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Transitions in RNA polymerase II elongation complexes at the 3′ ends of genes

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To understand the factor interactions of transcribing RNA polymerase II (RNApII) *in vivo*, chromatin immunoprecipitations were used to map the crosslinking patterns of multiple elongation and polyadenylation factors across transcribed genes. Transcription through the polyadenylation site leads to a reduction in the levels of the Ctk1 kinase and its associated phosphorylation of the RNApII C-terminal domain. One group of elongation factors (Spt4/5, Spt6/Iws1, and Spt16/Pob3), thought to mediate transcription through chromatin, shows patterns matching that of RNApII. In contrast, the Paf and TREX/THO complexes partially overlap RNApII, but do not crosslink to transcribed regions downstream of polyadenylation sites. In a complementary pattern, polyadenylation factors crosslink strongly at the 3′ ends of genes. Mutation of the 3′ polyadenylation sequences or the Rna14 protein causes loss of polyadenylation factor crosslinking and readthrough of termination sequences. Therefore, transcription termination and polyadenylation involve transitions at the 3′ end of genes that may include an exchange of elongation and polyadenylation/termination factors.

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Introduction

It is now clear that many steps in gene expression previously thought of as discrete are instead linked functionally and physically. Several mRNA processing events occur cotranscriptionally, and appropriate processing and packaging of mRNA is necessary for efficient export from the nucleus (Shatkin and Manley, 2000; Maniatis and Reed, 2002; Manley, 2002; Proudfoot *et al*, 2002; Reed and Hurt, 2002). One key component of such coupling is the C-terminal domain (CTD) of RNA polymerase II (RNApII). Transcripts synthesized by RNApII lacking the CTD are inefficiently capped, spliced, and polyadenylated (McCracken *et al*, 1997a, b), and many mRNA processing factors directly bind the CTD *in vitro* (Cho *et al*, 1997; Corden and Patturajan, 1997; McCracken *et al*, 1997a; Yue *et al*, 1997; Hirose and Manley, 1998; Hirose *et al*, 1999; Carty *et al*, 2000; Rodriguez *et al*, 2000; Morris and Greenleaf, 2000; Barilla *et al*, 2001; Dichtl *et al*, 2002a, b; Licatalosi *et al*, 2002). The CTD appears to be positioned near the RNA exit channel of RNApII, suggesting that mRNA processing factors bound there would have rapid access to the nascent mRNA (Cramer *et al*, 2000, 2001).

The CTD consists of multiple repeats of the amino-acid sequence YSPTSPS, which becomes multiply phosphorylated during transcription. The two major sites of phosphorylation are serine 2 and serine 5 (Zhang and Corden, 1991). We previously showed that the two phosphorylation sites predominate at different stages in the transcription cycle (Komarnitsky *et al*, 2000). Serine 5 is phosphorylated by basal transcription factor TFIIH at the promoter, and the mRNA capping enzyme is brought to the transcription complex via binding to this modified form of the CTD (Cho *et al*, 1997; McCracken *et al*, 1997b; Yue *et al*, 1997; Komarnitsky *et al*, 2000; Schroeder *et al*, 2000). As RNApII elongates, serine 5 phosphorylation drops and the capping enzyme dissociates. Meanwhile, increasing amounts of serine 2 phosphorylation are observed as RNApII moves further downstream (Komarnitsky *et al*, 2000). Serine 2 phosphorylation is dependent upon the Ctk1 kinase (Cho *et al*, 2001), which may be homologous to mammalian elongation factor P-TEFb. The Fcp1 CTD phosphatase also associates with transcription complexes, removing phosphates from serine 2 during elongation (Cho *et al*, 2001). It may also have a role in dephosphorylating serine 5 (Schroeder *et al*, 2000).

We predicted that mRNA processing, elongation, and/or termination factors might specifically bind to the serine 2 phosphorylated form of RNApII (Komarnitsky *et al*, 2000). In support of this hypothesis, efficient *in vivo* splicing and polyadenylation require an intact CTD (McCracken *et al*, 1997b), and the phosphorylated CTD stimulates these processes *in vitro* (Hirose and Manley, 1998; Hirose *et al*, 1999). Several polyadenylation factors interact with phosphorylated CTD *in vitro* (Rodriguez *et al*, 2000; Barilla *et al*, 2001; Dichtl *et al*, 2002a, b), and in at least one case specifically with the serine 2 phosphorylated form (Licatalosi *et al*, 2002).

Because associations with the CTD are dynamic during the transcription cycle, we used chromatin immunoprecipitation (ChIP) to survey the locations of various elongation and polyadenylation factors along transcribed genes. Just as we previously found a transition in CTD phosphorylation and association of capping enzyme at the 5′ ends of genes, we find evidence for another transition at the 3′ end of genes. RNApII transcribes past the polyadenylation site, and the mRNA is cleaved after the polyadenylation sequences emerge from RNApII (Birse *et al*, 1998; Dye and Proudfoot, 2001; Tran *et al*, 2001; Dichtl *et al*, 2002b; Orozco *et al*, 2002).

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Downstream of polyadenylation sites, the levels of serine 2 phosphorylation drop. Some elongation factors continue with RNApII past the polyadenylation site, but others are apparently released. In contrast, polyadenylation factors are strongly crosslinked to genes near the 3′ ends of genes. This crosslinking is dependent upon proper polyadenylation/termination sequences and functional Rna14 protein. The complementary patterns of some elongation and polyadenylation factors suggest that transcription termination may involve an exchange of factors bound to RNApII.

Results

ChIP analysis of events during elongation and at the 3′ end of genes

Having previously used ChIP to study transitions in transcription complexes during initiation and early elongation (Komarnitsky *et al*, 2000; Cho *et al*, 2001), we extended this analysis to the 3′ ends of genes. The first step was to identify several cleavage/polyadenylation sites. Three genes (*PMA1*, *ADH1*, and *PYK1*) were chosen for their relatively high transcription rates and long open reading frames. A 3′ rapid amplification of cDNA ends (3′-RACE) technique was used to clone DNA fragments corresponding to the mRNA/polyA tail junctions. Multiple clones were sequenced, and the major polyadenylation sites are shown in Figure 1. These results allowed us to design primers for ChIP that would amplify sequences within the transcribed regions downstream of the polyadenylation site.

To monitor the many factors involved in transcription elongation, termination, and polyadenylation, we used tandem affinity purification (TAP)-tagged fusion proteins (Rigaut *et al*, 1999; Puig *et al*, 2001; Gavin *et al*, 2002; Krogan *et al*, 2002). The TAP affinity tag contains a protein A module, so we used IgG-agarose to precipitate crosslinked chromatin.

