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Control of cleavage site selection during mRNA 3′ end formation by a yeast hnRNP

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Endonucleolytic cleavage of pre-mRNAs is the first step during eukaryotic mRNA 3′ end formation. It has been proposed that cleavage factors CF IA, CF IB and CF II are required for pre-mRNA 3′ end cleavage in yeast. CF IB is composed of a single polypeptide, Nab4p/Hrp1p, which is related to the A/B group of metazoan heterogeneous nuclear ribonucleoproteins (hnRNPs) that function as antagonistic regulators of 5′ splice site selection. Here, we provide evidence that Nab4p/Hrp1p is not required for pre-mRNA 3′ end endonucleolytic cleavage. We show that CF IA and CF II devoid of Nab4p/Hrp1p are sufficient to cleave a variety of RNA substrates but that cleavage occurs at multiple sites. Addition of Nab4p/Hrp1p prevents these alternative cleavages in a concentration-dependent manner, suggesting an essential and conserved role for some hnRNPs in pre-mRNA cleavage site selection.
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Introduction

Addition of a poly(A) tail to eukaryotic mRNA precursors (pre-mRNAs) requires a preliminary endonucleolytic step. An astonishingly large complex of multisubunit factors has been shown to be responsible for this apparently simple reaction (for reviews, see Wahle and Keller, 1996; Colgan and Manley, 1997; Wahle and Kühn, 1997). Five factors are necessary to cleave mammalian pre-mRNAs at their 3′ ends. Four of them were shown to bind directly to the primary transcript. The cleavage and polyadenylation specificity factor (CPSF) binds to the conserved AAUAAA upstream signal (Keller *et al*., 1991; Jenny *et al*., 1994); the cleavage stimulation factor (CstF) binds specifically to the downstream elements (Wilusz and Shenk, 1988; Beyer *et al*., 1997; Takagaki and Manley, 1997); cleavage factor I_m (CF I_m) preferentially cross-links to full-length RNA substrates (Rüegsegger *et al*., 1996); and poly(A) polymerase, which is not a cleavage factor *per se* but is required during cleavage of almost all pre-mRNAs, binds RNA with no specificity (Martin and Keller, 1996). CF II_m remains to be characterized. The requirement for so many essential RNA-binding factors during endonucleolytic cleavage may be the consequence of a lack of an absolute conservation of *cis*-acting elements. This complexity may be necessary to ensure cleavage at the correct poly(A) site (Rüegsegger *et al*., 1998).

In some instances, additional determinants are employed to define poly(A) sites. In *cis*, RNA structures may be an important element in the poly(A) site recognition by the polyadenylation machinery (Graveley *et al*., 1996). The shift from membrane-bound to secretion-specific forms during immunoglobulin (IgM) pre-mRNA processing is a good example of how *trans*-acting factors can modulate poly(A) site use. During differentiation of B cells, Ig heavy chain pre-mRNA is processed alternatively at the 3′ end, producing transcripts coding for either the membrane-bound antigen receptor or the secreted form. Regulation occurs at the level of cleavage efficiency at either poly(A) site. Increased use of the secretory poly(A) site correlates with enhanced binding activity and a higher intracellular concentration of CstF-64 (Edwalds-Gilbert and Milcarek, 1995; Takagaki *et al*., 1996).

In *Saccharomyces cerevisiae*, 3′ end formation requires CF IA, CF IB, CF II, polyadenylation factor I (PF I), poly(A) polymerase and the poly(A)-binding protein 1 (Pab1p) (Kessler *et al*., 1996; Amrani *et al*., 1997a,b; Minvielle-Sebastia *et al*., 1997; Preker *et al*., 1997; Zhao *et al*., 1997). Some subunits of these factors are significantly related to mammalian polypeptides involved in 3′ end processing (reviewed in Manley and Takagaki, 1996; Keller and Minvielle-Sebastia, 1997). CF IA contains four essential subunits including Rna14p, Rna15p, Clp1p and Pcf11p (Kessler *et al*., 1996; Amrani *et al*., 1997b; Minvielle-Sebastia *et al*., 1997). Pab1p has been reported to co-purify in sub-stoichiometric amounts associated with either CF IA (Minvielle-Sebastia *et al*., 1997) or CF IB (Kessler *et al*., 1997), and was shown to be required only for the polyadenylation step (Amrani *et al*., 1997a; Minvielle-Sebastia *et al*., 1997). CF IB consists of a single polypeptide encoded by the *HRP1* gene. Hrp1p is structurally related to mammalian heterogeneous nuclear ribonucleoprotein (hnRNP) A/B proteins (Kessler *et al*., 1997). Kessler *et al*. (1997) also reported that Hrp1p is necessary for both cleavage and polyadenylation of a GAL7 synthetic pre-mRNA. We independently identified the yeast nuclear polyadenylated RNA-binding protein Nab4p, which is identical to Hrp1p, as a new 3′-end processing factor. Here, we report that Nab4p/Hrp1p is an atypical 3′-end processing cleavage factor. Using mutant cell extracts, we show that *nab4* temperature-sensitive mutations do not significantly affect 3′ end cleavage and further demonstrate that purified CF IA and CF II devoid of Nab4p/Hrp1p can cleave a variety of pre-mRNAs efficiently and specifically *in vitro*. Thus, Nab4p/Hrp1p is not required for cleavage of precursor RNAs. However,

efficient and accurate 3′ end formation can only occur in its presence. We show that Nab4p/Hrp1p regulates cleavage site utilization in a concentration-dependent manner when alternative sites are present on the mRNA precursor. This is strongly reminiscent of the role of hnRNP A1 in 5′ splice site selection in mammals (Ge and Manley, 1990; Mayeda and Krainer, 1992). These results suggest that Nab4p/Hrp1p plays a role in the regulation of alternative 3′ end processing in yeast (e.g. *CBP1*; Mayer and Dieckmann, 1989; Sparks *et al*., 1997; Sparks and Dieckmann, 1998).

Results

Nab4p/Hrp1p is an atypical 3′-end processing factor

HnRNPs appear to play fundamental roles in mRNA biogenesis and nucleocytoplasmic transport (for reviews, see Dreyfuss *et al*., 1993; Swanson, 1995). In yeast, hnRNPs have been designated nuclear polyadenylated RNA-binding (Nab) proteins (Anderson *et al*., 1993b; Wilson *et al*., 1994). Recently, Kessler *et al*. (1997) reported that Nab4p/Hrp1p is the sole subunit of the cleavage factor CF IB and presented evidence that it is a required factor for cleavage and polyadenylation *in vitro* and *in vivo*. In contrast, our work suggested that Nab4p/Hrp1p was not required for cleavage *in vitro* or polyadenylation *in vivo*. To resolve this apparent discrepancy and examine further the role of Nab4p/Hrp1p in 3′ end processing, several *nab4* temperature-sensitive mutant strains were generated. Extracts were prepared from *NAB4*, *nab4-1*, *nab4-4* and *nab4-7* cells and assayed for iso-1-cytochrome c (CYC1) 3′ end endonucleolytic cleavage and polyadenylation activity *in vitro*. All of the *nab4* extracts produced near wild-type levels of cleavage products at the permissive growth temperature (24°C) and somewhat reduced, but detectable levels at the non-permissive temperature (36°C; Figure 1A). This result suggested that Nab4p/Hrp1p function may not be essential for CYC1 3′ end cleavage. Polyadenylation was more severely affected in all three *nab4* mutants although *nab4-4* extracts showed ~35% of the wild-type polyadenylation activity even at 34°C (higher temperatures eliminated polyadenylation in wild-type extracts) (Figure 1B). Unfortunately, it was difficult to interpret this result as a direct effect of Nab4p/Hrp1p loss of function since immunoblot analysis demonstrated that several proteins which affect polyadenylation *in vitro*, including Pab1p, underwent significant proteolysis during extract preparation despite repeated attempts to minimize this degradation (data not shown; see Materials and methods).

Utilization of downstream cleavage sites has been reported for several 3′ end processing mutants including *pap1-1*, *rna14-3* and *rna15-2* (Mandart and Parker, 1995). These mutants show rapid reductions (<5 min) in mRNA poly(A) tail length when cells are shifted to the non-permissive growth temperature (Minvielle-Sebastia *et al*., 1991; Patel and Butler, 1992). To test whether polyadenylation was affected in *nab4* mutants, the distribution of poly(A) tails generated *in vivo* was analyzed (Figure 1C). In contrast to previous results using the *hrp1-5* strain (Kessler *et al*., 1997), global poly(A) tail length distribution in *nab4-1* cells was only slightly affected up to 3 h following shift to the non-permissive temperature, in contrast to *rna15-2* which showed significant poly(A) tail loss by 30 min. Identical results were obtained with the *nab4-4* and *nab4-7* strains (data not shown). A possible reason for the discrepancy between our data and that of Kessler *et al*. (1997) was the difference between the *hrp1* and our *nab4* alleles. We therefore constructed a *nab4* allele, *nab4-9*, with a single $L^{205}S$ substitution which was identical to the *hrp1-5* allele, and placed it in our genetic background (see Materials and methods). The *hrp1-5* strain shows unusually short poly(A) tails at 23°C (maximum tail length of ~45 nucleotides) compared with a wild-type *HRP1* strain (~70 nucleotides) and further reduction to ~25 nucleotides within 3 h at 37°C (Kessler *et al*., 1997). Surprisingly, poly(A) tail length distribution in *nab4-9* cells at 24°C was very similar to that in wild-type *NAB4* cells (maximum tail length ~80 nucleotides). Shifting *nab4-9* cells to 37°C did not significantly alter this distribution although a relatively slow and general loss of $poly(A)^+$ RNA was observed characteristic of temperature-sensitive strains (Figure 1C). This analysis did not address the possibility that the polyadenylation of individual RNAs might be inhibited. Nevertheless, it appears that another gene (or genes) in the *hrp1-5* genetic background adversely influences polyadenylation, and its identification should provide important clues about the regulation of pre-mRNA processing *in vivo*. Alternatively, it is possible that our strain background may contain a specific suppressor that prevented us from seeing poly(A) tail shortening in a *nab4-9* mutant. However, the *nab4-1* allele integrated into a different genetic background (LDY133; see Materials and methods) gave the same results as shown before (Figure 1A–C) with regard to *in vitro* cleavage and polyadenylation, and *in vivo* poly(A) tail length (data not shown). In summary, *nab4* mutations which confer temperature-sensitive growth do not selectively affect cleavage of CYC1 precursor RNA *in vitro* nor poly(A) tail length distribution *in vivo*. Therefore, the role of Nab4p/Hrp1p in 3′ end processing appeared to be significantly different from that of other previously characterized cleavage and polyadenylation factors.

Multiple endonucleolytic cleavage sites are used by purified CF IA and CF II cleavage factors

To analyze further the role of Nab4p/Hrp1p *in vitro*, we used purified factors. Preker *et al*. (1997) reported the purification of PF I to near homogeneity from PJP14 cells expressing a tagged form of one of the PF I subunits, Fip1p (Preker *et al*., 1997). Ammonium sulfate-fractionated extracts from PJP14 cells were first subjected to chromatography on a Macro-Prep Q column. PF I-containing fractions eluted at 270 mM salt and its purification to near homogeneity was assayed by complementing polyadenylation-deficient *fip1-1* mutant extracts (Preker *et al*., 1997). Fractions containing cleavage activity eluted at 170 mM KCl and were used further to purify CF II. CF II-containing fractions were identified by their ability to cleave the 3′-untranslated region (3′-UTR) of the CYC1 precursor when combined with partially purified CF I fractions. CF II activity was purified by five additional chromatographic steps (see Materials and methods).

