protein (30 μg) were used for reactions and corresponding Western blots. Reaction products were separated on 6% polyacrylamide gels containing 8 M urea and visualized and quantitated by PhosphorImager analysis. Quantitation was performed by measuring the amount of radioactivity in the precursor and product bands in each lane and calculating the percentage of processed RNA. The efficiency of processing for each extract was determined by averaging the values from three separate experiments, and this average was used to calculate the fold-change in processing at 37°C relative to that at 24°C.

For preparation of extract from Degron-*brr5* strains, cells were grown in 1 L of selective media at 24°C to an OD₆₀₀ of 1, split in half, and collected by centrifugation. Each half was resuspended in 50 mL of 24°C (control) or preheated (37°C) media, and growth was continued at the permissive or nonpermissive temperatures, respectively, for an additional 90 min. After centrifugation, cells were resuspended in 30 mL of 1 M sorbitol, 50 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, and 10% glycerol, and frozen until extracts were made.

Transcription run-on assays were performed using the G-less cassette method (Steinmetz and Brow 2003).

Analysis of protein/protein interactions

For GST pull-downs, Brr5, Syc1, and Rna15 were in vitro translated using the TNT T7 Coupled Reticulocyte Lysate System (Promega) and labeled with [³⁵S]methionine according to the manufacturer's instructions. To prepare the beads, 20 μL of a 50% slurry of glutathione Sepharose 4B (Pharmacia) was incubated in 100 μL of buffer IP-150 (150 mM KCl, 20 mM Tris at pH 8, 0.1% NP-40). After incubation for 1 h at 4°C, a mixture of 500 ng of purified GST or GST-Pta1 (He et al. 2003) and in vitro translated Brr5 or Syc1 was added to the beads. This mixture was incubated for 2 h at 4°C, and beads were washed with 1 mL of IP-150 three times. The bound proteins were eluted in 50 mM glutathione and applied onto a 10% SDS-polyacrylamide gel. The resolved proteins were detected by autoradiography.

For coimmunoprecipitation, yeast whole-cell extracts (150 μg) were incubated with antibody against Brr5 bound to anti-rabbit IgG agarose (Sigma) at 4°C for 2–4 h in 200 μL of buffer IP-150 (He et al. 2003). Beads were washed four times with 300 μL of buffer IP-150. The bound proteins were eluted with 20 μL of SDS sample buffer, and analyzed on an SDS-10% polyacrylamide gel. Western blotting was performed according to standard procedures.

For two-hybrid assays, plasmid pGBD-PTA1 was constructed by insertion of the PTA1 open reading frame into vector pGBD-C2 (James et al. 1996). The yeast two-hybrid selection strain PJ69-4A was transformed with pGBD-PTA1 and pGAD plasmid library C1, C2, or C3 (James et al. 1996) containing fusions of GAD to yeast genomic DNA fragments. Transformants were selected on medium lacking leucine, tryptophan, and histidine in the presence of 2 mM 3-aminotriazole. Candidates were then tested for activation of the LACZ reporter dependent on the presence of the pGBD-PTA1 plasmid, and positive candidates were then sequenced.

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