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Npl3 is an antagonist of mRNA 3′ end formation by RNA polymerase II

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Proper 3′ end formation is critical for the production of functional mRNAs. Termination by RNA polymerase II is linked to mRNA cleavage and polyadenylation, but it is less clear whether earlier stages of mRNA production also contribute to transcription termination. We performed a genetic screen to identify mutations that decreased transcriptional readthrough of a defective *GAL10* poly(A) terminator. A partial deletion of the *GAL10* downstream region leads to transcription through the downstream *GAL7* promoter, resulting in the inability of cells to grow on galactose. Mutations in elongation factors Spt4 and Spt6 suppress the readthrough phenotype, presumably by decreasing the amount of polymerase transcribing through the downstream *GAL7* promoter. Interestingly, mutations in the mRNA-binding protein Npl3 improve transcription termination. Both *in vivo* and *in vitro* experiments suggest that Npl3 can antagonize 3′ end formation by competing for RNA binding with polyadenylation/termination factors. These results suggest that elongation rate and mRNA packaging can influence polyadenylation and termination.

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Introduction

In eukaryotes, transcription by RNA polymerase II (RNApII) and mRNA processing are highly concerted events. Nascent mRNAs are cotranscriptionally capped, spliced, and polyadenylated. Different phosphorylated states of the RNApII C-terminal domain (CTD) mediate coupling by acting as binding sites for the different mRNA processing factors (Cho *et al*, 1997; McCracken *et al*, 1997; Hirose and Manley, 1998; Ahn *et al*, 2004).

mRNA capping is linked to phosphorylation of CTD serine 5, a reaction catalyzed by the kinase subunit of the basal transcription factor TFIIH (Jove and Manley, 1984; Rasmussen and Lis, 1993; Cho *et al*, 1997; Yue *et al*, 1997; Cho *et al*, 1998; Ho and Shuman, 1999; Komarnitsky *et al*, 2000; Rodriguez *et al*, 2000; Schroeder *et al*, 2000). There is crosstalk between capping and the transition into transcription elongation (Cho *et al*, 1997; Ping and Rana, 2001; Kim *et al*, 2004a; Mandal *et al*, 2004). Several biochemical and genetic interactions between capping enzymes and the Spt4/5 complex (also known as DRB sensitivity-inducing factor or DSIF) have been documented (Wen and Shatkin, 1999; Pei and Shuman, 2002; Lindstrom *et al*, 2003).

The CTD of RNApII also couples events at the 3′ ends of genes (McCracken *et al*, 1997; Ahn *et al*, 2004; Kim *et al*, 2004b). Recruitment of polyadenylation factors to transcription complexes is mediated by a combination of two signals. One is the appearance of specific polyadenylation/termination sequences that are presumably recognized as RNA. In yeast, these sequences appear to be fairly degenerate. The second signal is phosphorylation of RNApII CTD on serine 2, a reaction dependent upon the Ctk1 kinase in yeast (Lee and Greenleaf, 1991; Cho *et al*, 2001; Skaar and Greenleaf, 2002; Ahn *et al*, 2004) or the analogous Cdk9 kinase in higher eukaryotes (Price, 2000; Shim *et al*, 2002; Ni *et al*, 2004). The phosphorylated CTD enhances transcript cleavage *in vitro* (Hirose and Manley, 1998). A cap-binding complex (CBC, composed of Cbp20 and Cbp80) binds to the cap structure, stabilizing the mRNA (Beelman and Parker, 1995) and promoting efficient 3′ end cleavage (Flaherty *et al*, 1997). This linkage, as well as dedicated RNA surveillance and degradation mechanisms, ensures that mRNAs have intact 5′ and 3′ ends.

In *Saccharomyces cerevisiae*, the galactose-responsive genes *GAL1*, *GAL7,* and *GAL10* are strongly induced upon growth in media containing galactose as the sole carbon source (Johnston, 1987; Lohr *et al*, 1995). All three genes are tightly regulated and required for galactose utilization. An interesting feature of these genes is their proximity to one another in the yeast genome (Figure 1A). The *GAL1* and *GAL10* genes are divergently transcribed and share promoter elements within their intergenic region. *GAL10* is upstream of *GAL7* and both are transcribed in the same direction. Mutations in the intergenic region between *GAL10* and *GAL7* reduce proper termination of the *GAL10* transcript, causing readthrough transcription that interferes with the downstream *GAL7* promoter (St John and Davis, 1981; Greger and Proudfoot, 1998). Therefore, these mutants are unable to grow in media containing galactose as the sole carbon source.

We carried out a genetic selection for mutations that could suppress a *GAL10* poly(A) site deletion. Suppression can be mediated by an event that interferes with readthrough of the mutated terminator, thereby restoring adequate expression of *GAL7,* but must also maintain expression of *GAL10.* We expected that extragenic mutations isolated in our selection would either reduce elongation or improve termination of the *GAL10* gene (Figure 1A). Four complementation groups of suppressors were identified. Two correspond to previously identified genes involved in elongation, *SPT6* and *SPT4*