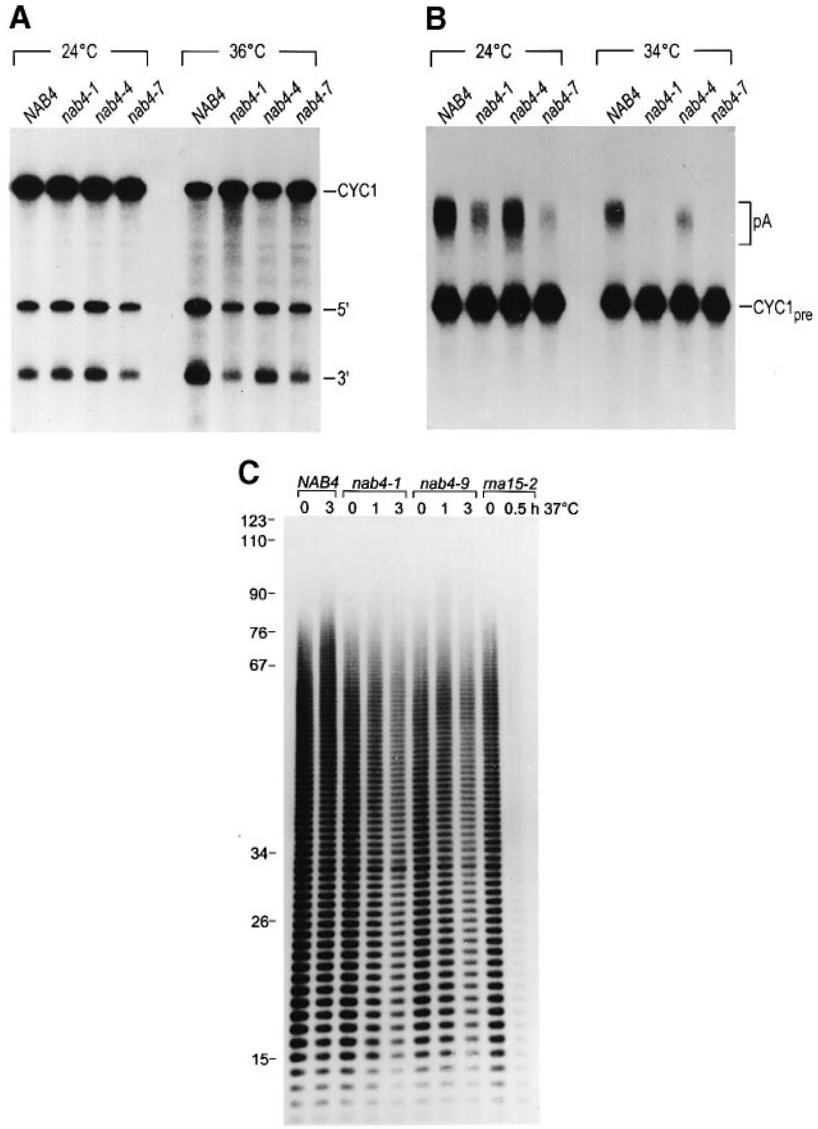

Fig. 1. 3′ End cleavage *in vitro* and poly(A) tail length distribution *in vivo* with *nab4* mutant strains. (**A**) Cleavage alone reactions of CYC1 precursor RNA, generated by *in vitro* transcription of pGYC1, were performed at either 24 or 36°C by replacing ATP with CTP and magnesium with EDTA. The positions of the pre-mRNA (CYC1), 5′ end (5′) and 3′ end (3′) cleavage products are indicated. (**B**) Polyadenylation alone reactions were performed using the pre-cleaved CYC1 precursor RNA generated in (A). The CYC1 pre-cleaved 5′ fragment (CYCpre) and polyadenylated product (pA) are indicated. (**C**) Poly(A) tail analysis of *NAB4*, *nab4-1*, *nab4-9* and *rna15-2* strains shifted to 37°C for 30 min, 1 h or 3 h. Size markers (indicated in nucleotides) are *Msp*I-cut pBR322.

We demonstrated previously that efficient and accurate cleavage of CYC1 transcripts occurred upon combination of purified CF IA and partially purified CF II/CF IB fractions (Minvielle-Sebastia *et al*., 1997). We thus examined whether the CF II fractions isolated with the purification procedure described here were able to perform specific endonucleolytic cleavage of CYC1 when added to purified CF IA fractions. Surprisingly, in addition to the normal 5′ cleavage product (CP I), a major fragment (CP III) and another, minor one (CP II) also accumulated (Figure 2A). It was remarkable that the abundance of CP III correlated with the purity of the CF II fractions used (Figure 2A, lanes 2–7). To test whether cleavage at additional sites occurs only with CYC1, we also performed *in vitro* reactions with the 295 nucleotide GAL7-1 transcript, derived from the 3′-UTR of the *GAL7* gene (Chen and Moore, 1992). Similarly to CYC1, the normal upstream cleavage product (CP I) was produced together with two additional smaller fragments (CP V and VI; Figure 2B, lane 2). The expected 3′ cleavage product (CP II) and two other smaller fragments (CP III and IV; Figure 2B, lane 3) accumulated only when EDTA replaced magnesium in the cleavage assay (see Materials and methods). This result suggested that the Mono S CF II fractions used in these assays still contained traces of the 5′–3′ exonucleolytic activity that is responsible for degrading the uncapped downstream fragment generated after cleavage at the poly(A) site (Butler and Platt, 1988). Nevertheless, in addition to the normal poly(A) site, purified CF IA and CF II allowed efficient cleavage at additional sites that partially purified CF I and CF II fractions would not otherwise recognize (Figure 2A) (Minvielle-Sebastia *et al*., 1997).

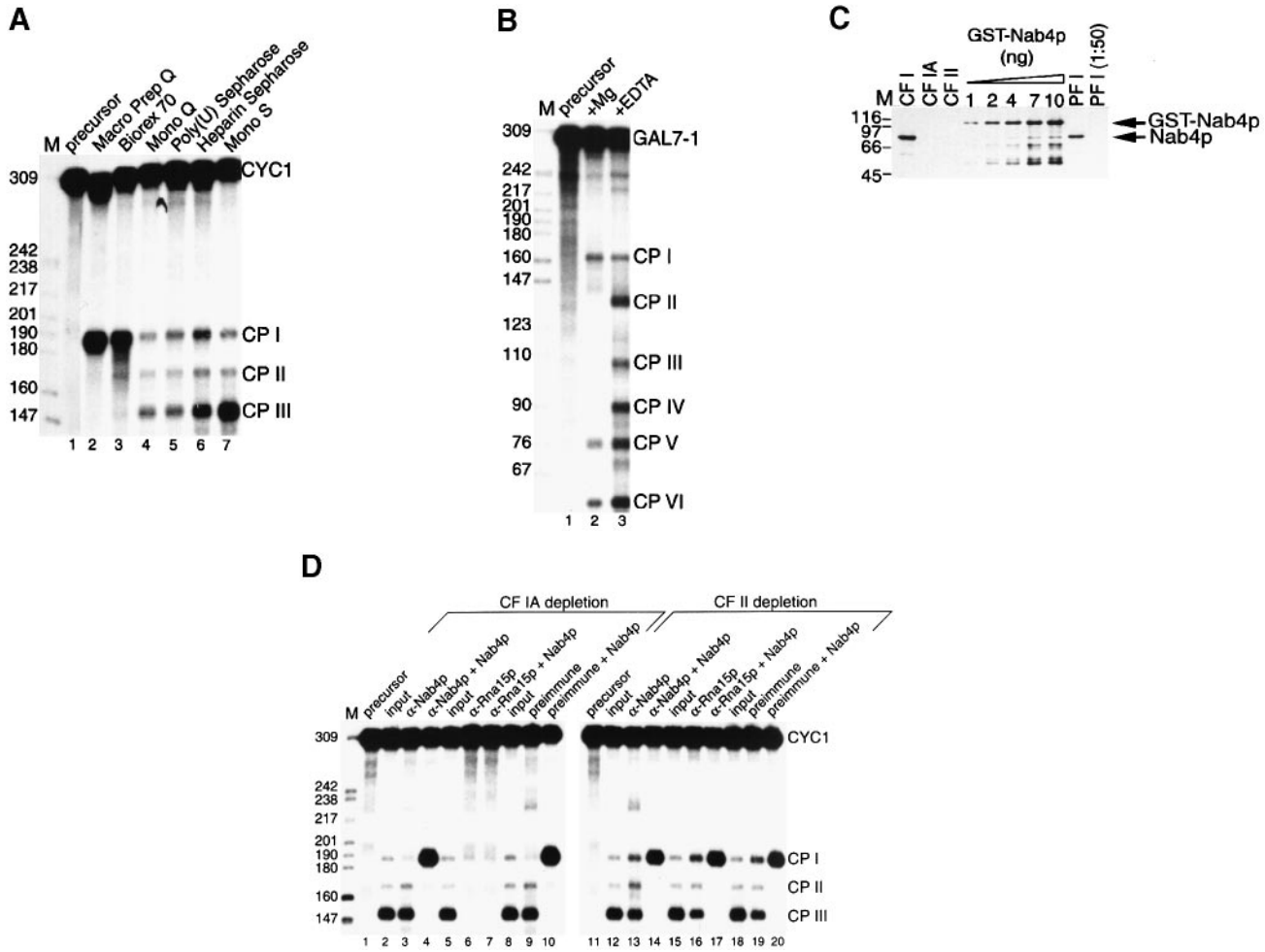

Fig. 2. Cleavage assays with purified CF IA and CF II devoid of Nab4p/Hrp1p result in cleavage at multiple sites. (**A**) Cleavage reactions with 1.5 μl of purified CF IA and 3 μl of the different steps of CF II purification (from the first column, lane 1, Macro Prep Q, to the last one, lane 7, Mono S). Lane 1, unreacted CYC1 precursor. The positions of the various cleavage fragments (CP I–CP III) are indicated. M, DNA markers; the sizes are indicated in nucleotides. (**B**) Cleavage reaction with purified CF IA and partially purified CF II (Mono S fraction). Lane 1, unreacted GAL7 wild-type transcript; lane 2, standard cleavage reaction performed with magnesium acetate; lane 3, EDTA replaces magnesium in the reaction mix. The positions of the various cleavage fragments (CP I–CP VI) are indicated. M, DNA markers (in nucleotides). (**C**) Immunoblot analysis of partially purified CF I (2 μl; Mono Q fraction from CF II purification, see above), pure CF IA (1.5 μl; 'Smart' Mono Q fraction), partially purified CF II (1 μl; Mono S fraction), partially purified PF I (1 μl; Mono S fraction or 1 μl of a 1:50 dilution of the same fraction) and increasing amounts of recombinant GST–Nab4p (1–10 ng). The mAb used was specific for Nab4p/Hrp1p (3H1). The smaller polypeptides migrating under recombinant Nab4p/Hrp1p (GST–Nab4p) are proteolysis products generated by *NAB4* overexpression. M, protein marker, in kDa. (**D**) Cleavage assays with immunodepleted CF IA and CF II fractions. The cleavage factors CF IA and CF II were subjected to immunodepletion with either anti(α)-Nab4p/Hrp1p or anti(α)-Rna15p antibodies, or with pre-immune serum prior to being used in cleavage reactions. Lanes 1 and 11, unreacted CYC1 precursor; lanes 2, 5, 8, 12, 15 and 18, control reactions performed with untreated CF IA and CF II fractions; lanes 3, 6 and 9, CF IA was immunodepleted with anti-Nab4p/Hrp1p, anti-Rna15p or pre-immune serum, respectively; lanes 4, 7 and 10, the same as lanes 3, 6 and 9, respectively, but recombinant Nab4p/Hrp1p was added to the reaction; lanes 13, 16 and 19, CF II was immunodepleted with anti-Nab4p/Hrp1p, anti-Rna15p or pre-immune serum, respectively; lanes 14, 17 and 20, the same as lanes 13, 16 and 19, respectively, but recombinant Nab4p/Hrp1p was added to the reaction. M, DNA markers (in nucleotides).