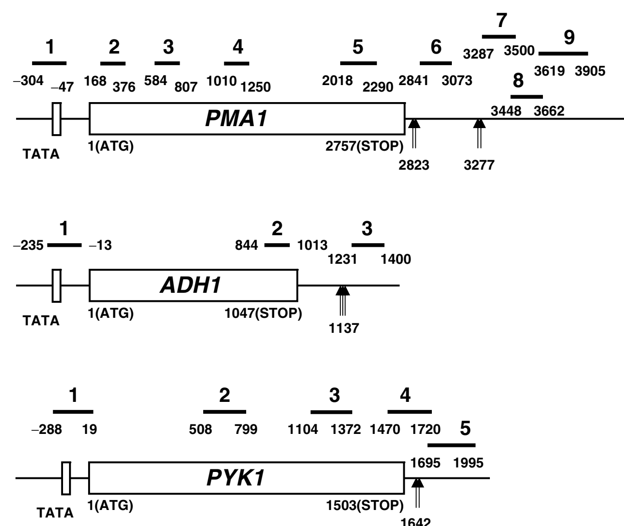

Figure 1 Schematic diagram of the *PMA1*, *ADH1*, and *PYK1* genes. Open reading frames are represented by the hatched box and the TATA box/promoter region by an open box. All nucleotide numbers are relative to the first nucleotide of the initiation codon (+1). Arrows indicate the position of the major polyadenylation sites as mapped by 3′ RACE. Bars above the gene show the positions of PCR products used in the ChIP analysis. The numbers above each PCR fragment are used for identification in all later figures.

Chromatin was prepared from strains in which the RNApII subunit Rpb3 or the basal factor TFIIF (Tfg1) was TAP-tagged. The ChIP patterns for these proteins (Figure 2; data not shown) exactly matched the patterns obtained when using antibodies that directly recognized the proteins or HA-epitope-tagged versions of the proteins (Komarnitsky *et al*, 2000; Cho *et al*, 2001; Krogan *et al*, 2002). Indeed, we find the TAP-tagged ChIPs superior to polyclonal antibodies or other tags because of better accessibility and reproducibility.

To analyze multiple factors involved in elongation, termination, and polyadenylation, strains containing TAP-tagged proteins were assayed by ChIP on the three genes whose polyadenylation sites were mapped. Several subunits from factors consisting of multiple proteins were tested. Because we were interested in events at the 3′ end of genes and their relation to the CTD, we also assayed levels of the Ctk1 kinase (using an HA-tagged version) and CTD serine 2 phosphorylation downstream of the open reading frame (Figure 2). Interestingly, both showed a partial drop downstream of the polyadenylation site.

Factors with crosslinking patterns that overlap that of RNApII

The first pattern of crosslinking observed matched that of RNApII (Figure 3). Many members of this group were elongation factor complexes containing Spt proteins (Suppressor of Ty Insertions;Winston and Carlson, 1992) which are united by many genetic and functional characteristics. Spt4 and Spt5 form a complex that copurifies with RNApII and other transcription factors. Spt4 and Spt5 mutants show extensive genetic and physical interactions with other elongation factors (Lindstrom and Hartzog, 2001; Krogan *et al*, 2002; Squazzo *et al*, 2002; Lindstrom *et al*, 2003; Rondon *et al*, 2003) and mRNA capping enzyme (Wen and Shatkin, 1999; Pei and Shuman, 2002; Lindstrom *et al*, 2003). The mammalian homolog of this complex, known as DSIF, was identified biochemically as affecting early steps in transcription elongation (Wada *et al*, 1998a, b; Yamaguchi *et al*, 1999). However, both Spt4 and Spt5 crosslink throughout transcribed regions (Figure 3; Pokholok *et al*, 2002) including downstream of the polyadenylation site.

In yeast, *SPT6* mutants behave similar to *SPT4* and *5* mutants in genetic assays (Winston and Carlson, 1992). Spt6 and Iws1 are associated with the Spt4/5-containing elongation complexes (Krogan *et al*, 2002; Lindstrom *et al*, 2003). On *Drosophila* chromosomes, the fly homologs of Spt4, 5, and 6 colocalize with phosphorylated RNApII (Andrulis *et al*, 2000; Kaplan *et al*, 2000). Consistent with these studies, in our higher resolution ChIP analysis, the Spt6 and Iws1 crosslinking patterns match those of the Spt4/5 complex (Figure 3).

Spt16 is tightly associated with Pob3 (Orphanides *et al*, 1999; Wittmeyer *et al*, 1999; Formosa *et al*, 2001; Krogan *et al*, 2002), and the mammalian homolog of this complex is known as FACT. FACT stimulates transcription on chromatin templates (Orphanides *et al*, 1999) and interacts functionally with DSIF (human Spt4/5) (Wada *et al*, 2000). The Nhp6A protein also copurifies with Spt6/Pob3 under some conditions (Brewster *et al*, 2001; Formosa *et al*, 2001). However, the ChIP crosslinking pattern for this protein was different from Spt16 and Pob3. Whereas Spt16 and Pob3 crosslinked to