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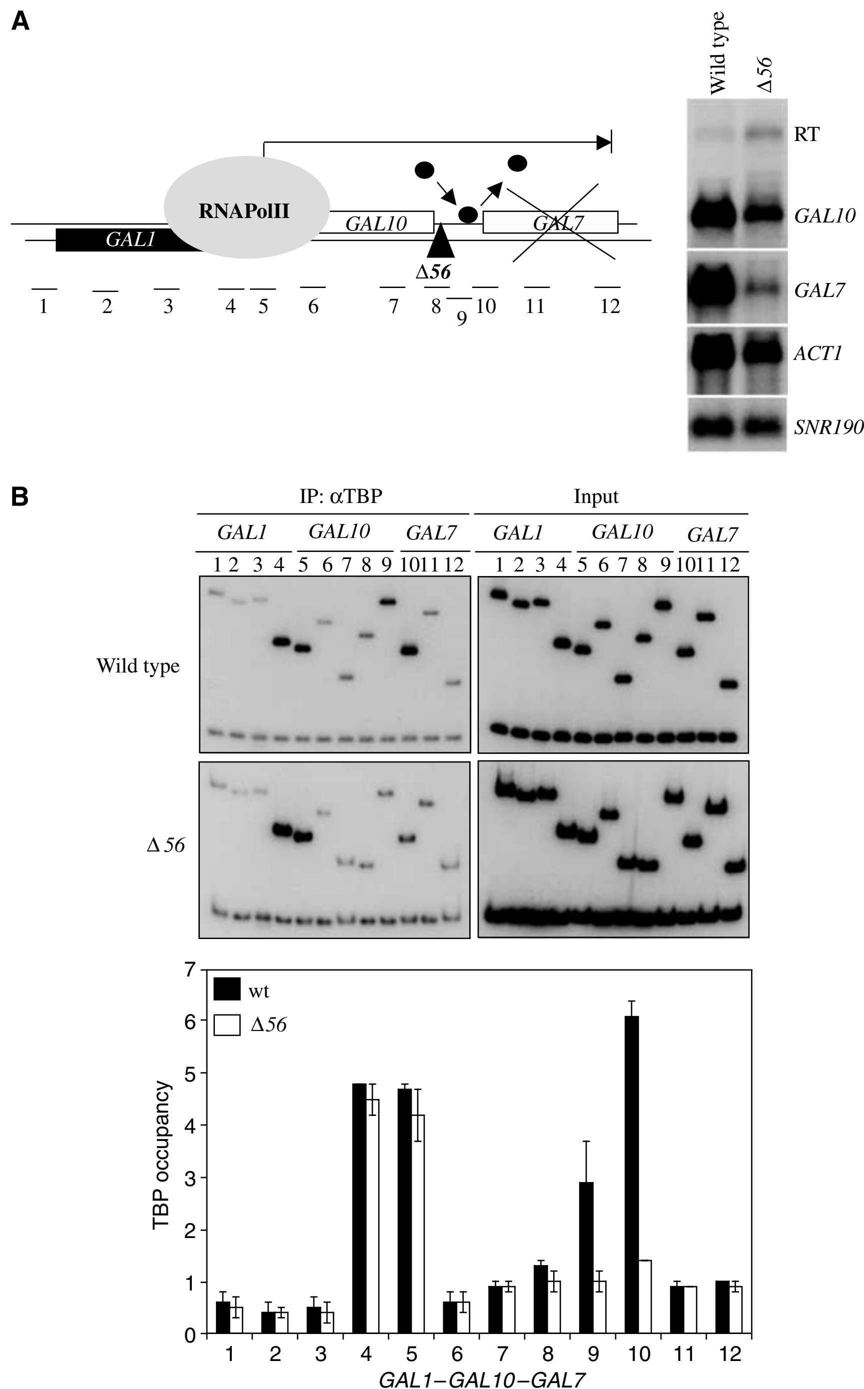

Figure 1 Characterization of the *gal10*Δ*56* locus. (**A**) Schematic representation of the *GAL1/GAL10/GAL7* locus depicting the increase of RNApII readthrough upon removal of the *GAL10* poly(A) site (Δ*56*). Readthrough precludes binding of transcription initiation factors (black circle) and expression of downstream *GAL7*. The locations of primers used for ChIP analysis are shown underneath. A Northern blot showing the reduction in the level of *GAL7* expression is also shown. RNA was prepared from wild-type (FY268) and *GAL10* poly(A)-deleted strain (CKY185) induced with 2% galactose overnight. A 20 μg portion of RNA was loaded for each strain. The blot was probed for the indicated transcripts, including the stable *SNR190* RNA (as a loading control). The readthrough transcript that hybridizes with both *GAL10* and *GAL7* sequences is marked (RT). (**B**) ChIP with anti-TBP antibody was performed in wild-type (FY268) and *gal10*Δ*56* (YSB1768) strains that were induced with galactose for 30 min. PCR reactions of the *GAL* locus are shown and are represented graphically (quantitation is described in Materials and methods) in the diagram (black bars for the wild type and white bars for *gal10*Δ*56*). The upper band in each lane is the primer pair as numbered in panel A. The lower band is a PCR product for a nontranscribed region that serves as a negative control. The values shown represent averages of three independent ChIP experiments and error bars show standard deviation.

(Winston *et al*, 1984; Hartzog *et al*, 1998; Rondon *et al*, 2003; Endoh *et al*, 2004; Kaplan *et al*, 2004). We also identified suppressor mutations in the *NPL3* gene. Npl3 was originally identified as an effector of rRNA biogenesis (Russell and Tollervey, 1992). Later, it was shown that Npl3 is an RNA-binding protein recruited cotranscriptionally to RNApII-transcribed genes, contributing to mRNA transport out of the nucleus (Lei *et al*, 2001). More recently, it was reported

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that Npl3 can act as a negative regulator of translation (Windgassen *et al*, 2004). Here, we provide genetic and biochemical evidence that Npl3 antagonizes termination of transcription.