CF IB is not required for pre-mRNA cleavage but for poly(A) addition in vitro

To determine whether Nab4p/Hrp1p was present in the CF IA and CF II fractions used in this study, immunoblotting was performed with monoclonal antibodies generated against this polypeptide. The Nab4/Hrp1 protein was not detectable in either the purified CF IA or CF II fractions, in contrast to unfractionated CF I and partially purified PF I (Mono S fraction; see Figure 3C) (Preker *et al*., 1997). This was surprising since we could obtain efficient cleavage of both the CYC1 and GAL7 transcripts in the absence of CF IB (Figure 2A and B). This differs from the previous study by Kessler *et al*. (1997) which reported that Nab4p/Hrp1p is essential for the cleavage step. To rule out the possiblity that the protein was present at a level not detectable by immunoblotting, we carried out two rounds of immunodepletion on CF IA and CF II fractions with antibodies to Nab4p/Hrp1p (see Materials and methods). Whereas immunodepletion of CF IA and CF II with anti-Nab4p/Hrp1p did not detectably affect cleavage activity (Figure 2D, compare lanes 3 and 13 with lanes 2 and 12, respectively), antibodies directed against Rna15p, a component of CF IA, completely abolished CF IA activity in the cleavage reaction (Figure 2D, compare lanes 5 and 6). As controls, depletion of CF II fractions with anti-Rna15p, and CF IA and CF II with pre-immune serum did not inactivate the cleavage reaction (Figure 2D, lane 16, and lanes 9 and 19, respectively). The slight decrease observed in lanes 16 and 19 compared with

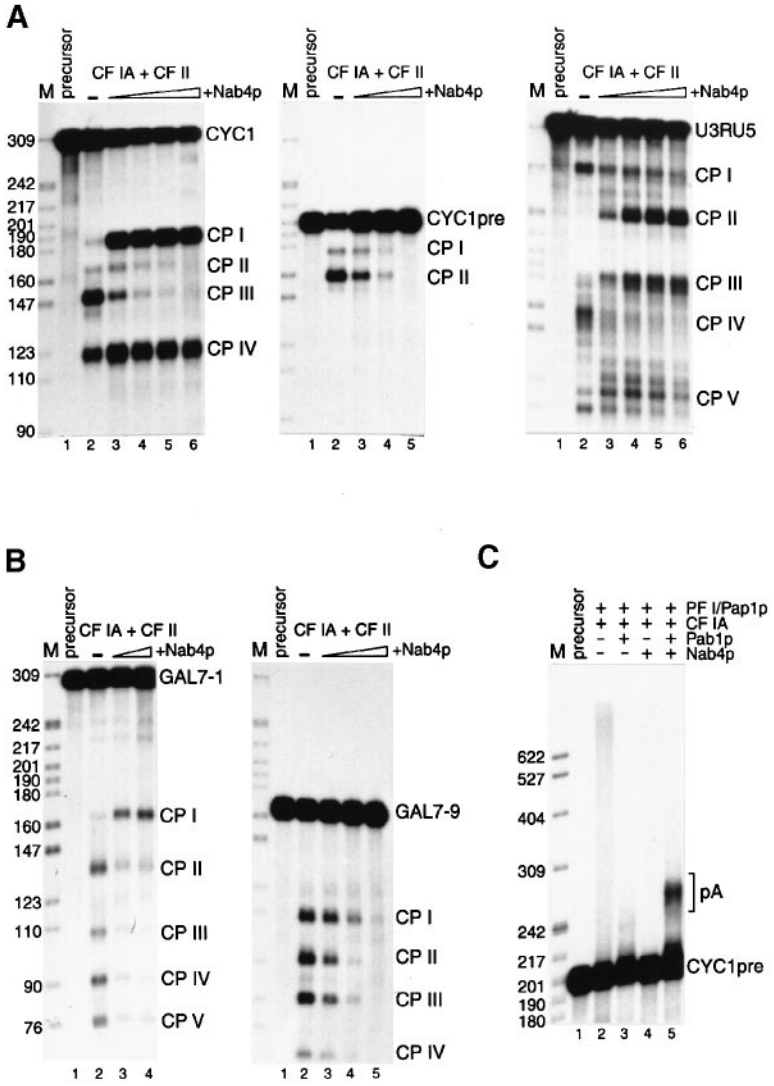

Fig. 3. Effect of Nab4p/Hrp1p on 3′ end endonucleolytic cleavage of a variety of transcripts. Cleavage reactions with purified CF IA, CF II and 0–80 ng of GST–Nab4p. The precursor RNAs used were (**A**) CYC1 (left panel), CYC1 pre-cleaved (middle panel) and U3RU5 (right panel), and (**B**) GAL7 full-length (GAL7-1; left panel) and pre-cleaved (GAL7-9; right panel) transcripts. The positions of the various cleavage products (CP I–V) are indicated. M, DNA markers (in nucleotides). Lanes 1, unreacted precursors; lanes 2, no Nab4p/Hrp1p was added. With CYC1 and U3RU5, 10, 25, 50 or 80 ng of Nab4p/Hrp1p were added (lanes 3–6, respectively); with CYC1 pre-cleaved and GAL7-9, 25, 50 or 80 ng of Nab4p/Hrp1p were used (lanes 3–5, respectively); with GAL7-1, 25 or 50 ng of Nab4p/Hrp1p were added (lanes 3 and 4, respectively). (**C**) Polyadenylation reactions with the CYC1 pre-cleaved precursor. Various combinations of PF I, CF IA, Pab1p and GST–Nab4p are indicated at the top. pA, specific polyadenylated product.

the activity found with the untreated input, lanes 15 and 18, respectively, is likely to be attributable to the dilution of the fractions during the immunodepletion. This experiment showed that cleavage can occur in the absence of Nab4p/Hrp1p.

To test for the precise role of Nab4p/Hrp1p in cleavage, we produced recombinant GST–Nab4p in *Escherichia coli*. This recombinant polypeptide was recognized specifically by the monoclonal antibody to Nab4p/Hrp1p (see Figure 2C). Remarkably, the addition of increasing amounts of recombinant Nab4p/Hrp1p to cleavage assays with CYC1 resulted in the accumulation of CP I and CP IV only, with a concomitant reduction of CP II and CP III (Figure 3A, left panel). The amount of the 119 nucleotide downstream fragment (CP IV) was only slightly affected. A careful titration of Nab4p/Hrp1p (from 1 to 10 ng) showed that the accumulation of CP I was progressive and resulted from a shift of the major cleavage product CP III to CP I (data not shown). Consistent with this result, the addition of Nab4p/Hrp1p to the CF IA and CF II fractions immunodepleted with antibodies directed against this yeast hnRNP or with the pre-immune serum resulted in the accumulation of CP I only (see Figure 2D, lanes 4, 10, 14, 17 and 20). As expected, Nab4p/Hrp1p could not restore the activity of the anti-Rna15p-immunodepleted CF IA fraction (Figure 2D, lane 7). Elimination of the aberrant cleavage products by recombinant Nab4p/Hrp1p was likely to be specific because addition of recombinant Pab1p or Rna15p had no effect on the accumulation of CP II and CP III (data not shown). The simplest interpretation of this result was that CP II and CP III were derived from the upstream fragment CP I by cleavage at alternative sites. To verify this conclusion, cleavage assays were performed with the CYC1 pre-

cleaved substrate (CYC1pre) which lacks sequences located downstream of the normal poly(A) site (Minvielle-Sebastia *et al*., 1994). A similar cleavage pattern was produced in the absence of Nab4p/Hrp1p, with two major cleavage products, CP I and CP II. Formation of both CP I and CP II was inhibited by increasing concentrations of recombinant Nab4p/Hrp1p (Figure 3A, middle panel). Another substrate RNA, U3RU5, derived from the yeast Ty retrotransposon element that contains *cis*-acting sequences similar to those of CYC1 (Hou *et al*., 1994), was also cleaved in the absence of Nab4p/Hrp1p, and addition of GST–Nab4p shifted the major but aberrant cleavage products CP I and CP IV to the normal ones CP II and CP III (Figure 3A, right panel). Although GAL7 cleavage could occur with CF IA and CF II only, we observed that complementation with Nab4p/Hrp1p could restore utilization of the major poly(A) site producing predominantly CP I, although the aberrant cleavage products CP III–V were not completely eliminated (Figure 3B, left panel). Finally, the GAL7-9 pre-cleaved substrate (Zhelkovsky *et al*., 1995) was also cleaved in the absence of Nab4p/Hrp1p into four major products (CP I–IV) that addition of Nab4p/Hrp1p readily removed. These results demonstrated that CF IB is not an essential cleavage factor *in vitro*, but that multiple cleavage sites are used in its absence.

We next examined if Nab4p/Hrp1p is required for the formation of the poly(A) tail. The partially purified PF I/Pap1p fraction (Mono S fraction) (Preker *et al*., 1997) did not contain detectable Nab4p/Hrp1p at the concentration used in this assay (1:50; see immunoblot, Figure 2C), whereas other PF I components (Ysh1p and Yth1p; Barabino *et al*., 1997; Preker *et al*., 1997) were readily detectable by immunoblotting (data not shown). Addition of pure CF IA to PF I did not inhibit the non-specific elongation of extremely long poly(A) tails to CYC1pre that previously was shown to occur with pure PF I alone (Figure 3C, lane 2) (Minvielle-Sebastia *et al*., 1997; Preker *et al*., 1997). Additions of both recombinant Pab1p, required for poly(A) tail length control in yeast (Minvielle-Sebastia *et al*., 1997), and GST–Nab4p were necessary to obtain normal polyadenylated product (Figure 3C, lane 5). This result shows that although not required for the cleavage step, Nab4p/Hrp1p is essential for regulated poly(A) tail synthesis *in vitro*.