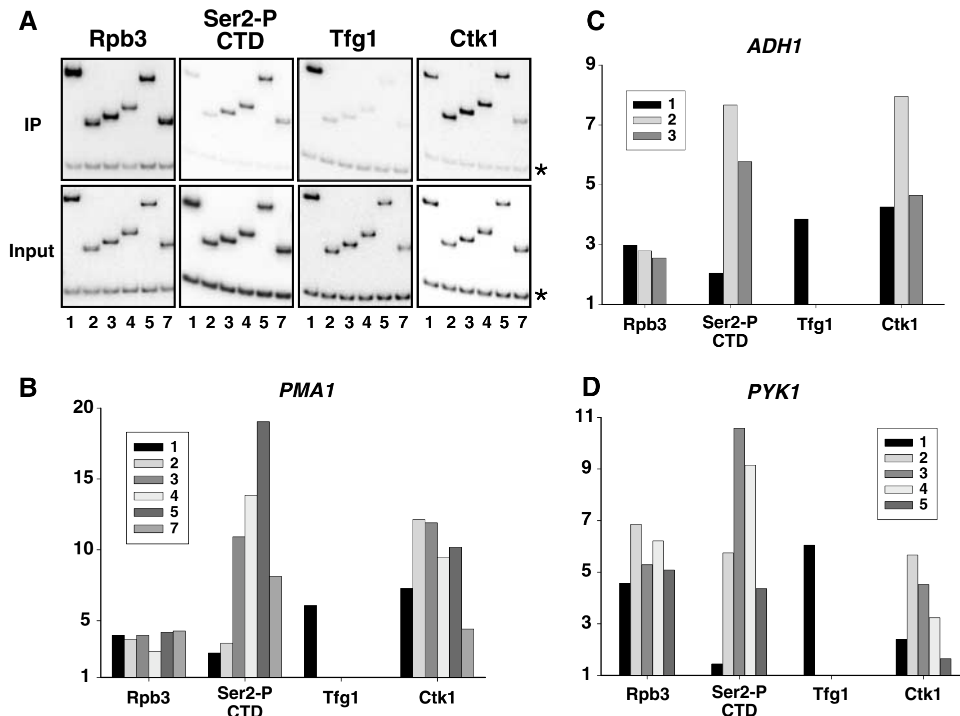

Figure 2 A drop in phosphorylation of CTD serine 2 and Ctk1 kinase occurs after transcription through polyadenylation sites. (**A**) Strains containing TAP-tagged Rpb3, Tfg1, and HA-tagged Ctk1 were analyzed by ChIP of the *PMA1* gene. Sheared chromatin was precipitated with IgG-agarose. In addition, chromatin was immunoprecipitated with the H5 monoclonal antibody, which recognizes CTD phosphorylated at serine 2. Precipitated chromatin was used for PCR amplification (upper panels) with primers as diagrammed in Figure 1. The top band is the *PMA1*-specific band, while the common lower band (marked by an asterisk) is an internal background control from a nontranscribed region on chromosome V. The bottom panels show the input control. (**B**) Quantitation of the data from (A). Signals are expressed as *x*-fold over the background, calculated as described in Materials and methods. (**C**,**D**) A similar analysis was carried out on the *ADH1* and *PYK1* genes.

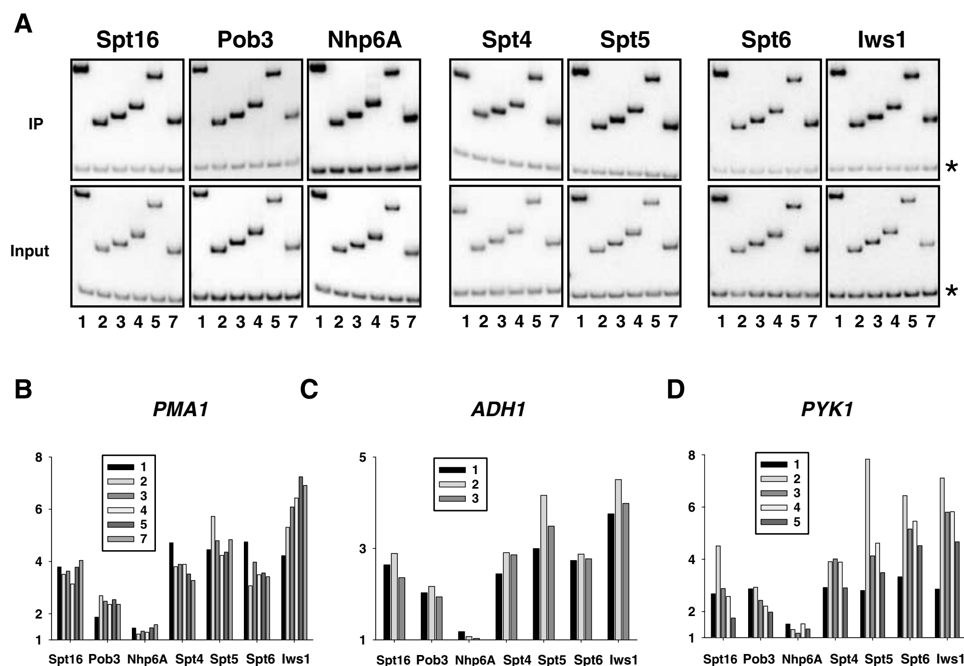

Figure 3 Chromatin-related elongation factors crosslink similarly to RNApII. (**A**) ChIP analysis was carried out using strains carrying the indicated TAP-tagged proteins as described in Figure 2 and Materials and methods. PCR analysis of immunoprecipitated chromatin was performed on the *PMA1* gene. (**B**–**D**) Results of ChIP analysis on the *PMA1*, *ADH1*, and *PYK1* genes were quantitated as described in Figure 2 and Materials and methods. Note the efficient crosslinking downstream of the polyadenylation site, similar to the pattern seen with Rpb3 (Figure 2).

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transcribed regions specifically, the HMG box protein Nhp6A crosslinking was also observed at the nontranscribed control region (Figure 3). This suggests that Nhp6 may be a general chromatin component that interacts with Spt16/Pob3 within transcribed regions.

Transition at the 3′ end

A different pattern of crosslinking was observed with elongation factors making up the Paf and TREX complexes (Figures 4 and 5). These two complexes crosslinked to coding regions of genes, but only weakly to transcribed regions downstream of the polyadenylation site. This pattern suggests that these factors are released from the elongation complex upon passage through a polyadenylation sequence. This dissociation could be one of the events that renders RNApII competent for termination.

The Paf complex is a set of proteins associated with RNApII (Krogan *et al*, 2002; Mueller and Jaehning, 2002; Squazzo *et al*, 2002). Recent genetic studies suggest that the Paf complex functions to regulate transcription elongation (Mueller and Jaehning, 2002; Squazzo *et al*, 2002). Members of the Paf complex show strongest crosslinking to coding regions, but much less to regions downstream of the polyadenylation site (Figure 4). The complex members crosslinked to promoters, although usually less than to coding regions. This was particularly notable with Paf1. This may reflect the association of the Paf complex during early elongation.

The TREX complex consists of proteins identified in two very distinct ways. One subset (the THO complex) was identified using genetic screens for transcription-stimulated recombination (Chavez and Aguilera, 1997; Piruat and Aguilera, 1998). Members of this group include Hpr1, Tho2, Mft1, and Thp2. The THO complex can also be found in association with the Sub2 and Yra1 proteins (Jimeno *et al*, 2002; Strasser *et al*, 2002). These two RNA-binding proteins are necessary for proper export of mRNA out of the nucleus (Luo *et al*, 2001; Lei and Silver, 2002; Strasser *et al*, 2002). It is likely that these proteins contribute to mRNA-specific packaging. Previous ChIP experiments demonstrated that Sub2 and Aly1 (Lei and Silver, 2002) as well as Hpr1 and Tho2 (Strasser *et al*, 2002) can be crosslinked to coding regions of genes. We also find that these factors crosslink weakly or not at all to promoter regions, but with a strong signal in transcribed regions further downstream. TREX complex crosslinking drops dramatically downstream of polyadenylation sites, suggesting that it is no longer associated with the elongating RNApII in this region (Figure 5).