Results

Suppressors of a transcription terminator deletion

A deletion of 55 base pairs (bp) within the intergenic region between the *GAL10* and *GAL7* genes causes readthrough transcription from *GAL10* into the *GAL7* promoter (Greger and Proudfoot, 1998; Kaplan *et al*, 2004). Reduction in *GAL7* expression results in a gal^{-} phenotype. Chromatin immunoprecipitation (ChIP) for TATA-binding protein (TBP) confirmed that binding of basal transcription factors to the downstream *GAL7* promoter was compromised in the *gal10Δ56* strain (Figure 1B). Control primers for the entire *GAL* cluster showed that TBP binding to the *GAL1* and *GAL10* promoters was not affected (Figure 1B). Using 3′-RACE, we detected some polyadenylated transcripts terminated immediately downstream of the deleted poly(A) sequence (data not shown). We believe that this termination is likely not sufficient since *GAL10/GAL7* readthrough transcript is increased and *GAL7* mRNA is reduced (Figure 1A).

Beginning with this *gal10Δ56* strain (kindly provided by C Kaplan), mutations were selected that could suppress the lack of growth in galactose. In this scheme, suppression requires both re-establishment of expression of the downstream *GAL7* gene and also maintenance of *GAL10* expression. Three categories of suppressors were predicted: (1) intragenic mutations that either improve *GAL10* termination or increase *GAL7* promoter activity, (2) extragenic mutations that reduce readthrough due to a transcription elongation defect, or (3) extragenic mutations that reduce readthrough through enhanced termination. Our prediction of class 2 suppressors was supported by the ability of the *gal10Δ56* strain to grow slowly on galactose plates when the elongation-inhibiting drugs mycophenolic acid (MPA) or 6-azauracil (6-AU) (Exinger and Lacroute, 1992; Shaw and Reines, 2000) are added (Figure 2).

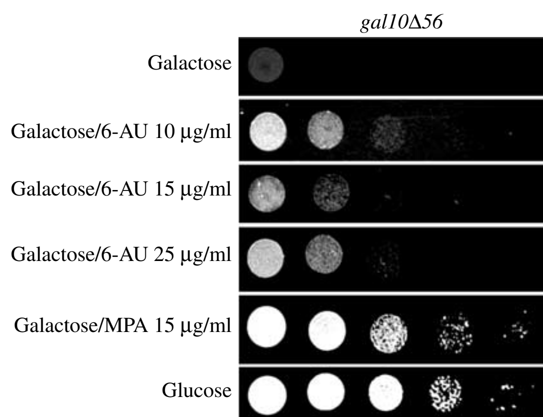

Figure 2 6-AU and MPA partially suppress the *gal10Δ56* galactose growth defect. Serial 10-fold dilutions of a *gal10Δ56* strain (YSB1768) were spotted on 2% galactose plates supplemented with 6-AU (10, 15, or 25 μg/ml) or MPA (15 μg/ml) as indicated. Plates were incubated for 4 days at 30°C. *gal10Δ56* strains grown on 2% galactose or 2% glucose plates are shown as controls.

Cells carrying the *gal10Δ56* allele were mutagenized and mutants were isolated that were able to grow on galactose media. Four independent complementation groups of suppressors of the galactose^{-} phenotype (referred to as *sog*1–4) were identified (Table I). Attempts to clone the *sog4* gene have so far been unsuccessful. The suppressor genes *sog2* (two alleles) and *sog3* (two alleles) were identified through candidate plasmid complementation as *SPT4* and *SPT6*, respectively. We note that *SPT4* and *SPT6* mutants have previously been shown to suppress the readthrough of the *gal10Δ56* allele (Kaplan *et al*, 2004). Plasmids complementing *sog1* were isolated from a genomic library and were found to contain the *NPL3* gene. Linkage and plasmid complementation confirmed that *NPL3* is allelic with *sog1*.

Suppression by spt4 is caused by defective elongation

The identification of suppressors *sog2* and *sog3* as *SPT4* and *SPT6* confirmed our genetic predictions, since both Spt4 and Spt6 modulate transcription elongation (Swanson and Winston, 1992; Hartzog *et al*, 1998; Wada *et al*, 1998). Our suppressing allele of *SPT4* was designated *spt4-301* (Figure 3C). Previously characterized *spt4* alleles were tested to see if they also suppress the *gal10Δ56* galactose growth phenotype. Four previously isolated alleles of *spt4*, including *spt4Δ::URA3,* were crossed into the *gal10Δ56* background. Genetic analysis showed that *spt4-289* strongly suppresses the *gal10Δ56* gal^{-} phenotype. *spt4-6*, *spt4-3,* or *spt4Δ::URA3*(Winston *et al*, 1984) restores growth on galactose (data not shown), but growth is slow (it should be noted that these *spt4* mutations cause slow growth on their own). Next, we analyzed the transcription patterns of the suppressing *spt4* alleles. RNA was isolated from cells (wild type, *gal10Δ56*, *spt4-289*, *spt4-289* in the *gal10Δ56* background, and our *spt4-301* in *gal10Δ56*) that had been grown in galactose. Northern blotting shows that the level of readthrough transcription is decreased in both *spt4-289* and *spt4-301* as compared to *gal10Δ56* (Figure 3A and data not shown). Accordingly, *GAL7* transcription is restored. ChIP of TBP on the *GAL* locus at 30 min (Figure 3B) and 4 h (not shown) postinduction showed that TBP occupancy at the *GAL7* promoter was restored in the *spt4-301* suppressor strain, although not to wild-type levels. This lower occupancy of TBP in the *spt4-301* strain correlates with slower growth on galactose. Therefore, suppression is partial but sufficient for the selection.