Evidence for a specific cleavage complex devoid of CF IB

The cleavage assays presented above, which were performed in the absence of Nab4p/Hrp1p, showed that the major cleavage product that accumulated after 60 min at 30°C was not the normal 5′ fragment. We thus examined the kinetics of cleavage with purified CF IA and CF II devoid of Nab4p/Hrp1p (Figure 4). First, it is remarkable that cleavage occurs very rapidly with both the CYC1 and GAL7 transcripts since cleavage products accumulated within 30 s after the reactions were started. Secondly, accumulation of CP I that resulted from cleavage at the normal poly(A) site within CYC1 (Figure 4A, lower panel, large arrowhead marked ‘1’) was present early in the reaction and drastically reduced at later time points. This loss coincided with the appearance of CP II then CP III at 1–2 min. Similarly to the CP I kinetics, the amount of CP II increased within the first 20–30 min, then dramatically decreased by 90 min. Only CP III and the normal 3′ fragment (CP IV) accumulated throughout the entire time course. These results suggested that cleavage initially occurred at the normal poly(A) site, with subsequent cleavage of the normal 5′ fragment into CP II and CP III, indicating a precursor–product relationship. However, replacing magnesium acetate with EDTA in this experiment had the advantage of stabilizing minor uncapped fragments, as suggested in the earlier experiment with GAL7 (Figure 2B). Consequently, an additional fragment labeled CP V in Figure 4A significantly accumulated with time. The size of CP V, 142 nucleotides, corresponded to cleavage at position 2 as the first cleavage event. Interestingly, a faint band running below CP II was also visible that could correspond to the 156 nucleotide cleavage product that would arise if the first cleavage of the CYC1 precursor occurred at the most distal site (position 3; Figure 4A). It is likely that these minor products are not stable and can be cleaved further into the end products CP II and CP IV. However, primary cleavage at sites 2 and 3 resulted in the accumulation of cleavage products after 2 min, whereas CP I was the most abundant fragment visible after only 30 s. Hence, it appeared that in the absence of Nab4p/Hrp1p, the cleavage complex formed by CF IA and CF II preferentially recognized and cleaved at the authentic poly(A) site first, and that additional cleavage sites were utilized further sequentially. An alternative to this processive model would be that the endonucleolytic cleavage complex utilized the normal poly(A) site followed by degradation of the 5′ fragment CP I by an exonuclease activity to produce CP II and CP III. This possibility was eliminated by running longer gels which resolved the short fragments generated by cleavage between cleavage sites 1 and 2 (23 nucleotides), and cleavage sites 2 and 3 (14 nucleotides; data not shown). Also, it is very unlikely that a putative Mg^{2+}-independent 3′–5′ and/or 5′–3′ exonucleolytic activity would stop at cleavage site 3, allowing accumulation of CP III and leaving the uncapped CP IV intact. This experiment suggested that the alternative cleavage sites 2 and 3 were specific. The schematic representation of the cleavage reactions with CYC1 is depicted below the gel in Figure 4A.

Similarly to CYC1, the time course for GAL7 cleavage in the absence of Nab4p/Hrp1p revealed that the authentic poly(A) site (numbered 1 in Figure 4B, lower panel) is utilized preferentially first, resulting in the accumulation of CP I within 30 s. However, CP I was also unstable and was substantially reduced by 90 min. In contrast to CYC1, the kinetics with GAL7 appeared to be more complicated. First, unlike the CYC1 downstream fragment CP IV, the GAL7 3′ cleavage product CP II that was expected to appear concomitant with CP I accumulated only at later time points (5–7 min), and accumulation of CP III and CP IV was delayed even further. Secondly, careful inspection of the cleavage time course showed that CP V was produced shortly after CP I, and that CP VI accumulated at later time points. Because we provided evidence that an exonuclease activity was responsible for the absence of CP II–IV when Mg^{2+} was used in the cleavage assays (Figure 2B), we concluded that cleavage occurred at the additional specific cleavage sites 4 and 5 located within

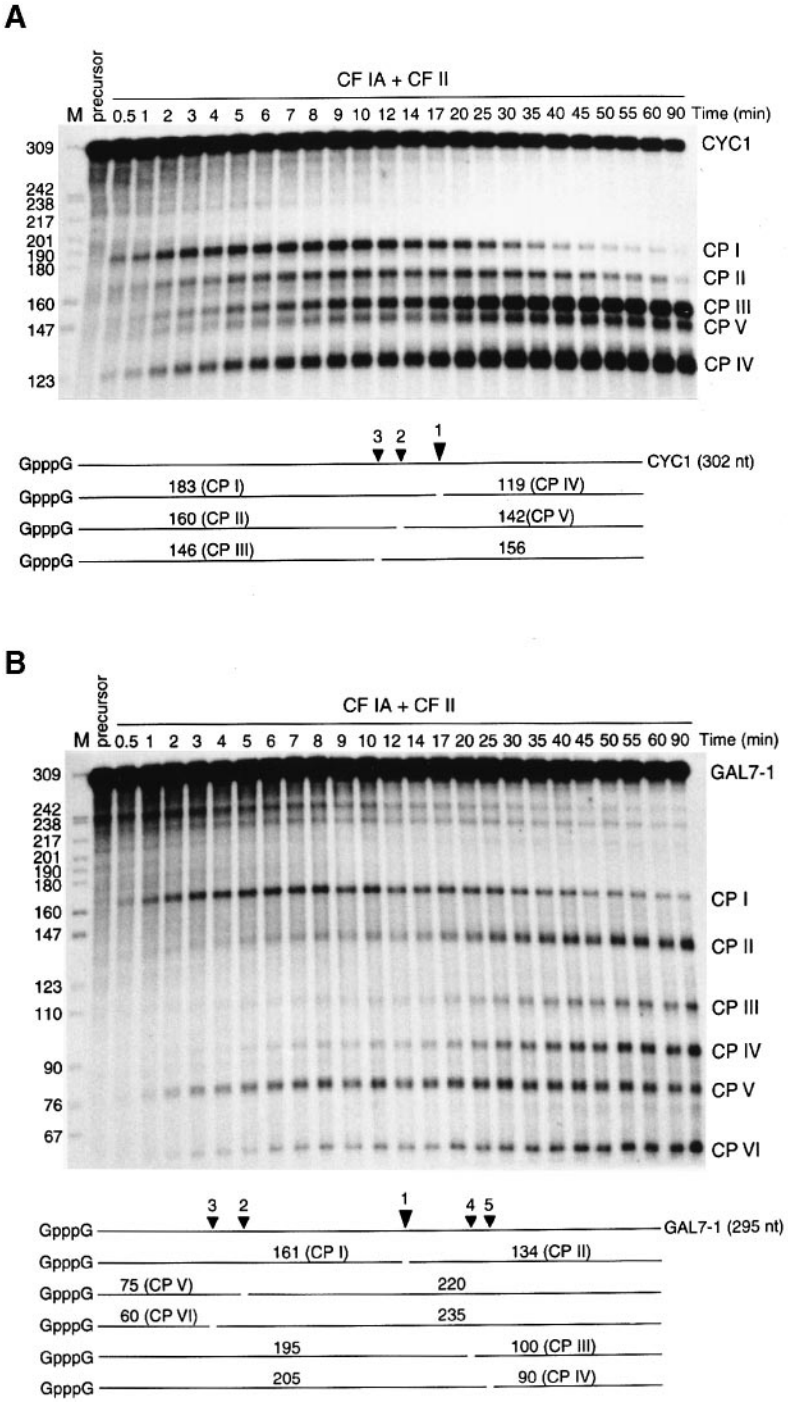

Fig. 4. Kinetics of cleavage in the absence of Nab4p/Hrp1p. Standard reactions were carried out with CF IA and CF II and stopped at the times indicated. (**A**) Endonucleolytic cleavage time course with the CYC1 full-length precursor. The positions of the various cleavage products that significantly accumulate are indicated (CP I–V). A schematic representation of the different cleavage products is diagrammed below the autoradiogram of the gel. Numbers refer to their sizes, in nucleotides. The positions of the cleavage sites that generate CP I–V are indicated (arrowheads topped with numbers). (**B**) Kinetics of cleavage with the GAL7 full-length precursor. The cleavage events are diagrammed below the gel as described in (A).

the GAL7-1 downstream fragment CP II in the time course experiment performed with EDTA. Because it appeared that CP V and CP VI were stable in either Mg^{2+} or EDTA conditions, and hence were capped, it is reasonable to assume that CP I was cleaved into CP V, the latter being shortened further into CP VI. As with CYC1, accumulation of upstream cleavage products seemed to be a function of time, with CP I being cleaved sequentially into CP V and CP VI. However, we cannot rule out that cleavage sites 2 and 3, depicted in the bottom panel of Figure 4B, are also recognized directly by the cleavage complex. Supporting this possibility, fragments corresponding in size to a single cut at either cleavage site 2 (220 nucleotides) or site 3 (235 nucleotides) can be observed (Figure 4B,

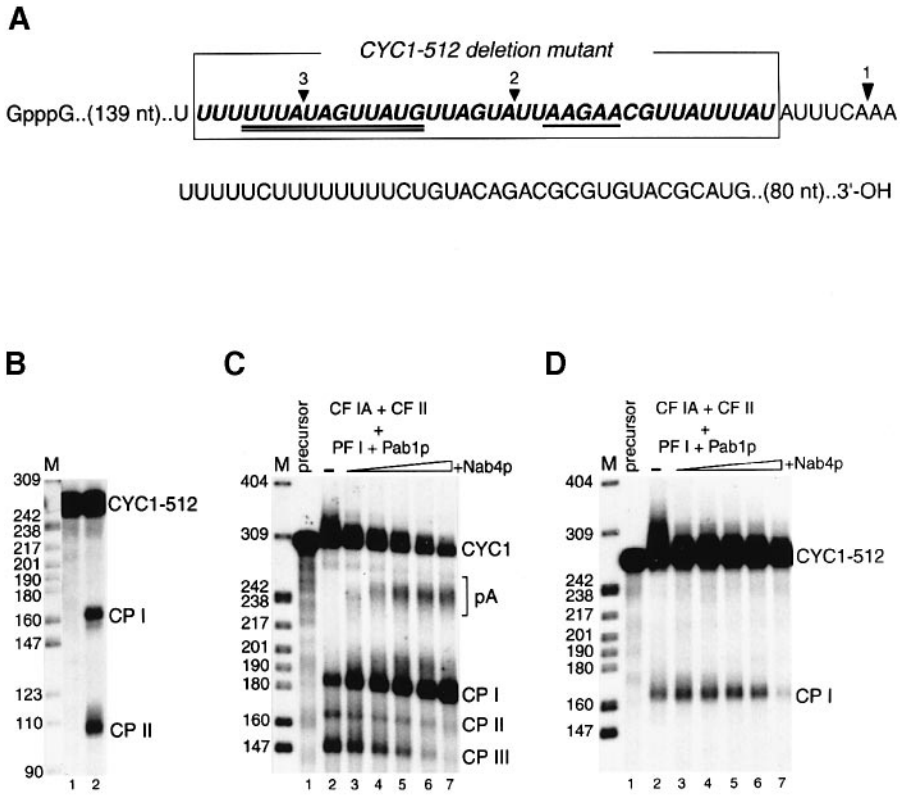

Fig. 5. The cyc1-512 mutant RNA is cleaved efficiently but not polyadenylated *in vitro*. (**A**) Partial sequence of the CYC1 precursor RNA and mapping of the cleavage sites by RT–PCR and cDNA sequencing. The positions of the mapped cleavage sites that generate CP I, CP II and CP III are indicated (arrowheads topped with numbers). Cleavage site 1 corresponds to the major poly(A) site mapped *in vivo* (Zaret and Sherman, 1982) and *in vitro* (Butler and Platt, 1988). The 38 nucleotide deletion identified in the cyc1-512 mutant is boxed. The putative efficiency and positioning elements directing 3′ end formation at the major poly(A) site 1 are double underlined and single underlined, respectively. (**B**) Cleavage assay with CF IA, CF II and the mutant cyc1-512 substrate. The positions of the two major cleavage products are indicated (CP I and CP II). Lane 1, unreacted precursor. (**C**) Reconstitution of cleavage–polyadenylation reactions with CYC1 wild-type substrate, and purified CF IA, CF II, PF I, Pab1p and 0–50 ng of GST–Nab4p. Lane 1, unreacted precursor; lane 2, no added Nab4p/Hrp1p; lanes 3–7, addition of Nab4p/Hrp1p (2, 5, 10, 25 and 50 ng, respectively). CP I–III, pA, cleavage and polyadenylated products, respectively. (**D**) Same as in (C), but with the cyc1-512 mutant substrate.

doublet below the precursor; see also Figures 2B and 3B). These results demonstrated that the cleavage complex CF IA–CF II is competent for specific recognition of the authentic poly(A) sites in CYC1 and GAL7 RNAs, but that it can cleave further at specific alternative sites in the absence of Nab4p/Hrp1p.