The apparent loss of the Paf and TREX complexes from the elongation complex upon passage through polyadenylation sites contrasts with the pattern seen with the Spt family elongation factors. That the two complexes share this behavior is interesting, since the TREX complex component Hpr1 has also been found in association with the Paf complex (Chang *et al*, 1999). Therefore, the two complexes may interact directly or indirectly via the RNApII.

Two distinct, but not mutually exclusive, models could explain the elongation factor transitions at the 3′ end. First, dissociation of elongation factors may be functionally linked to termination. Transcription through a cleavage/polyadenylation site is necessary to trigger termination downstream, but termination does not require the actual cleavage or polyadenylation reactions (Birse *et al*, 1998; Dye and Proudfoot, 2001; Tran *et al*, 2001; Dichtl *et al*, 2002b; Orozco *et al*, 2002). The termination signal is presumed to include the actual mRNA cleavage site that is recognized by the polyadenylation/termination machinery. The signaling

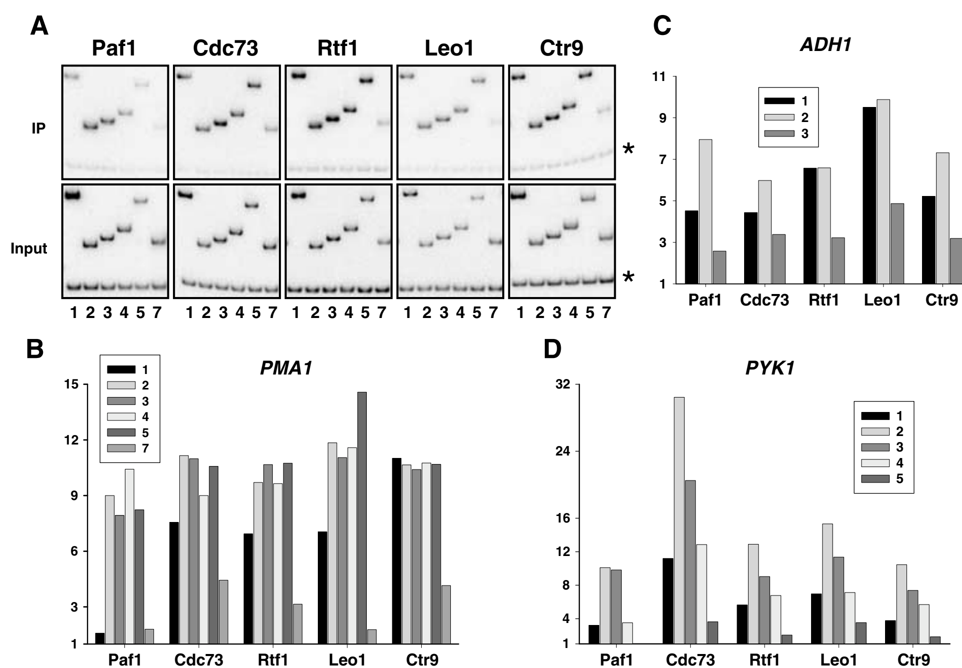

Figure 4 Crosslinking of PAF complex subunits to transcribed regions upstream of the polyadenylation site. (**A**) ChIP analysis was carried out using strains carrying the indicated TAP-tagged proteins as described in Figure 2 and Materials and methods. PCR analysis of immunoprecipitated chromatin was performed on the *PMA1* gene. (**B–D**) Results of ChIP analysis on the *PMA1*, *ADH1*, and *PYK1* genes were quantitated as described in Figure 2 and Materials and methods.

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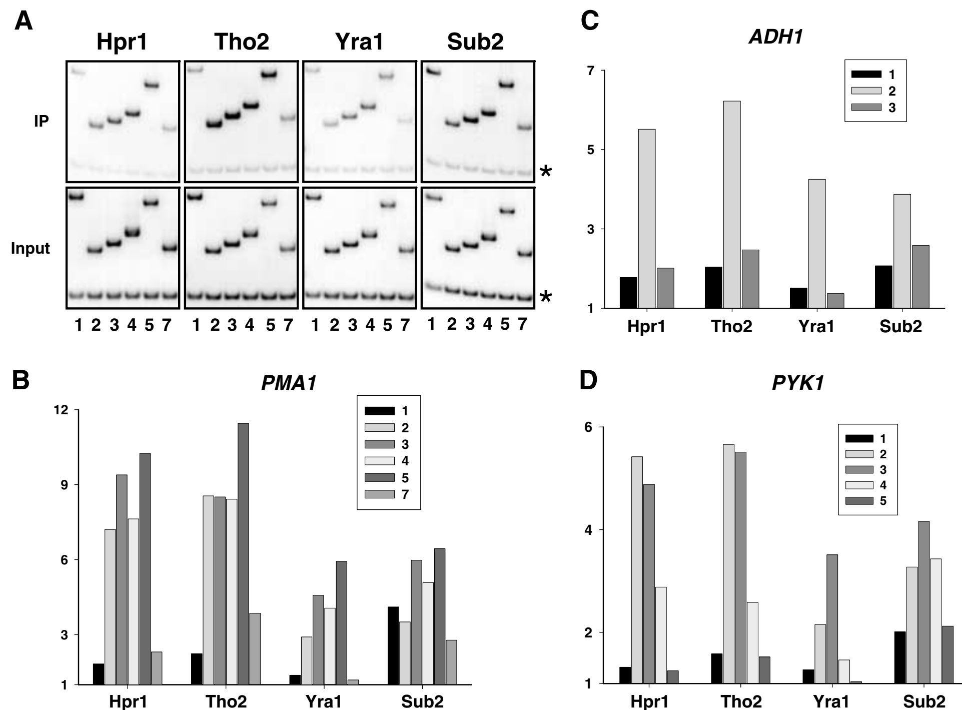

Figure 5 Crosslinking of TREX complex subunits to transcribed regions upstream of the polyadenylation site. (**A**) ChIP analysis was carried out using strains carrying the indicated TAP-tagged proteins as described in Figure 2 and Materials and methods. PCR analysis of immunoprecipitated chromatin was performed on the *PMA1* gene. (**B–D**) Results of ChIP analysis on the *PMA1*, *ADH1*, and *PYK1* genes were quantitated as described in Figure 2 and Materials and methods.

event may trigger an exchange of elongation (i.e. antitermination) factors for polyadenylation factors.