To probe the mechanism of the *spt4-301* suppression, *in vitro* transcription extracts were prepared and analyzed for both elongation and termination defects. We used the previously described templates pG-Leu-CYCds (here designated pG^{-}) and pG-Leu-CYCpAmax (designated pG^{-}+pA) to measure elongation and termination (Steinmetz and Brow, 2003). These templates contain two G-less cassettes separated by the *CYC1* downstream region either without (pG^{-}) or with (pG^{-}+pA) the poly(A) and termination sequences included. Although poly(A) site-dependent termination is not completely efficient in the yeast extract, a similar mammalian system produced quantitatively similar results (Tran *et al*, 2001). Using extracts from *spt4-301* and the parent strain, the ratios of the G-less cassettes were compared (Table II). With a template lacking a poly(A) site, the ratio of the downstream to upstream G-less cassette is decreased for *spt4-301* (38% readthrough to the second cassette) compared to wild

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Table I Phenotypes of suppressor alleles

Complementation groups	Ts	Cs	Caffeine (15 mM)	Spt phenotype	Dominance	Genes
sog1	−	+/−	−	+	Recessive	*NPL3*
sog2	+	+	+	−	Recessive	*SPT4*
sog3	−	+	+	−	Recessive	*SPT6*
sog4	+	+	+	−	Recessive	*NA*

+, wild-type phenotype; −, mutant phenotype.

type (68% readthrough). This result indicates that defects in Spt4 result in less efficient elongation, a result also seen in mammalian transcription systems (Wada *et al*, 1998; Kim *et al*, 2003) and in a yeast system (Rondon *et al*, 2003). The magnitude of the decrease was not changed by the presence of the poly(A) site (26% in the mutant versus 56% in the wild type), indicating that the *spt4-301* mutation does not have an additional major effect on termination or polyadenylation. Based on this result, we believe that less efficient elongation in *spt4-301* cells suppresses the termination defect in *gal10Δ56* by reducing readthrough into the *GAL7* promoter.

A severe elongation defect in spt6 mutant cells suppresses gal10Δ56

Two alleles of *spt6* were isolated in the genetic selection (Figure 3C and data not shown), one of which (*spt6-401*) was selected for further analysis. Similar to the previously characterized allele *spt6-50*, *spt6-401* is sensitive to 6-AU (Hartzog *et al*, 1998). As assayed by ChIP, TBP occupancy at the *GAL7* promoter is restored near wild-type levels in *spt6-401* cells (Figure 3B). Similar results were obtained when chromatin was immunoprecipitated with antibodies against the TFIIH subunit Tfb3 (not shown). TBP occupancy at *GAL*-induced promoters (*GAL1, GAL2, GAL10,* and *GAL7*), and also the constitutive *ADH1, PYK1,* and *GAL3,* was similar in both *spt6-401* and wild-type cells (not shown), indicating that the effect at *gal10Δ56* is not due to general changes in TBP occupancy.

Extracts from *spt6-401* cells were assayed for elongation and termination defects *in vitro* as described above for *spt4-301*. Compared to wild type, extracts from *spt6-401* cells display a reduction in the ratio of the second to first G-less cassette (11% readthrough in the mutant versus 68% in the wild-type extract) using the template lacking a poly(A) site, indicating less efficient elongation (Table II). An even greater reduction in readthrough is seen when the poly(A) and termination sequences are present (5% in the mutant versus 56% in the wild-type extract), possibly suggesting increased termination in the mutant extract. Therefore, suppression of *gal10Δ56* in *spt6-401* cells is likely to be mediated through a defect in elongation that also enhances termination by RNApII. Similar conclusions have been made by Kaplan *et al* (2004).

Increased termination efficiency in npl3 suppressors

The Npl3 protein contains two RNA recognition motifs (RRM1 and RRM2) and a glycine/arginine-rich repeat (GAR) (Birney *et al*, 1993). Mutations found in three *npl3* suppressor alleles map near the RRM2 motif (L225S (isolated twice) and E244K). All alleles of *npl3* identified in our selection are sensitive to high temperature, caffeine, and 6-AU (Figure 4 and Table I). These phenotypes, as well as the reported association of Npl3 with transcription elongation complexes (Lei *et al*, 2001; Lei and Silver, 2002), led us to suspect that the mutations in *npl3* that suppress *gal10Δ56* were doing so through effects on transcription. As with the other suppressors, the *GAL* locus was analyzed for TBP occupancy by ChIP using a representative allele of *npl3*. The distribution of TBP in *npl3* suppressor alleles along the locus is similar to that of *spt4* suppressor cells. The level of TBP occupancy in *npl3-120* is increased at the *GAL7* promoter when compared to *gal10Δ56* (Figure 3B), suggesting an effect on transcription. Therefore, the suppressing effects of the *npl3* mutants are unlikely to be mediated at later steps involving Npl3, such as RNA export or stability.