Cryptic 3′-end-forming signals are used in the cyc1-512 mutant RNA

To determine the exact cleavage sites in the CYC1 RNA utilized by the CF IA–CF II cleavage complex, CP I–CP III were isolated, subjected to RT–PCR, cloned, and the 3′ ends determined by DNA sequencing. CP II and CP III corresponded to cleavage at sites 2 and 3, respectively, which were located as predicted within 37 nucleotides upstream of the normal poly(A) site 1 (Figure 5A). Notably, cleavage site 2 was adjacent to the previously described positioning element (Figure 5A, single underlined), whereas cleavage site 3 was within the efficiency element (double underlined). Interestingly, cleavage sites 2 and 3 were located in a region of the RNA that is deleted in the cyc1-512 mutant transcript (boxed in Figure 5A). It has been shown that this mutation abolishes 3′ end formation both *in vivo* and *in vitro* in yeast cell-free extracts (Zaret and Sherman, 1982; Butler and Platt, 1988). However, another region of the RNA located upstream of this 38 nucleotide sequence may be recognized by the cleavage complex CF IA–CF II when it processes the CYC1 transcript at positions 2 and 3. Therefore, we performed cyc1-512 cleavage reactions with the purified factors CF IA–CF II (Figure 5B). As predicted, in the absence of the alternative cleavage sites 2 and 3, efficient cleavage of the mutant RNA occurred at one position only. When coupled cleavage–polyadenylation reactions were carried out, increasing concentrations of Nab4p/Hrp1p were able to restore efficient and accurate processing of wild-type CYC1 pre-mRNA (Figure 5C). In contrast, no polyadenylation of the unique cyc1-512 upstream cleavage product CP I occurred, and addition of Nab4p/Hrp1p even inhibited the cleavage reaction (Figure 5D). These results suggested that although cryptic 3′-end processing signals can be used by the cleavage complex, they might be too weak or non-specific to allow the polyadenylation complex to function *in vitro*.

The cleavage complex has no strict sequence specificity

The ability of CF IA–CF II to cleave RNAs that lacked the 3′-end-forming signals otherwise essential *in vivo* and *in vitro* in unfractionated cell-free systems suggested that the cleavage complex can recognize and bind to related sequences. To test this possibility, we performed UV cross-

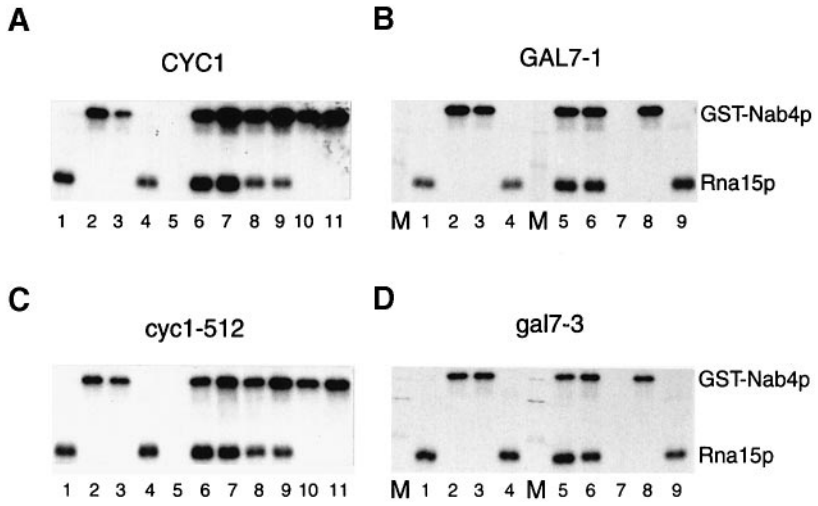

Fig. 6. CF IA and recombinant Nab4p/Hrp1p cross-link to both wild-type and mutant substrates. (**A**) UV cross-linking of different proteins with the wild-type CYC1 substrate. Lane 1, recombinant Rna15p; lane 2, GST–Nab4p; lane 3, GST–Nab4-9 mutant protein; lane 4, CF IA; lane 5, CF II; lane 6, recombinant Rna15p with 50 ng of GST–Nab4p; lane 7, recombinant Rna15p with 80 ng of GST–Nab4p; lane 8, CF IA with 50 ng of Nab4p/Hrp1p; lane 9, CF IA with 80 ng of Nab4p/Hrp1p; lane 10, CF II with 50 ng of Nab4p/Hrp1p; lane 11, CF II with 80 ng of Nab4p/Hrp1p. (**B**) UV cross-linking with the wild-type GAL7-1 substrate. Lanes 1–4, as lanes 1–4 in (A); lane 5, recombinant Rna15p with 50 ng of Nab4p/Hrp1p; lane 6, CF IA with 50 ng of Nab4p/Hrp1p; lane 7, CF II alone; lane 8, CF II with 50 ng of Nab4p/Hrp1p; lane 9, CF II with CF IA. (**C**) The same reactions as in (A) performed with mutant cyc1-512. (**D**) The same reactions as in (B) performed with mutant gal7-3. M, protein marker. The positions of Rna15p and GST–Nab4p are indicated.

linking experiments with wild-type and mutant RNAs in the presence of tRNA as a non-specific competitor (see Materials and methods). With *E.coli*-expressed recombinant Rna15p and GST–Nab4p alone, we found polypeptides that corresponded in size to Rna15p (42 kDa) and GST–Nab4p (~100 kDa) with both CYC1 and GAL7-1 wild-type substrates (Figure 6A and B, lanes 1 and 2, respectively). This result was expected because these proteins contain one (Rna15p) or two (Nab4p/Hrp1p) canonical RNP-type RNA-binding domains (RBDS) (Minvielle-Sebastia *et al*., 1991; Henry *et al*., 1996), and were previously shown to cross-link to wild-type RNAs (Kessler *et al*., 1996, 1997). A GST fusion of the Nab4-9 mutant protein was also produced which contained a single $L^{205}S$ substitution in the first RBD as previously described (Kessler *et al*., 1997). Nab4-9p was also able to bind and cross-link to wild-type RNAs, although with a slightly decreased efficiency (Figure 6A and B, lanes 3). With purified CF IA, a single radioactive polypeptide the size of which corresponded to Rna15p was found, which is consistent with the polypeptide composition of this purified factor (Figure 6A and B, lanes 4) (Kessler *et al*., 1996; Minvielle-Sebastia *et al*., 1997). With combinations of proteins, we could still observe cross-linking of the RBD-containing polypeptides, showing that their binding to wild-type RNAs was not mutually exclusive (Figure 6A, lanes 6–9, and B, lanes 5 and 6). However, in contrast to previous reports (Kessler *et al*., 1997; Zhao *et al*., 1997), none of the CF II subunits was cross-linked detectably, and no significant stimulation or inhibition of Nab4p/Hrp1p and CF IA binding by the addition of CF II was visible (Figure 6A, lanes 5, 10 and 11, and B, lanes 7–9). Moreover, efficient cross-links were also found when the mutant cyc1-512 and gal7-3 were substituted for the wild-type CYC1 and GAL7-1 transcripts, respectively (Figure 6C and D). These results demonstrated that Nab4p/Hrp1p and the RNA-binding component of the cleavage

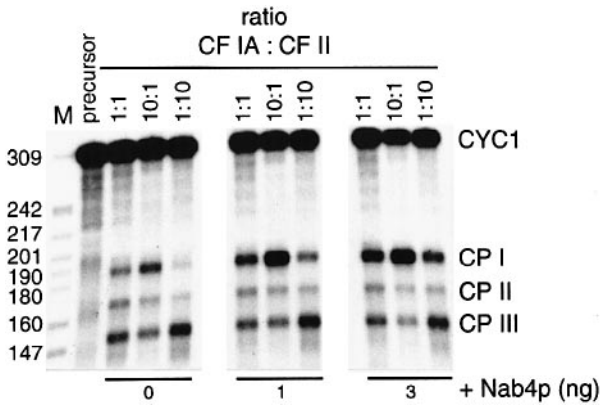

Fig. 7. Antagonistic action of CF IA–Nab4p/Hrp1p versus CF II for poly(A) site selection. Cleavage reactions were performed with CYC1 and relative CF IA:CF II ratios (1:1, 10:1 or 1:10) in the presence of 0, 1 or 3 ng of Nab4p/Hrp1p. The positions of the various cleavage fragments (CP I–III) are indicated. M, DNA markers; the sizes are indicated in nucleotides.

complex can bind RNA with no exclusive specificity for the sequences that have been shown to be essential 3′-end-forming signals *in vivo* and in cell-free extracts *in vitro*.

The CF IA + Nab4p/CF II ratio modulates cleavage site selection

We have shown in the experiments described above that increasing amounts of Nab4p/Hrp1p completely inhibited endonucleolytic cleavage at alternative sites (Figure 3). To determine more precisely which of the CF IA and/or CF II activities were antagonized by Nab4p/Hrp1p, we tested relative concentrations of all three components in cleavage assays with CYC1. These experiments were carried out starting with a CF IA:CF II ratio (1:1) that allowed production of virtually equal amounts of the three cleavage products CP I–III (Figure 7). Significantly, in

the absence of Nab4p/Hrp1p, a 10-fold increase of CF IA favored CP I accumulation, whereas a 10-fold excess of CF II resulted in the accumulation of CP III and a concomitant decrease in the amounts of CP I and II. However, we could never completely switch to the exclusive utilization of the normal poly(A) site 1 by augmenting CF IA concentrations (data not shown). As expected, addition of Nab4p/Hrp1p increased the use of poly(A) site 1, which was enhanced further by a 10-fold excess of CF IA. At all Nab4p/Hrp1p concentrations tested, an excess of CF II consistently antagonized the combined effect of both CF IA and Nab4p/Hrp1p, in that it inhibited accumulation of CP I. Therefore, the yeast hnRNP Nab4p/Hrp1p, and to a lesser extent CF IA, can modulate endonucleolytic cleavage site selection during pre-mRNA 3′ end processing.