A second model is that some elongation factors associate specifically with the nascent mRNA, but not with the RNA transcribed from the region downstream of the polyadenylation site. Upon cleavage, the nascent mRNA may be released or displaced such that the elongation factors can no longer be crosslinked. The 3′ fragment of the cleaved transcript may be targeted for degradation because it lacks the proper protein packaging (as well as cap and polyA tail) that would identify it as mRNA. This model is particularly attractive for the TREX complex, which binds mRNA and is required for proper export (Luo *et al*, 2001; Lei and Silver, 2002; Strasser *et al*, 2002).

Recruitment of polyadenylation factors to the 3′ end of genes

To determine whether polyadenylation factors associate with the transcription elongation complex *in vivo*, ChIP experiments using TAP-tagged polyadenylation factors were carried out (Figure 6). Strikingly, these factors crosslinked very strongly near the polyadenylation sites of the genes. Signals were strongest with members of the CFIA (Rna14, Rna15, and Pcf11) complex, although 3′ end localization was also seen with CPF components (Cft1, Cft2, Fip1, Pta1, and Yth1). On *PMA1*, some polyadenylation factors showed a low level of crosslinking to promoter and early coding regions. However, this crosslinking was 5- to10-fold less than that seen at 3′ ends and was not observed at *ADH1* and *PYK1*.

The one polyadenylation factor that showed a different pattern was Hrp1 (not to be confused with Hpr1 of the TREX complex), which is the sole component of the activity known as CFIB (Kessler *et al*, 1997; Minvielle-Sebastia *et al*, 1998). Hrp1 crosslinked throughout coding regions of genes and to the 3′ end downstream of the polyadenylation site as previously observed (data not shown; Komarnitsky *et al*, 2000). Hrp1 (also known as Nab4) is an RNA-binding protein that has also been implicated in nonsense-mediated decay (NMD) and mRNA export (Kessler *et al*, 1997; Minvielle-Sebastia *et al*, 1998; Gonzalez *et al*, 2000; Gross and Moore, 2001). Hrp1 is required for proper 3′ cleavage site selection. We suspect that the role of Hrp1 is to package RNA in the appropriate configuration for cleavage/polyadenylation and transport.

To determine the functional significance of 3′ crosslinking of polyadenylation factors, ChIP was carried out on two plasmids carrying either the intact *CYC1* termination region or a mutant *cyc1-512* allele that has defective 3′ end formation (Zaret and Sherman, 1982). In agreement with earlier nuclear run-on experiments (Birse *et al*, 1998), transcription (defined by Rpb3 crosslinking) terminated within a few hundred base pairs of the *CYC1* polyA site. In contrast, transcription continued past this site in the *cyc1-512* allele (Figure 7B). Defective termination correlated with loss of Rna15 crosslinking near the polyadenylation site (Figure 7C). ChIP experiments were also carried out in an *rna14-1* mutant, which is defective for polyadenylation at the nonpermissive temperature (Bonneaud *et al*, 1994; Minvielle-Sebastia *et al*, 1994). In an *RNA14* strain, Rna15 crosslinks to the 3′ region of the *PMA1* gene at both 23 and 37°C. In the *rna14-1* mutant background, Rna15 crosslinking is normal at 23°C, but lost at 37°C (Figures 8A and B). Immunoblotting shows that this effect is not due to degradation of the Rna15 protein at the nonpermissive temperature (Figure 8C). Supporting an

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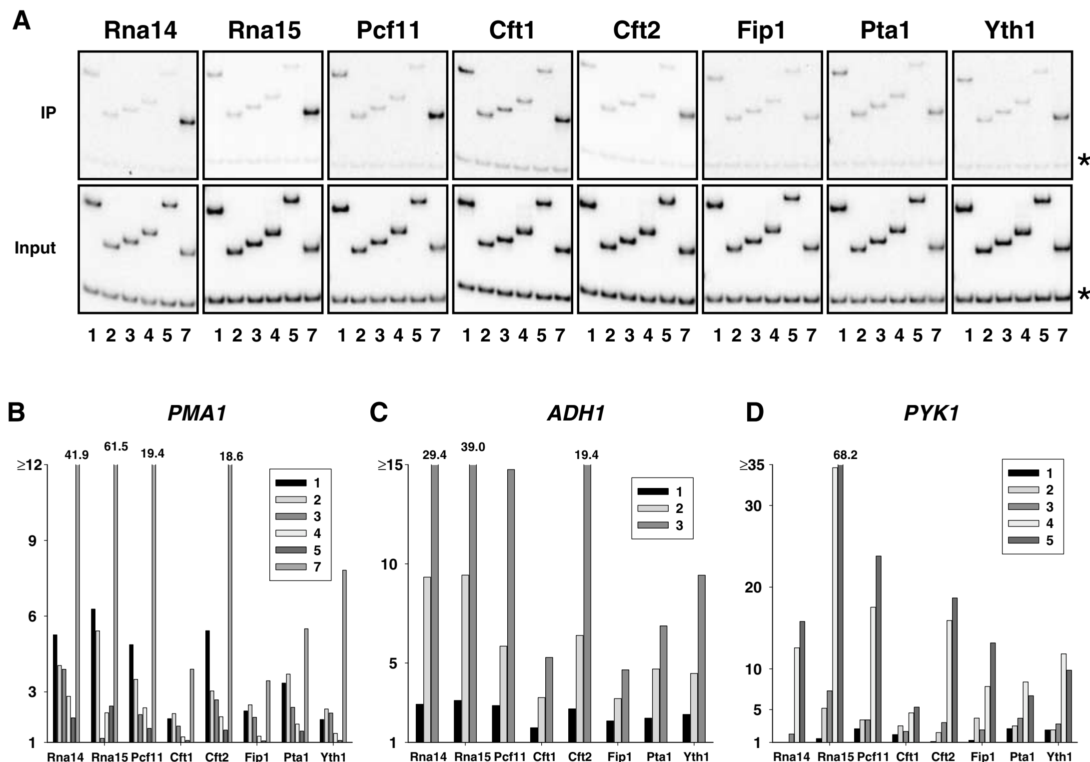

Figure 6 Crosslinking of polyadenylation factors at the 3′ end of genes. (**A**) ChIP analysis was carried out using strains carrying the indicated TAP-tagged proteins as described in Figure 2 and Materials and methods. PCR analysis of immunoprecipitated chromatin was performed on the *PMA1* gene. (**B–D**) Results of ChIP analysis on the *PMA1*, *ADH1*, and *PYK1* genes were quantitated as described in Figure 2 and Materials and methods.