To explore whether the *npl3* suppression was due to elongation or termination effects, the *in vitro* transcription assay described above was used (Figure 5A). On a template without a poly(A) site between cassettes, *npl3-120* extract had a slightly reduced ratio of readthrough to the second cassette (39% mutant versus 58% in wild type, less than a two-fold difference). When a poly(A) site is included between cassettes, the difference is more pronounced (13% readthrough in the mutant versus 33% in wild type). Therefore, this allele may affect both elongation and termination. In a second set of transcription experiments, the *npl3-210* allele (assayed in the original *gal10*Δ56 background) only showed an effect on the template containing the terminator insert (28 versus 56% readthrough) (Table II). In both experiments, the Npl3 mutants actually had more efficient termination, suggesting that Npl3 may antagonize a step necessary for termination.

To test this idea directly, recombinant Npl3 was added back to mutant transcription extracts to see whether it would promote transcription readthrough. Figure 5B shows a representative experiment using *npl3-210* extract supplemented with increasing concentrations of native or heat-inactivated Npl3 added. Using the template without a poly(A) site, additional Npl3 does not change the readthrough (always 50–60%) compared to the negative control (100HI). However, using the template with a poly(A) site, additional Npl3 increases the ratio of the second G-less cassette to the first. Reactions containing 100 ng of heat-denatured Npl3 show about 23% readthrough, while 10–100 ng of native Npl3 increase readthrough to greater than 40%. Therefore, Npl3 antagonizes termination *in vitro*.

To test whether the *npl3* suppressors also exhibit termination defects *in vivo*, a series of termination reporter plasmids (as described in Materials and methods) were used (Hyman *et al*, 1991). All contained a LacZ reporter gene downstream of the *rp51* intron. Plasmids with the *ADH2* terminator (pL101) or *CYC1* terminator (pL201) cloned into the intron show much less expression of the downstream β-galactosidase gene compared to the construct with no terminator