Discussion

We have demonstrated in this study that Nab4p, previously described as CF IB/Hrp1p (Kessler *et al*., 1996, 1997), is not an essential factor for pre-mRNA 3′ end endonucleolytic cleavage *in vitro*, but plays an important role in the regulation of poly(A) site selection. This function is very specific since cleavage site selection is not influenced by other RNA-binding proteins, such as Pab1p or Rna15p (data not shown). In the absence of Nab4p/Hrp1p, purified CF IA and CF II primarily recognized the authentic poly(A) sites of a variety of transcripts. However, the cleavage complex also cleaved at additional specific sites. Considering the CYC1 transcript, only three cleavage products were obtained and the overall cleavage efficiency was comparable in the absence or in the presence of Nab4p/Hrp1p. The presence of short fragments (~23 and 14 nucleotides; data not shown) and the fact that the cleavage products were not degraded further by exonucleases demonstrated that the endonucleolytic complex CF IA–CF II can cleave at specific sites. Therefore, the cleavage complex can recognize additional *cis*-acting RNA sequences that lie upstream of the proposed efficiency and positioning elements (Guo and Sherman, 1995, 1996). Efficient cleavage of mutant cyc1-512, which lacks these elements, supports this conclusion. Although GAL7 3′-end-forming signals belong to a different class of motifs with UA repeats as essential sequences (Abe *et al*., 1990; Chen and Moore, 1992; Guo and Sherman, 1995), GAL7-1 wild-type transcript could also be cleaved at the normal cleavage site in the absence of Nab4p/Hrp1p. However, similarly to CYC1, additional sites were also used. Our results contradict previous data showing that Nab4p/Hrp1p acts as an essential cleavage factor, with specificity for the $(UA)_6$ efficiency element (Kessler *et al*., 1997). Since the same RNA substrate was used in both studies, these discrepancies could be explained by subtle differences in the ratio of cleavage factors employed, the threshold of sensitivity of the *in vitro* assays or other experimental procedures. However, Kessler *et al*. (1997) did report a stimulation of the 3′-end processing reaction by Nab4p/Hrp1p which is consistent with our conclusions that Nab4p/Hrp1p acts in a concentration-dependent manner.

How does Nab4p/Hrp1p inhibit utilization of alternative cleavage sites? With CYC1, we observed that increasing concentrations of Nab4p/Hrp1p sequentially suppressed upstream cleavage products. One possible model is that Nab4p/Hrp1p prevents the cleavage complex from using the upstream sites by anchoring CF IA and CF II to the major poly(A) site 1. This explanation is unlikely because Nab4p/Hrp1p as well as CF IA can bind mutant RNAs that lack the specific positioning and/or efficiency elements (Figure 6). Alternatively, Nab4p/Hrp1p might alter RNA structure and create a higher order structure, such as a stable stem–loop, that blocks movement of the complex. We have attempted to test the latter possibility by preincubation of CYC1 precursor RNA under cleavage conditions with GST–Nab4p but in the absence of CF IA and CF II, followed by Nab4p/Hrp1p removal and incubation with CF IA and CF II (data not shown). Although this pre-treatment with Nab4p/Hrp1p failed to inhibit the use of upstream cleavage sites, we cannot rule out the possibility that alternative CYC1 precursor RNA structures may not be stable to RNA extraction and precipitation.

A plausible mechanism of Nab4p/Hrp1p action is that this yeast hnRNP prevents the cleavage complex from using additional sites by binding to upstream sequences, therefore blocking the access of the cleavage complex by masking potential cleavage sites. In the absence of Nab4p/Hrp1p, the cleavage complex CF IA–CF II primarily associates at the major poly(A) site, but cleavage at alternative sites is not prohibited. When the last potential cleavage site is utilized, the complex dissociates (Figure 8A). Cumulatively, our results show that the efficiency and positioning elements are neither required for cleavage nor necessary for Nab4p/Hrp1p binding (Figures 5 and 6). However, these elements are essential for the polyadenylation reaction, suggesting that PF I requires them for specific binding and activity (Figures 3C and 5D). We propose that specificity for the 3′-end-forming signals in yeast is not conferred by a single polypeptide, but rather by the subtle association of multiple RNA-binding activities. It is conceivable that Nab4p/Hrp1p acts by stabilizing the binding of the cleavage complex on the pre-mRNA, protecting the efficiency and positioning elements from cleavage. It thus offers PF I optimal conditions to bind to the specificity elements, which further enhances the stability of the processing complex and allows polyadenylation to occur (Figure 8B). In this view, careful RNA-binding analyses with variable concentrations of CF IA, CF II, PF I, Nab4p/Hrp1p and Pab1p will have to be tested. This model is consistent with the fact that 3′ end processing *in vivo* and in whole-cell extracts is dependent upon the efficiency and positioning elements because the physiological ratio of the different factors is maintained.

Nab4p/Hrp1p is a yeast hnRNP structurally related to metazoan hnRNP A/B proteins (Henry *et al*., 1996; Kessler *et al*., 1997; A.M.Krecic, R.E.Hector, L.Minvielle-Sebastia, M.R.Paddy, K.Beyer, W.Keller and M.S.Swanson, in preparation). In human cells, alternative 5′ splice site selection is regulated by relative amounts of hnRNP A1 and SF2/ASF proteins (Ge and Manley, 1990; Ge *et al*., 1991; Krainer *et al*., 1991; Mayeda and Krainer, 1992). An excess of SF2/ASF favors utilization of proximal 5′ splice sites whereas an excess of hnRNP A1 results in the use of distal 5′ splice sites. From the data shown in our study, a similar mechanism may be responsible for the

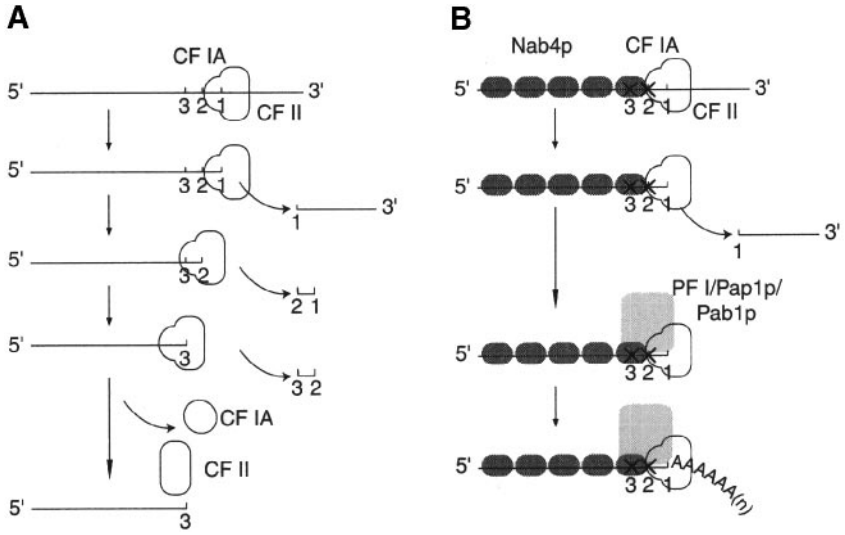

Fig. 8. Model of Nab4p/Hrp1p function in 3′ end processing. (**A**) Pre-mRNA 3′ end processing in the absence of Nab4p/Hrp1p. The cleavage complex CF IA–CF II preferentially recognizes the natural poly(A) site first (denoted 1) and then cleaves at additional sites (2 and 3). In the absence of this yeast hnRNP, the cleavage complex dissociates when no more sites are found on the pre-mRNA. (**B**) Pre-mRNA 3′ end processing in the presence of Nab4p/Hrp1p. Binding of the cleavage complex is stabilized by Nab4p/Hrp1p, which also masks the alternative cleavage sites. Following cleavage, the polyadenylation factors PF I–Pap1p–Pab1p can join and further stabilize the complex, which is now proficient for polyadenylation.

use of alternative endonucleolytic sites in yeast pre-mRNA 3′ end processing by Nab4p/Hrp1p. It is indeed known that a variety of yeast genes produce multiple transcripts that differ at their 3′ end by alternative polyadenylation. Some of these genes even give rise to transcripts that terminate within the open reading frame (ORF). The best characterized one, *CBP1*, produces two mRNAs that are regulated reciprocally by the carbon source (Mayer and Dieckmann, 1989; Sparks *et al*., 1997; Sparks and Dieckmann, 1998). It is therefore conceivable that, rather than being a strict fidelity factor, Nab4p/Hrp1p functions by regulating the use of alternative 3′ end cleavage sites in a concentration-dependent manner *in vivo*. The role of Nab4p/Hrp1p we demonstrated *in vitro* may reflect the mechanism that gives rise to multiple transcripts from *CBP1* and from a variety of other yeast genes (Mayer and Dieckmann, 1989; Finnegan *et al*., 1991; Minvielle-Sebastia *et al*., 1991; Stone *et al*., 1991; Mandart and Parker, 1995; Sparks *et al*., 1997; Mandart, 1998; Sparks and Dieckmann, 1998).

We showed that CF IA functions synergistically with Nab4p/Hrp1p to cleave at the normal poly(A) site whereas CF II acts as an antagonistic factor (see Figure 7). It has been reported that variations in the concentration and binding efficiency of the 64 kDa subunit of mammalian CstF modulate poly(A) site selection of the IgM heavy chain gene that occurs during differentiation of B lymphocytes (Edwalds-Gilbert and Milcarek, 1995; Takagaki *et al*., 1996). It is thus reasonable to believe that variations in the relative amounts of the different components of the cleavage complex may also occur *in vivo* in yeast and modulate the endonucleolytic step of the 3′ end processing reaction. Interestingly, Rna15p, which is the RNA-binding component of CF IA, is the putative yeast homolog of CstF-64 (Takagaki and Manley, 1994; Keller and Minvielle-Sebastia, 1997), and our results showed that CF IA enhances the use of the major poly(A) site. It is therefore possible that modulation of the intracellular concentrations of polyadenylation factors may be a specific regulatory mechanism for the expression of some genes.

Table I. Yeast strains

Designation	Genotype
YJA213	*MATα leu2 his3Δ200 trp1-289 ura3-52 nab2Δ1::LEU2* [pNAB2.13]
YJA513	*MATα leu2Δ2 ura3-52 nab2Δ1::LEU2* [pNAB2.15]
LDY133	*MATα ade2 ade3 leu2 trp1 ura3 his3*
YAO403	*MATα**a** leu2Δ2/leu2Δ2 ura3-52/ura3-52 NAB4/nab4Δ2::LEU2*
YAO405-2A	*MATα leu2 ura3-52 trp1-289 his3Δ200 nab4Δ2::LEU2* [pNAB4.7]
YAO417-1D	*MAT**a** leu2Δ2 ura3-52 nab4Δ2::LEU2* [pNAB4.46]
YAO418-1D	*MAT**a** leu2Δ2 ura3-52 nab4Δ2::LEU2* [pNAB4.47]
YAO421	*MATα ade2 ade3 leu2 trp1 ura3 his4 nab4-1*
YAO428-1B	*MAT**a** leu2Δ2 ura3-52 nab4Δ2::LEU2* [pNAB4.55]
YAO431-1C	*MAT**a** leu2Δ2 ura3-52 nab4Δ2::LEU2* [pNAB4.58]
YAO467-1A	*MAT**a** leu2Δ2 ura3-52 nab4Δ2::LEU2* [pNAB4.95]
LM45	*MAT**a** ade2-1 leu2-3,112 ura3-1 rna15-2*

In this view, the concentration-dependent regulation of cleavage site selection would be an evolutionarily conserved function for some hnRNPs. It may be interesting to consider whether mammalian hnRNPs can modulate alternative pre-mRNA 3′ end processing as well.