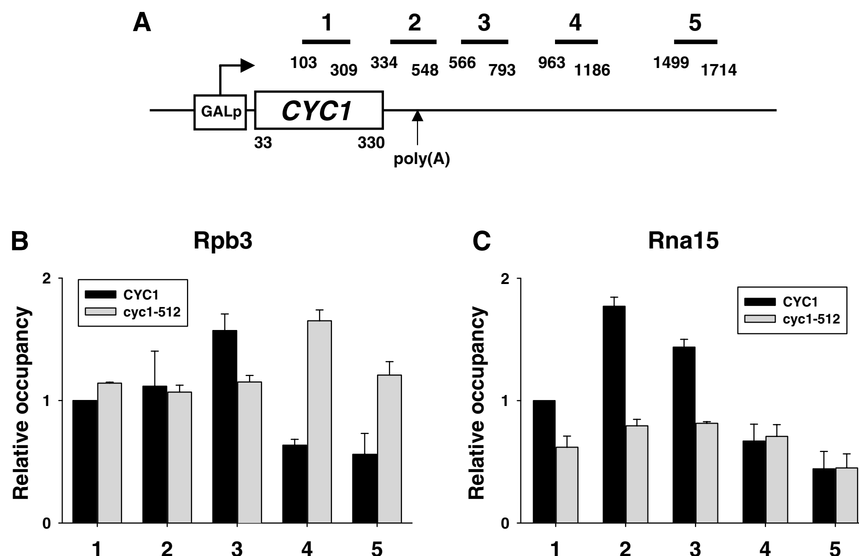

Figure 7 Transcription termination and localization of polyadenylation factors are dependent upon functional polyadenylation sequences. (**A**) Schematic diagram of the pGCYC1 plasmid. This high-copy plasmid (Birse *et al*, 1998) contains the GAL1 promoter upstream of the CYC1 gene and terminator region. The arrow indicates the normal polyadenylation site. Bars above the gene indicate positions of the primer pairs used for ChIP analysis. The pGcyc1-512 plasmid is identical except that it carries a small deletion of critical polyadenylation sequences (Zaret and Sherman, 1982). (**B**) Localization of RNApII was determined by ChIP of the Rpb3-TAP protein. The signal from primer pair 1 was set to an arbitrary value of 1.0, and signals from the other primer pairs (denoted on the *x*-axis) were expressed relative to that value on the *y*-axis (see Materials and methods). Black bars are for pGCYC1 and gray bars are for PGcyc1-512. Note that RNApII drops after primer pair 3 on wild-type *CYC1* but continues transcribing well downstream in the mutant. (**C**) Localization of Rna15-TAP was determined by ChIP as in (B). Note that Rna15 shows a peak over the polyadenylation region (primers 2 and 3) with wild-type *CYC1* but not in the mutant.

additional role for the polyadenylation complex in transcription termination, Rpb3 crosslinking did not drop downstream of the polyA site in the *rna14-1* mutant (Figure 8D). Therefore, 3′ crosslinking of polyadenylation factors, like polyadenylation itself, is sensitive to both mutations in the transcribed sequence and in the factors themselves. In further experiments, we also found that this crosslinking is dependent upon the CTD serine 2 kinase Ctk1 (Ahn *et al*, in press).

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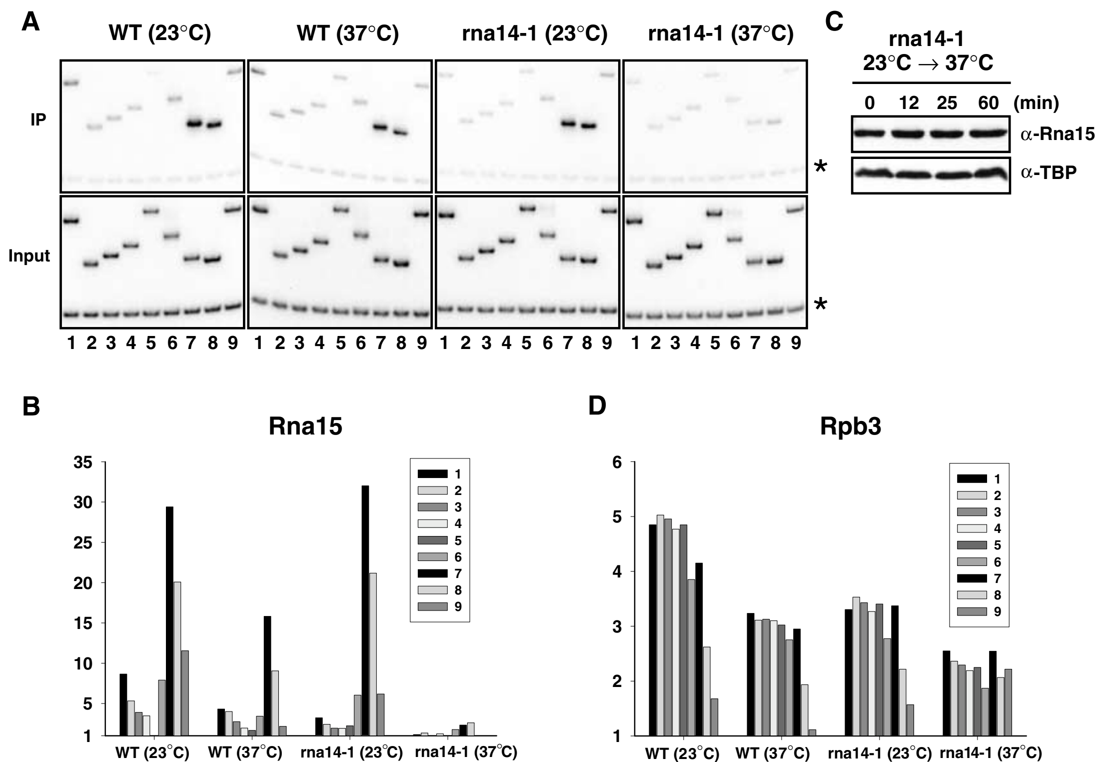