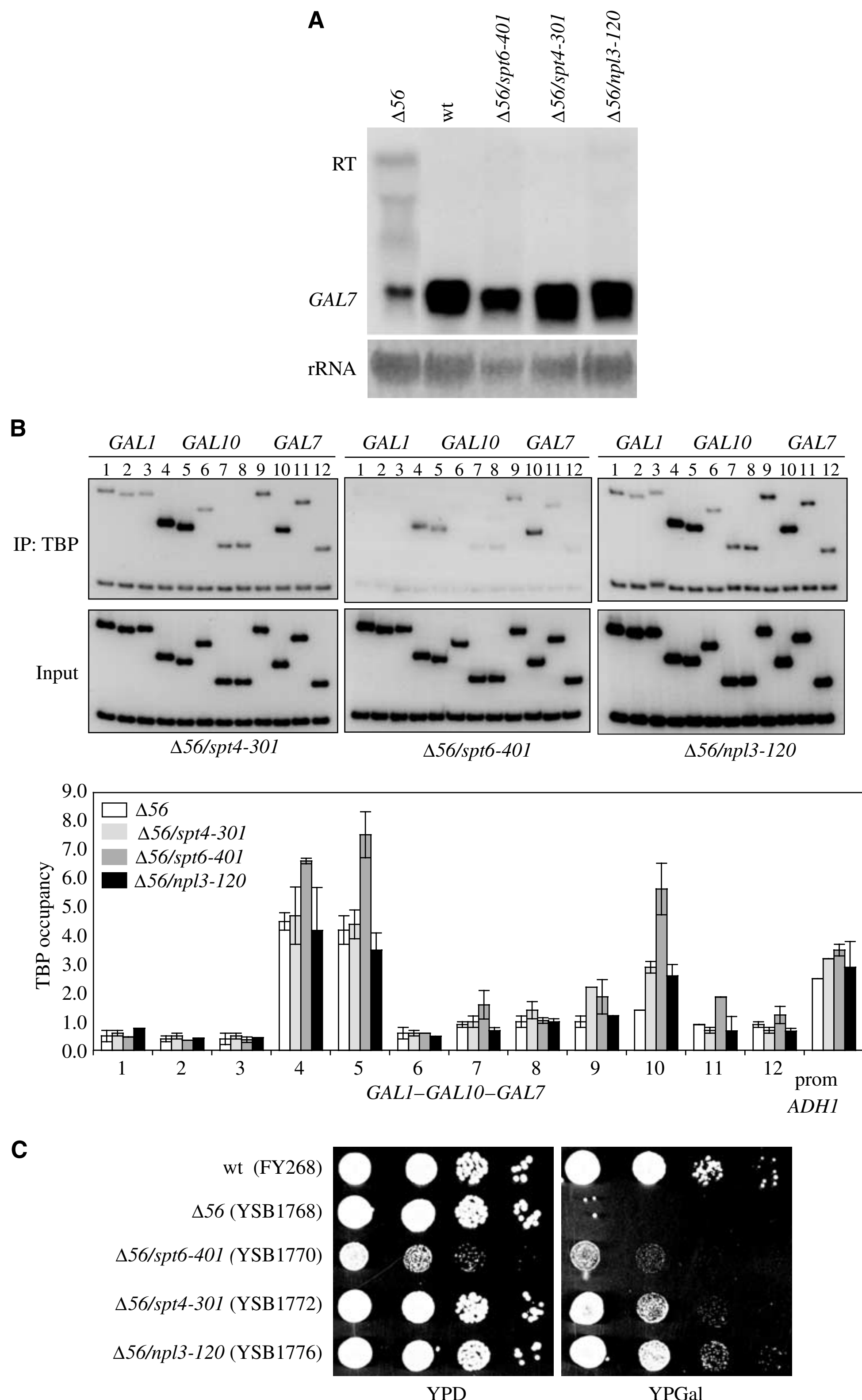

Figure 3 Suppression of readthrough at *gal10*Δ*56*. (**A**) RNA samples from *gal10*Δ*56* strains with no suppressor (YSB1768), *spt4-301* (YSB1772), *spt6-401* (YSB1770), or *npl3-120* (YSB1776) suppressing alleles were analyzed for GAL7 expression by Northern analysis. RT designates the readthrough transcript. In the bottom strip, the blot was stained for rRNA as a loading control. (**B**) ChIPs for TBP were performed for *gal10*Δ*56* strains as described in panel A. Strains were induced with galactose for 30 min and primers are as in Figure 1. Representative PCR reactions are shown and results (average of two independent ChIP experiments) are represented graphically underneath. The *ADH1* promoter was used as an unaffected control and shown at the right of the graph. (**C**) Serial dilutions of wild-type (FY268), *gal10*Δ*56* (YSB1768), and *gal10*Δ*56* strains containing the suppressing alleles *spt6-401* (YSB1770), *spt4-301* (YSB1772), and *npl3-120* (YSB1776) were spotted onto YPD or YPGal plates and grown for 2 days at 30°C.

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insert (pHZ18Δ2Sma). These plasmids were transformed into *NPL3* or *npl3-120* strains and then spotted into SC-gal plates and allowed to grow overnight. Strains were then replica plated onto filters for the β-galactosidase assay (Figure 6). For further quantitation, the β-galactosidase assays were also carried out with protein extracts derived from liquid cultures (Figure 6, bottom panel). Expression levels for the construct with no terminator were slightly lower in the *npl3-120* strain (1.192 versus 2.236 β-gal units/μg protein, less than a two-fold effect). In contrast, the constructs containing the terminators had much lower levels of β-galactosidase in the *npl3-120* strain. The *ADH2*-3′end showed a 23-fold decrease in expression (0.003 U/μg in the mutant versus 0.079 wild type) and the *CYC1*-3′end had a 7.5-fold decrease (0.034 versus 0.254) in the mutants relative to wild-type cells (Figure 6). Since these strong effects are only seen when a terminator sequence is present, it appears that termination is more efficient in the *npl3* suppressor strain. Considering these results and the *in vitro* experiments, we suggest that the *gal10Δ56* allele is suppressed by partial loss of an Npl3 function that antagonizes termination.

To test whether the *npl3-120* suppressor affected the recruitment of the polyadenylation/termination machinery, ChIP experiments were carried out on the *gal10Δ56* locus (Figure 7A). Crosslinking of RNApII (assayed with the antibody 8WG16) did not reveal any gaps between *GAL10* and *GAL7* that might signify termination, presumably because the two genes are too close together. Npl3 levels were slightly reduced in the suppressor strain. In the wild-type parent strain, the polyadenylation/termination factor Rna15 crosslinked near the *GAL10* 3′ end. This crosslinking was greatly reduced in the *gal10Δ56* strain, as expected from the increased readthrough at this locus. Strikingly, in the *npl3-120* suppressor strain, Rna15 crosslinking was restored to levels at least as high as in the wild-type strain. To determine