Temperature-sensitive mutations in the Rna14p, Rna15p and Pcf11p subunits of CF IA recently have been shown to affect termination of transcription by RNA polymerase II (Birse *et al*., 1998). Moreover, *rna14* and *rna15* mutants alter poly(A) site choice for *ACT1* mRNA (Mandart and Parker, 1995), and mutations in *NAB4* result in longer than normal RNA polymerase II transcripts and in higher intracellular Nab4p/Hrp1p levels (A.M.Krecic, R.E.Hector, L.Minvielle-Sebastia, M.R.Paddy, K.Beyer, W.Keller and M.S.Swanson, in preparation). These results suggest that alteration of components of the 3′-end processing apparatus may also change their relative amounts and affect the 3′ end endonucleolytic step, perturbing transcription termination by RNA polymerase II. We currently are testing whether cellular variations in yeast

pre-mRNA 3′-end processing components may regulate alternative polyadenylation *in vivo*.

Materials and methods

Yeast strains and plasmids

Table I lists the yeast strains used in this study. The *NAB4* gene was isolated using a mutant *nab2* strain (YJA213). The pNAB2.13 plasmid was isolated by selecting *in vivo* recombinants between gapped pNAB2.19 (digested with *Nar*I–*Msc*I) and the *NAB2* gene pool which were obtained by mutagenic PCR amplification (Muhlrad *et al*., 1992) using primers MSS44 (5′-TAATACGACTCACTATAGGGAGA-3′) and MSS57 (5′-ATTAACCCTCACTAAAG-3′), and pNAB2.15 (Anderson *et al*., 1993b) as template. The PCR products were purified and co-transformed with gapped pNAB2.19 into YJA513. The pNAB2.19 was constructed by subcloning the blunt-ended 2.46 kb *Pvu*I–*Nsi*I fragment from pRNP327 (Anderson *et al*., 1993b) into *Sma*I-cut pRS314 (Sikorski and Hieter, 1989). Transformants were replicated onto plates containing 5-fluoro-orotic acid (5-FOA) to select against pNAB2.15, and incubated at 24 and 37°C. One of the resulting temperature-sensitive strains (YJA213) was used to isolate additional *NAB* genes, including *NAB4*, using a previously described procedure (Anderson *et al*., 1993b). Briefly, YJA213 cells (20 l of OD_{600} = 2–3) were grown at 30°C, and cells were exposed to UV light for 5 min at 4°C to induce the formation of covalent poly(A)$^+$ RNA–protein complexes *in vivo*. These complexes were purified by oligo(dT) chromatography, and subsequently were employed to immunize mice as described (Anderson *et al*., 1993b). The *NAB4* gene was isolated by expression screening of a λgt11 genomic library (Snyder *et al*., 1987) using a 1:400 dilution of the resulting anti-RNP antiserum. One genomic clone (RNP2-13.1) encoded a partial novel ORF (starting at nucleotide 96 of the *NAB4* coding region) and the 2.3 kb *Eco*RI insert was subcloned into *Eco*RI-cut pSP72 (Promega) to generate pRNP4.0. The pNAB4.0 clone, containing the full-length *NAB4* gene within an ~7 kb *Bam*HI genomic DNA fragment, was obtained by hybridization screening of a YCp50-based yeast genomic DNA library (Yeast Genetic Stock Center) using an 800 nucleotide *Eco*RI–*Hin*cII fragment from pRNP4.0 and previously described hybridization and wash conditions (Wilson *et al*., 1994). To determine whether *NAB4* was essential for cell viability, one chromosomal allele was transplaced in the diploid strain YJA501 (Anderson *et al*., 1993b) using a subclone of RNP2-13.1 in which 40% of the ORF was eliminated. Briefly, pRNP4.6 was generated by subcloning the *Eco*RI–*Hin*dIII fragment of pRNP4.0 into *Eco*RI–*Hin*dIII-cut pSP72. The *NAB4* coding region of pRNP4.6 was disrupted by replacing the internal *Sty*I fragment with the 2.2 kb *Xho*I–*Sal*I *LEU2* fragment of YEp13 (Broach *et al*., 1979) by blunt end ligation, yielding pNAB4.9. Genomic DNA blot analysis confirmed the desired recombination event, and segregation analysis of a leucine prototroph gave a 2:2 pattern of viable to non-viable spores. All viable spores were leucine auxotrophs, indicating that *NAB4* was essential. Haploid leucine prototrophs could be rescued with pNAB4.7. This complementing plasmid consisted of a 2.7 kb *Bam*HI–*Sal*I genomic DNA fragment from pNAB4.0 subcloned into *Bam*HI–*Sal*I-cut pRS316 (Sikorski and Hieter, 1989). The *nab4* null allele was also complemented by the single-copy pNAB4.46, containing the same genomic DNA fragment subcloned into *Bam*HI–*Sal*I-cut YCp50.

To isolate anti-Nab4p monoclonal antibodies (mAbs), the partial *NAB4* ORF was subcloned in-frame with GST. pRNPex4.7 was generated by subcloning the *Eco*RI–*Sal*I fragment of pRNP4.0 into *Eco*RI–*Sal*I-cut pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ). The resulting Nab4p fusion protein was used to generate the 3H1 mAb using a previously described procedure (Wilson *et al*., 1994).

Generation of nab4 mutant strains

Mutant *nab4* alleles were created by random PCR mutagenesis. Briefly, the entire *NAB4* coding region was amplified by PCR for 30 cycles under mutagenic conditions using primers MSS194 (5′-GGTATACT-CAATAAACA-3′) and MSS177 (5′-CTAATACACGTACATGT-3′), 2 mM $MgCl_2$ and *Taq* polymerase. Four separate reactions were performed by lowering the concentration of each of one of the four nucleotides to 40 μM, with the remaining nucleotides at 200 μM and $MnCl_2$ included at 15 μM. Mutagenic PCR products were pooled and amplified under normal PCR conditions using MSS192 (5′-GCA-GTATACACAAGTCT-3′) and MSS181 (5′-TGGTAGCCATTATTACG-3′). This PCR product was then purified by agarose gel electrophoresis and co-transformed with gapped and gel-purified pNAB4.8 into YAO405-2A. pNAB4.8 was generated by subcloning the 2.7 kb *Bam*HI–*Sal*I fragment of pNAB4.0 into *Bam*HI–*Sal*I-cut pRS314, and gapped by digesting the plasmid with *Aat*II and *Age*I, eliminating ~40% of the *NAB4* coding region. Transformants were replicated onto plates containing 5-FOA to select against pNAB4.7, and incubated at 24 or 36°C. Putative mutant alleles were recovered by plasmid rescue and sequenced. Single-copy mutant *nab4* plasmids were created by subcloning the 2.7 kb *Bam*HI–*Sal*I fragment of *nab4-1*, *nab4-4* and *nab4-7* into *Bam*HI–*Sal*I-cut YCp50 to yield pNAB4.47, pNAB4.55 and pNAB4.58, respectively. These YCp50 clones were transformed into the diploid strain YAO403 and all were recessive to *NAB4* at 36°C. Diploid cells harboring each of the mutants were sporulated and dissected at 24°C. Leu$^+$Ura$^+$ haploid cells containing *nab4-1*, *nab4-4* or *nab4-7* were Ts$^-$ at 36°C. The missense mutations in these alleles are: *nab4-1* ($N^{167}D$, $F^{179}Y$, $P^{194}H$, $Q^{265}L$); *nab4-4* ($S^{34}C$, $I^{163}T$, $M^{368}K$, $D^{442}G$, $D^{448}G$); and *nab4-7* ($S^{64}R$, $K^{92}M$, $T^{125}A$, $I^{163}T$). The *nab4-9* Ts$^-$ allele encoding a single $L^{205}S$ missense mutation was generated by PCR amplification using mutagenic oligonucleotides. First, 5′- and 3′-mutagenized fragments were generated by PCR amplification using MSS198 (5′-CCCGAATTCGCAATGAG-CTCTGACGA-3′) and MSS565 (5′-CACTAGAAGGTTTTTCAAAA-GATGAGAAACCGAACCCTCTAG-3′), or MSS222 (5′-CCCAAG-CTTGACAGCTGTACTTC-3′) and MSS564 (5′-CTAGAGGGTT-CGGTTTCTCATCTTTTGAAAAACCTTCTAGTG-3′), respectively. Next, these 5′ and 3′ products were combined in a second PCR amplification using MSS198 and MSS222. The DNA sequence of the 1.1 kb *Sst*I–*Bst*EII fragment from this secondary PCR was confirmed then subcloned into pNAB4.46 to generate the single-copy *nab4-9* plasmid pNAB4.95. Transformation of YAO403 with pNAB4.95 revealed that *nab4-9* was also recessive. Sporulation and dissection of the resulting diploid strain, YAO467, yielded the *nab4-9* haploid strain YAO467-1A, which was Ts$^-$ at 37°C.

The *nab4-1* allele was integrated into the *NAB4* chromosomal locus by subcloning the 2.7 kb *Bam*HI fragment of *nab4-1* into the yeast integrative vector YIp5 (Struhl *et al*., 1979) to generate pNAB4.45. This construct was linearized within *nab4-1* using *Bst*EII and transformed into LDY133 to target integration into the *NAB4* locus by homologous recombination. Ura$^+$ transformants were grown in YPD overnight to allow loss of the integrated *URA3* marker. These cultured cells were plated on 5-FOA to select for loss of *URA3* then tested for the Ts$^-$ phenotype. To confirm the integration of *nab4-1* at the *NAB4* locus, the plasmid pNAB4.7 was digested with *Aat*II and *Age*I, gel purified and transformed into the Ts$^-$ strain YAO421. The plasmid recovered from the resulting Ura$^+$ cells was sequenced, revealing only those point mutations found in *nab4-1*. Southern analysis of genomic DNA from YAO421 confirmed the integration of a single copy of *nab4-1*.

In vitro 3′ end cleavage and polyadenylation assays

Cell extracts for *in vitro* polyadenylation were prepared using either a previously described spheroplast protocol (Butler and Platt, 1988) or a liquid nitrogen lysis procedure designed to minimize Nab4p/Hrp1p proteolysis. For the liquid nitrogen procedure, 1 l of cells was grown to OD_{600} = 4.5–5.5, pelleted at 1000 *g* for 5 min at 4°C and resuspended in 8 ml of 50 mM HEPES (pH 7.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM ε-amino-*n*-caproic acid, 1 mM *p*-aminobenzamidine, 2 μg/ml pepstatin, 2 mM Pefabloc SC (Boehringer Mannheim). Resuspended cells were pipeted into a 15 cm mortar filled with liquid nitrogen and ground with a pestle for ~7 min to a very fine powder. Additional liquid nitrogen was added 2–3 times during this procedure to keep the cell suspension frozen. The cell powder was placed in a 40 ml Dounce homogenizer and rapidly warmed using a 24°C water bath while stirring with a 1 ml pipet. All subsequent procedures were performed at 4°C. The viscous cell suspension was homogenized using six strokes of the tight 'A' pestle, and the lysate subsequently processed according to a described method (Butler and Platt, 1988). *In vitro* reactions for 3′ end cleavage and polyadenylation were done essentially as described previously (Butler and Platt, 1988). The 3′ endonucleolytic cleavage reactions were performed at either 24 or 36°C in a 25 μl volume which included 1 U/μl of recombinant placental RNase inhibitor (Gibco-BRL).