Figure 8 Transcription termination and localization of polyadenylation factors are dependent upon functional Rna14. (**A**) ChIP analysis was carried out on the *PMA1* gene in *RNA14* (WT) and *rna14-1* strains carrying TAP-tagged Rna15. Strains were grown at the permissive temperature (23°C) or shifted to the nonpermissive temperature (37°C) for 25 min. Each PCR reaction contained a specific primer pair (see Figure 1 for the numbering scheme) and a control primer from a nontranscribed region (marked by an asterisk). Crosslinking of Rna15 was lost in the *rna14-1* strain at the nonpermissive temperature. (**B**) Quantitation of results shown in (A). (**C**) Immunoblotting for Rna15-TAP was carried out on samples from the rna14-1 strain shifted to the nonpermissive temperature for the indicated amount of time. Note that Rna15 is not degraded under these conditions. Immunoblotting for TBP on the same gel was carried out as a loading control (lower panel). (**D**) Quantitation of ChIP results for Rpb3-TAP carried out as in (A). Note that transcription normally terminates between primer pairs 8 and 9 (consistent with the lack of Rna15 at primer pair 9). However, crosslinking of Rpb3 at primer pair 9 is seen in the *rna14-1* mutant at the nonpermissive temperature, indicating read-through of the terminator.

We conclude that the observed crosslinking reflects cotranscriptional polyadenylation.

Discussion

Just as our earlier results showed a dynamic pattern of factors associating with RNApII at the 5′ ends of genes, we find further changes occurring at 3′ ends during the elongation and termination phases of transcription.

Spt4/5 (DSIF in humans) interacts functionally with capping enzyme (Wen and Shatkin, 1999; Pei and Shuman, 2002; Lindstrom *et al*, 2003), and human DSIF functions early during the transcription cycle (Wada *et al*, 1998a, b; Ping and Rana, 2001). The crosslinking patterns of Spt4 and Spt5 are consistent with these results, showing a strong signal near promoter regions. However, Spt4/5 also crosslinks throughout the coding and 3′ regions of genes (Figure 3), indicating that it probably has other roles during elongation.

Spt4/5 shows the same crosslinking pattern as two other elongation factors, the Spt16/Pob3 and Spt6/Iws1 complexes. These three complexes contain proteins that have been implicated in transcription on chromatin templates. Members of this group of histone-related Spt proteins (Winston and Carlson, 1992) have very similar phenotypes and show extensive genetic interactions with each other (Winston and Carlson, 1992; Lindstrom and Hartzog, 2001; Squazzo *et al*, 2002). Affinity purification experiments in yeast have shown that these three complexes can be copurified, probably through simultaneous interactions with RNApII and perhaps also via direct interactions (Krogan *et al*, 2002; Lindstrom *et al*, 2003). This group of elongation factors shows a crosslinking pattern that exactly overlaps that of RNApII, including the transcribed region downstream of the polyadenylation site.

Several transitions are observed at the 3′ ends of genes. Although RNApII continues transcribing downstream of the polyadenylation site, the level of CTD serine 2 phosphorylation shows a partial drop (Figure 2). This drop parallels a reduction in crosslinking of the serine 2 kinase, Ctk1. We also found that levels of Bur1 kinase drop after the polyadenylation site (Keogh *et al*, 2003). Several elongation factors (Paf and TREX complexes, TFIIS, Chd1, and Rad26) also appear to dissociate from elongation complexes downstream of the polyadenylation site (Figures 4 and 5 and data not shown). Therefore, the ChIP experiments can distinguish two classes of elongation factors: those that continue traveling with RNApII downstream of polyadenylation sites and those that do not. It will be interesting to determine whether the loss of positive elongation factors is important for transcription termination.

The polyadenylation factors strongly crosslink at and just downstream of the polyadenylation site (Figures 6–8). This

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crosslinking is dependent upon a functional polyadenylation sequence (Figure 7) and the Rna14 protein (Figure 8), correlating perfectly with the requirements for transcription termination as well as mRNA cleavage and polyadenylation. What is the significance of strong polyadenylation factor crosslinking at the 3′ ends of genes? A simple interpretation is that these factors are efficiently recruited to the elongation complex only in this region (a 3′ loading model). Recruitment of polyadenylation factors could be triggered by the appearance of the appropriate RNA sequences and/or the loss of the Paf and TREX complexes.

Alternatively, it has been proposed that at least some polyadenylation components are delivered to the promoter by transcription factor TFIID and transferred to the RNApII CTD (Dantonel *et al*, 1997; Licatalosi *et al*, 2002). This 5′ loading model is based on the observation that several polyadenylation factor subunits copurified with TFIID subunits from mammalian extracts (Dantonel *et al*, 1997). However, this observation may not extend to yeast. Several distinct purifications of yeast TFIID and polyadenylation complexes (Dichtl *et al*, 2002a; Gavin *et al*, 2002; Ho *et al*, 2002; Sanders *et al*, 2002) failed to observe any copurification. In a quantitative mass spectroscopy analysis, no polyadenylation factors were identified as stoichiometric components of yeast RNApII initiation complexes (Ranish *et al*, 2003).

In the 5′ loading model, polyadenylation factors should associate with the elongation complex throughout the coding regions of genes. A recent paper (Licatalosi *et al*, 2002) observed some crosslinking of Pcf11 and Fip1 to both the 5′ and 3′ parts of the *ENO2* and *TEF1* coding regions. However, the downstream probes used in this paper were approximately 100 base pairs upstream of the stop codons and are therefore well upstream of the polyadenylation sites. The signal reported may be similar to the weak crosslinking of polyadenylation factors that we observed in some coding regions. The crosslinking they reported was not dependent upon CTD phosphorylation by the Ctk1 kinase (Licatalosi *et al*, 2002). In contrast, the 3′ crosslinking of polyadenylation factors we see is abrogated in a strain lacking Ctk1 (Ahn *et al*, in press).

Genetic interactions between the polyadenylation factors Ssu72 and Sub1 and the basal transcription factor TFIIB (Knaus *et al*, 1996; Wu *et al*, 1999; Calvo and Manley, 2001; Dichtl *et al*, 2002a; He *et al*, 2003; Nedea *et al*, 2003) have also been cited as support for the presence of polyadenylation factors at the promoter (Calvo and Manley, 2003). However, these results should be interpreted with caution. Although these factors copurify with polyadenylation factors, they may also have distinct roles as components of other complexes. Two other proteins that copurify with the polyadenylation factors are known to have other functions. The Glc7 phosphatase has many substrates in both the nucleus and cytoplasm (see the *Saccharomyces* Genome Database: http://www.yeastgenome.org/). The Swd2 protein is also a component of the Set1 histone methyltransferase complex (Roguev *et al*, 2001). Sub1 may fall into this class of proteins with multiple functions. Sub1 and its mammalian homolog PC4 bind single-stranded nucleic acids and stimulate transcription in the absence of any other polyadenylation factors (Ge and Roeder, 1994; Kaiser *et al*, 1995; Henry *et al*, 1996). Sub1 has a ChIP pattern distinct from the other polyadenylation factors, crosslinking to both promoters and 3′ ends but not to coding regions (Nedea *et al*, 2003).