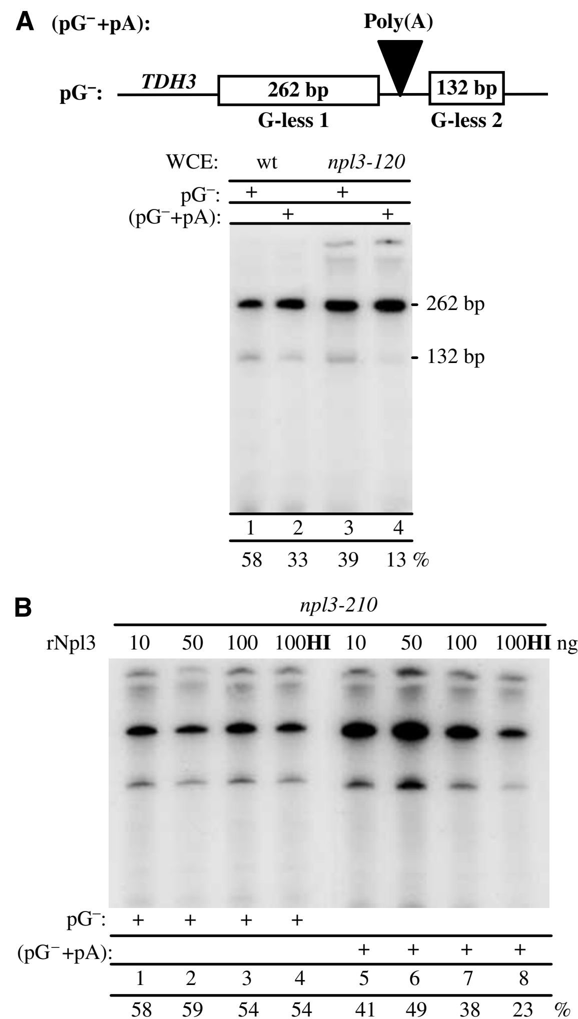

Figure 5 Npl3 antagonizes termination *in vitro*. (**A**) Mutations in *NPL3* enhance transcription termination *in vitro*. At top is a schematic diagram of transcription templates pG⁻ and pG⁻ + pA (Steinmetz and Brow, 2003). Two G-less cassettes are separated by a spacer with no insert (pG⁻) or containing the *CYC1* termination sequence (pG⁻ + pA). Whole-cell extracts (WCEs) from *NPL3* (YSB1799, lanes 1 and 2) and *npl3-120* (YSB1800, lanes 3 and 4) strains were used in transcription reactions with either pG⁻ (lanes 1 and 3) or pG⁻ + pA (lanes 2 and 4). The percent of polymerases that read through (i.e. the ratio of downstream G-less cassette 2 to upstream G-less cassette 1 normalized for labeled U content) is shown below each lane. (**B**) Recombinant wild-type Npl3 reverses the termination-enhancing effect of *npl3-210*. Npl3 was purified from *E. coli* and added to transcription extracts from *npl3-210* (YSB1771). Templates were either pG⁻ (lanes 1–4) or pG⁻ + pA (lanes 5–8). Heat-inactivated Npl3 (HI, incubated at 95°C for 5 min) was added to WCEs as negative control and is shown in lanes 4 and 8. The percentage of polymerases that read through is shown under each lane.

Table II Transcription readthrough caused by different mutations

Strain	pG⁻ (no terminator)		pG⁻ + pA	
GAL10 background				
NPL3 (YSB1799)	0.58	±0.03	0.33	±0.07
npl3-120 (YSB1800)	0.39	±0.06	0.13	±0.03
gal10Δ56 background				
NPL3/SPT4/SPT6 (YSB1768)	0.68	±0.02	0.56	±0.01
npl3-210 (YSB1771)	0.63	±0.07	0.28	±0.03
spt4-301 (YSB1772)	0.38	±0.02	0.26	±0.05
spt6-401 (YSB1770)	0.11	±0.07	0.05	±0.03

Values shown are average of three independent experiments ± standard deviation.

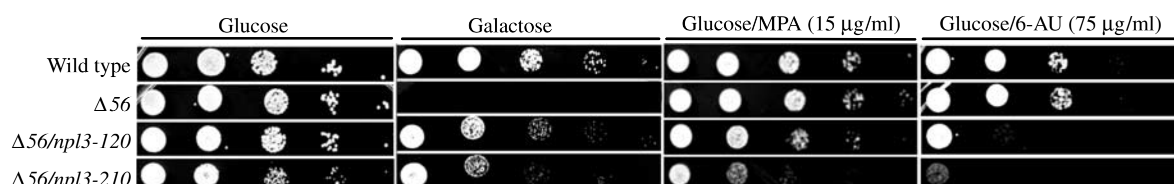

Figure 4 Suppressing alleles of *NPL3* are sensitive to 6-AU and MPA. Serial dilutions of cells were spotted on YP plates containing 2% glucose, 2% galactose alone, or 2% galactose supplemented with 6-AU (75 μg/ml) or MPA (15 μg/ml). Strains assayed were wild type (FY268), *gal10Δ56* with no suppression (YSB1768), *npl3-120* (YSB1776), or *npl3-210* (YSB1771).

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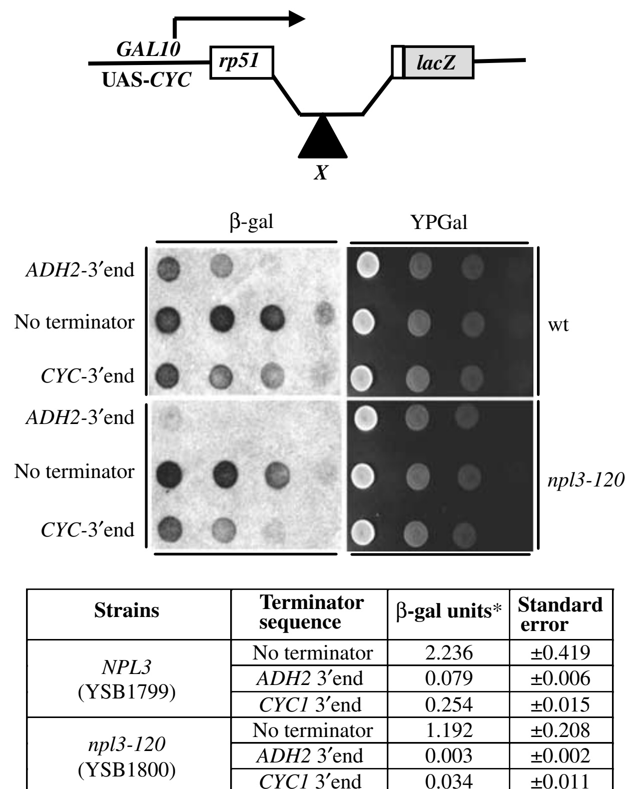