Poly(A) tail length analysis

The length distribution of poly(A) tails was determined by following a previously described procedure with a few modifications (Minvielle-Sebastia *et al*., 1991). Briefly, 1 μg of total yeast RNA was 3′ end labeled for 16–18 h on ice. Following digestion of non-poly(A)$^+$ RNA, reactions were brought up to 200 μl with diethylpyrocarbonate (DEPC)-treated water prior to extraction. Poly(A) tails were precipitated using

5 µg of RNase-free glycogen (Boehringer Mannheim) as carrier, washed quickly in –20°C 70% ethanol, and resuspended in DEPC-treated water. Poly(A) tails were separated on 8% denaturing polyacrylamide gels.

Purification of 3′-end processing factors

Purified CF IA and CF II were isolated as previously described with several modifications (Minvielle-Sebastia *et al*., 1997; Zhao *et al*., 1997). For CF IA, poly(U)–Sepharose fractions (Minvielle-Sebastia *et al*., 1997) were purified further on a Mono Q column (PC 1.6/5; Smart System, Pharmacia). These Mono Q fractions are free of detectable Pab1p, as assessed by Western blotting with an mAb to Pab1p (Anderson *et al*., 1993a). The CF II fractions were partially purified according to the following procedure; this purification method is adapted from a previously published scheme (Zhao *et al*., 1997). Ammonium sulfate-fractionated extracts prepared from strain PJP14 were applied to a Macro-Prep High Q column as described (Preker *et al*., 1997). Fractions containing combined CF I/CF II activities, which eluted at 100–200 mM KCl, were applied to a Bio-Rex 70 column (Bio-Rad) and the proteins were eluted with a 50–500 mM KCl gradient. CF II-containing fractions were chromatographed on a 1 ml Mono Q HR 5/5 column (Pharmacia) as described (Zhao *et al*., 1997). The CF II-containing sample was applied to a poly(U)–Sepharose column (Pharmacia) which was developed with a gradient from 0.1 to 1 M KCl. CF II activity eluted at ~250 mM KCl. This CF II activity was purified further on a 1 ml heparin column (HiTrap column, Pharmacia) as described (Zhao *et al*., 1997). Active CF II fractions were loaded onto a 1 ml Mono S HR 5/5 column (Pharmacia) and proteins were eluted with a gradient from 50 to 500 mM KCl. The CF II activity eluted between 250 and 300 mM KCl. The active CF II Mono S fractions used in this study do not contain detectable CF IA, CF IB or PF I activities. Interestingly, the final Mono S CF II fractions showed, in addition to the four components described in Zhao *et al*. (1997), the 53 kDa subunit Pfs2p (cited in Keller and Minvielle-Sebastia, 1997; Preker *et al*., 1997) and Yth1p, homologous to CPSF-30K (Barabino *et al*., 1997). The actual involvement of Pfs2p and Yth1p in the cleavage reaction currently is being tested (S.M.L.Barabino, M.Ohnacker, M.Sadowski, L.Minvielle-Sebastia and W.Keller, unpublished results).

Partially purified PF I Mono S fractions (Preker *et al*., 1997) were used in polyadenylation assays at a 1:50 dilution. As reported previously, poly(A) polymerase is an integral component of this factor (Preker *et al*., 1997).

Recombinant Rna15p (Minvielle-Sebastia *et al*., 1997) was purified as a His_6-tagged fusion on an Ni^{2+}-NTA column, as recommended by the manufacturer (Qiagen). For expression of recombinant full-length Nab4p/Hrp1p, the *NAB4* ORF (nucleotides 3–1605) was amplified by PCR using MSS197 (5′-CCCGGATCCTTTTTACCTATTATATG-3′) and MSS198, and cloned into *Bam*HI–*Eco*RI-cut pGEX-4T-1 to generate pNAB4.84. Preliminary immunoblotting results using mAb 3H1 demonstrated that GST–Nab4p was very sensitive to proteolysis. A protease-deficient bacterial strain (CAG629) was transformed with pNAB4.84 for optimal expression of full-length protein. Cells were grown at 30°C in 2× YT and 500 µg/ml carbenicillin to OD_{600} = 0.6 and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. The GST–Nab4p fusion protein was purified by glutathione–Sepharose chromatography according to the manufacturer's instructions (Novagen) followed by dialysis in 50 mM Tris–HCl (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF). The GST–Nab4-9p was prepared using pNAB4.97, which was constructed by subcloning the *Sst*I–*Hin*dIII fragment from pNAB4.95 into *Sst*I–*Hin*dIII-cut pNAB4.84, and the recombinant protein purified as described for GST–Nab4p.

RNA synthesis

Capped and labeled RNAs were synthesized from restriction enzyme-linearized templates by transcription with bacteriophage RNA polymerases (Stratagene, Boehringer) and purified on 6% polyacrylamide–8.3 M urea gels. CYC1 and CYC1 pre-cleaved RNAs were transcribed from the pG4-CYC1 and pG4-CYC1pre plasmids, respectively (Minvielle-Sebastia *et al*., 1994; Preker *et al*., 1995); GAL7 full-length wild-type and mutant RNAs, and GAL7 pre-cleaved RNAs were obtained from the pJCGAL7-1, pJCGAL7-3 and pJCGAL7-9 plasmids, respectively (Chen and Moore, 1992; Zhelkovsky *et al*., 1995); the Ty-derived RNAs were transcribed from the U3RU5 plasmid (Hou *et al*., 1994).

In vitro 3′-end processing assays with purified factors

For cleavage assays with purified factors, a standard cleavage reaction (performed in a final volume of 20 µl) contained 1.5 µl of purified CF IA ('Smart' Mono Q fraction), 0.5 µl of partially purified CF II (Mono S fraction), 2% polyethylene glycol 8000, 75 mM potassium acetate, 2 mM magnesium acetate, 2 mM cordycepin triphosphate, 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.01% NP-40, 1 mM DTT, 0.1 mg/ml purified bovine serum albumin (BSA), 0.2 U of RNAguard (Pharmacia) and 15 fmol of radiolabeled transcript. In order to inhibit degradation of uncapped cleavage products, EDTA replaced magnesium acetate at the same final concentration. Reactions were processed for 60 min at 30°C, terminated by addition of a stop solution, and the RNAs recovered as described previously (Minvielle-Sebastia *et al*., 1994). Half of the reaction products were separated on 6% polyacrylamide–8.3 M urea gels and visualized by autoradiography.

For cleavage kinetics in the absence of Nab4p/Hrp1p, amounts of CF IA and CF II corresponding to 20 times a standard reaction were mixed and pre-incubated at 30°C for 15 min in the absence of the labeled transcripts. Cleavage reactions were started by adding the RNAs. Aliquots of 16 µl were withdrawn at the times indicated, and reactions were stopped and analyzed as described above.

Polyadenylation reactions with CYC1pre (20 µl final volume) were processed for 60 min at 30°C and contained 1.5 µl of purified CF IA, 0.5 µl of partially purified PF I (Mono S fraction, 1:50 dilution), 80 ng of recombinant Pab1p (Minvielle-Sebastia *et al*., 1997) and 25 ng of GST–Nab4p. The reaction mixture was the same as for the standard cleavage reactions, except that 2 mM ATP replaced cordycepin triphosphate. In the coupled cleavage–polyadenylation assays, CYC1 or cyc1-512 replaced CYC1pre, and 0.5 µl of CF II was added.

3′-RACE and cDNA cloning of CYC1 cleavage products

The CP I, II and III fragments produced during 3′ end processing reactions described above were fractionated by electrophoresis, excised from the denaturing gel and polyadenylated *in vitro* with Pap1p (Martin and Keller, 1998). Briefly, polyadenylation was performed for 5 min at 30°C in a 15 µl reaction volume which contained 20 mM Tris–HCl (pH 7.0), 1.6 mM $MgCl_2$, 60 mM KCl, 10% glycerol, 1 mM DTT, 0.8 mg/ml acetylated BSA, 1 mM ATP, 15 U of RNasin and 500 U of poly(A) polymerase. Polyadenylated fragments were reverse transcribed and amplified by 3′-RACE using a CYC1 gene-specific primer (5′-GCAGGTCGATATCATGTAATTAGTTATG-3′) and a cDNA amplification kit (Marathon, Clontech, Palo Alto, CA). Amplified fragments were subcloned into pSP72, and five individual isolates of each cleavage product were sequenced to determine the site of poly(A) addition.

Immunoblot analysis

Proteins were resolved on SDS–10% polyacrylamide gels, transferred to nitrocellulose membranes and probed with a 1:500 dilution of mAb 3H1. Reactive antigens were visualized by enhanced chemiluminescence (ECL, Amersham) with horseradish peroxidase-conjugated anti-mouse secondary antibodies at 1:1000 dilution.

Immunodepletion of CF IA and CF II fractions

Protein A–Sepharose beads (CL4B; Pharmacia) were washed three times with 1 ml of NET_{300} buffer [50 mM Tris–HCl (pH 7.9), 300 mM NaCl, 0.05% NP-40, 0.5 mM DTT]. A 5 µl aliquot of either mAb 3H1, polyclonal antibodies to Rna15p (Minvielle-Sebastia *et al*., 1997) or pre-immune serum was coupled to 40 µl of packed protein A–Sepharose beads in 100 µl of NET_{300} buffer overnight at 4°C. The beads were then washed once with 1 ml of NET_{300} buffer, and three times with 1 ml of buffer E_{50} [20 mM Tris–HCl (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF, 0.7 µg/ml pepstatin, 0.4 µg/ml leupeptin hemisulfate]. CF IA and CF II fractions (~40 µl in buffer E_{50}) were applied separately on the protein A–Sepharose-bound antibodies for 4 h at 4°C. The supernatants were subjected to a second round of immunodepletion with antibodies freshly coupled to protein A–Sepharose beads.

UV cross-linking assays

UV cross-linking reactions were performed in microtiter plates and contained (in 10 µl) 100 fmol of labeled transcripts in 75 mM potassium acetate, 2 mM magnesium acetate, 2 mM ATP, 0.01% NP-40, 1 mM DTT, 0.1 mg/ml purified BSA, 2 µM tRNA (0.5 µg), and proteins as follows: 25 ng of recombinant Rna15p; 50 or 80 ng of GST–Nab4p; 50 ng of GST–Nab4-9p; 1.5 µl of CF IA ('Smart' Mono Q); and 5 µl of CF II (Mono S). When proteins were combined, they were pre-incubated for 10 min at 30°C. They were then added to the reaction mixture and incubated further for 20 min at 30°C. The samples were irradiated on ice in a UV Stratalinker at 500 mJ, and then digested with RNase A (100 ng per reaction) for 45 min at 37°C. The proteins

were mixed with SDS-loading buffer, and separated on SDS–12% polyacrylamide gels. The gels were fixed, dried and visualized by autoradiography after an overnight exposure.

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