Mutations in *SSU72* exacerbate the growth and transcription start site defects seen in select alleles of the TFIIB gene (Sun and Hampsey, 1996), but *ssu72* mutants do not shift transcription start sites on their own. Synthetic lethal and slow growth phenotypes can occur between factors that operate at different steps in gene expression, so this cannot be taken as evidence of a direct connection. Although *in vitro* coimmunoprecipitation of Ssu72 and TFIIB has been reported, this could not be confirmed by yeast two-hybrid assay (Wu *et al*, 1999; Dichtl *et al*, 2002a). TFIIB was not found in association with Ssu72 and other polyadenylation factors in several proteomic studies (Dichtl *et al*, 2002a; Gavin *et al*, 2002; Ho *et al*, 2002). Ssu72 crosslinks specifically to the 3′ ends of genes (Nedea *et al*, 2003).

Although we favor a simpler model in which polyadenylation factors are recruited to the transcription elongation complexes at the 3′ ends of genes, it should be noted that our ChIP results are compatible with both models. It is possible that the polyadenylation factors are associated with RNApII throughout elongation, but become competent for *in vivo* crosslinking only upon passage through a polyadenylation site. If so, the 3′ transition we observe would indicate a major conformation change, perhaps reflecting a transfer of polyadenylation factors from the CTD to the nascent mRNA. Future experiments will help to distinguish between these two models.

It is remarkable that so many factors associate with the transcription elongation complex. It remains to be seen whether all the factors are simultaneously bound to the same RNApII. It is equally possible that dynamic, transient interactions lead to overlapping crosslinking patterns. Another important question is whether there are interdependent relationships between factors for association with the transcription complex. Future *in vitro* and *in vivo* experiments will be required to answer these questions.

Materials and methods

Yeast strains

Strains used in this study are listed in Supplementary Table 1.

RNA analysis and 3′-RACE mapping of polyadenylation sites

RNA was extracted from cells using hot phenol. Total RNA (2 μg) was reverse transcribed using 200 U of Superscript II reverse transcriptase (Invitrogen) at 42°C for 50 min. The reaction was primed with 10 pmol of the oligo d(T) adapter primer (5′-AATTCCCGGGAGCGGGCGTCGACTTTTTTTTTTTTTTTTT-3′), which is specific for polyA+ mRNA. After RNase H treatment at 37°C for 20 min, one-tenth of the cDNA product was amplified by PCR (30 amplification cycles). Each PCR reaction contained a gene-specific 5′ primer and a 3′ universal amplification primer (UAP, 5′-GGGAATTCCCGGGAGCGGGCGTCG-3′). A second nested PCR was performed with the 3′UAP and a different gene-specific primer located downstream of the one used in the first PCR reaction. RT–PCR products were cloned into pGEM-T Easy Vector (Promega) and the mRNA/poly(A) junctions were determined by DNA sequencing of multiple isolates for each gene.

Chromatin immunoprecipitations

Preparation of chromatin was as previously described (Komarnitsky *et al*, 2000). For precipitation of TAP-tagged proteins, rabbit IgG-agarose was washed twice with TE (10 mM Tris, pH 8.0, 1 mM EDTA). In all, 10 μl of beads were mixed with 800 μl of chromatin solution and incubated overnight at 4°C. Beads were then washed sequentially with 1.4 ml each of FA lysis buffer + 275 mM NaCl,

FA lysis buffer + 500 mM NaCl, wash buffer (10 mM Tris, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na deoxycholate), and TE. Immunoprecipitated chromatin was eluted from the beads by heating for 10 min at 65°C in 200 μl of 50 mM Tris, pH 7.5, 10 mM EDTA, and 1% SDS. After recovery of the supernatant, beads were washed with 200 μl TE that was then added to the first supernatant. Reversal of crosslinking and PCR reactions were as described (Komarnitsky *et al*, 2000). Oligonucleotide primer sequences are available upon request.

For 12CA5 (α-HA antibody) immunoprecipitations, antibody was preincubated with protein A–sepharose CL-4B (Amersham) for 1 h at room temperature and washed twice with TE. Chromatin solution was then added and incubated at 4°C overnight. Elution and processing were as described above.

PCR signals were quantitated by a phosphoimager. The ratio of each gene-specific product to that of a nontranscribed region of chromosome V in the immunoprecipitations was determined after normalization for amplification efficiency (determined from the input sample signals). Therefore, numbers on the *y*-axis of each graph show the fold enrichment of the ChIP signal over the background signal. Note that a value of 1 represents a signal equal to background. In multiple PCR reactions, variability was typically no more than 10–20% of the signal.

For Figure 8, both *RNA14* and *rna14-1* cells were incubated at 23°C until OD_{595} reached 0.8. To shift temperature, an equal volume of media prewarmed to 51°C was added and cells were further incubated at 37°C for 25 min. Formaldehyde crosslinking and chromatin preparation were performed as described above.

For Figure 7, the ChIP protocol was modified for target sequences on a plasmid. The Rpb3-TAP (NJK12) and Rna15-TAP (YEN4) strains were transformed with either pGCYC1 or pGcyc1-512 plasmids (Birse *et al*, 1998). Cells were incubated in the presence of 2% raffinose until OD_{595} reached 0.3. Galactose was added to 2% and cells were further incubated for 3 h. Formaldehyde crosslinking and chromatin preparation were performed as usual. For PCR reactions, chromatin DNAs from both immunoprecipitated and input samples were diluted 1:50. Parallel reactions were also carried out with strains containing no plasmid, and any detectable signal was subtracted as background from the endogenous copy of CYC1. Because the pGCYC1 plasmids have 2 μm origins, they are at high copy and the signal from the endogenous *CYC1* gene is negligible. To normalize for plasmid copy number and chromatin preparation, a segment of bacterial ampicillin resistance gene within the plasmid was used as an internal PCR control within each reaction. The relative occupancy of Rpb3 and Rna15 along the CYC1 terminator region was plotted by setting the primer pair 1 of pGCYC1 to an arbitrary value of 1.0 and calculating the relative amount of the other primer pairs. Note that this quantitation differs from that in the other figures.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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