Strains	Terminator sequence	β-gal units*	Standard error
NPL3 (YSB1799)	No terminator	2.236	±0.419
	ADH2 3′end	0.079	±0.006
	CYC1 3′end	0.254	±0.015
npl3-120 (YSB1800)	No terminator	1.192	±0.208
	ADH2 3′end	0.003	±0.002
	CYC1 3′end	0.034	±0.011

*Values from β-gal assay in liquid cultures

Figure 6 An *npl3* mutation enhances transcription termination *in vivo*. The top panel shows a schematic diagram of reporter constructs. The parent construct (pHZ18Δ2Sma) contains an intron upstream of a *lacZ* reporter gene. Inserted within the intron (designated by X) is either the *ADH2*-3′end (pL101) or the *CYC1*-3′end (pL201). *NPL3* (YSB1799) and *npl3-120* (YSB1800) strains were transformed with the indicated constructs and spotted in serial dilutions on YP plates containing galactose (right panels). These were replica plated onto filters and assayed for β-galactosidase activity (β-gal, left panels). The reduced intensity of the color indicates less readthrough of the terminator. The same strains were assayed quantitatively from liquid cultures and the results are presented as units per microgram protein in the table.

whether this effect was specific to the *GAL10/7* locus or was a general effect, Rna15 levels at the *ADH1*, *PYK1,* and *PMA1* genes were assayed (Figure 7B). Although levels of RNApII were similar in the wild-type and *npl3-120* strains (Figure 7B and data not shown), crosslinking of Rna15 was markedly increased in the suppressor strain. Although the *PMA1* terminator is efficient, we noted that RNApII crosslinking was reduced in the *npl3-120* strain downstream of the cleavage site (primers 6 and 7), consistent with improved termination. These results strongly support a model in which Npl3 normally acts to antagonize cotranscriptional recruitment of polyadenylation/termination factors to the mRNA.

Discussion

Using a genetic selection based on a deletion in the *gal10* poly(A) site, we isolated mutations in three genes that decrease readthrough into the downstream *GAL7* promoter. This genetic approach has some similarity to two previously described screens used to identify transcription factors: the Ty/solo δ insertion screen (Winston *et al*, 1984) and the *cyc1-512* screen (Zaret and Sherman, 1982; Winston *et al*, 1984). In the Ty screen, a transcript from an inserted transposon element disrupts transcription from a nearby cellular gene. Suppression typically reduces the transcription from the Ty promoter or instead favors the downstream promoter, allowing reactivation of the selectable downstream gene. In Sherman's *cyc1-512* screen, a deletion of 38 bp upstream of the normal poly(A) site results in unstable readthrough transcripts and downregulation of *CYC1*. Using conditions that require strong *CYC1* expression, mutations that restore adequate expression of *CYC1* were isolated in this screen (e.g. *CBC1* and *UPF1*).

Our approach differs from both screens in that both the upstream and downstream promoters (*GAL10* and *GAL7*) must remain transcriptionally active, since both are required for growth on galactose. This approach presumably reduces the isolation of suppressors affecting transcription initiation and should instead produce mutations that affect the readthrough transcript. As predicted, the selection resulted in the isolation of mutations that either enhance termination or impede elongation.

The isolation of elongation factor mutants such as *spt4* and *spt6* in the selection was expected, and agrees with a similar finding from Kaplan *et al* (2004). Mutations in these factors probably reduce the number of transcripts reading through from *GAL10*. By reducing the rate of elongation in the *gal10Δ56* background, there is likely to be an increased opportunity for basal factors to bind to and initiate transcription from the downstream *GAL7* promoter. This effect can be observed in the increased crosslinking of TBP to the *GAL7* promoter in the suppressor mutants. Note that the *spt6* mutant actually shows better TBP binding at the *GAL7* promoter than *spt4*, but weaker suppression (Figure 3). This is presumably due to the additional severe elongation defect when Spt6 is defective. Since Spt5 has been shown to enhance capping (Wen and Shatkin, 1999; Mandal *et al*, 2004), and Spt4 and Spt5 are interacting partners of DSIF, we cannot formally rule out an additional effect of capping upon termination in the *spt4*-mediated suppression *gal10Δ56.*

More surprising was the isolation of Npl3 mutants as suppressors of defective termination. Previous indirect evidence links Npl3 to mRNA processing events. Npl3 is an RNA-binding protein (Russell and Tollervey, 1995) and may also bind directly to CBC components (Shen *et al*, 2000). A functional interaction between Npl3 and 3′ end processing proteins (Hrp1 and Rna15) has been suggested by genetic interactions. Mutations in *HRP1* and *RNA15* suppress the Ts^- phenotype of *npl3-1* (Henry *et al*, 1996). Hrp1 and Rna15 are required for cleavage and polyadenylation of mRNAs (Chen and Moore, 1992; Kessler *et al*, 1996). Kessler *et al* (1997) reported that recombinant Npl3 did not affect cleavage or polyadenylation in a purified *in vitro* system. However, both our *in vivo* and *in vitro* results indicate that Npl3 antagonizes transcription termination, which is linked to cleavage and polyadenylation. Our results are not contradictory, since in the Kessler experiments cleavage/polyadenylation was not coupled to transcription.

All of these results are consistent with a model in which cotranscriptional binding of Npl3 to the nascent mRNA transcript antagonizes the recognition of RNA sequences by the polyadenylation/termination machinery. This may help

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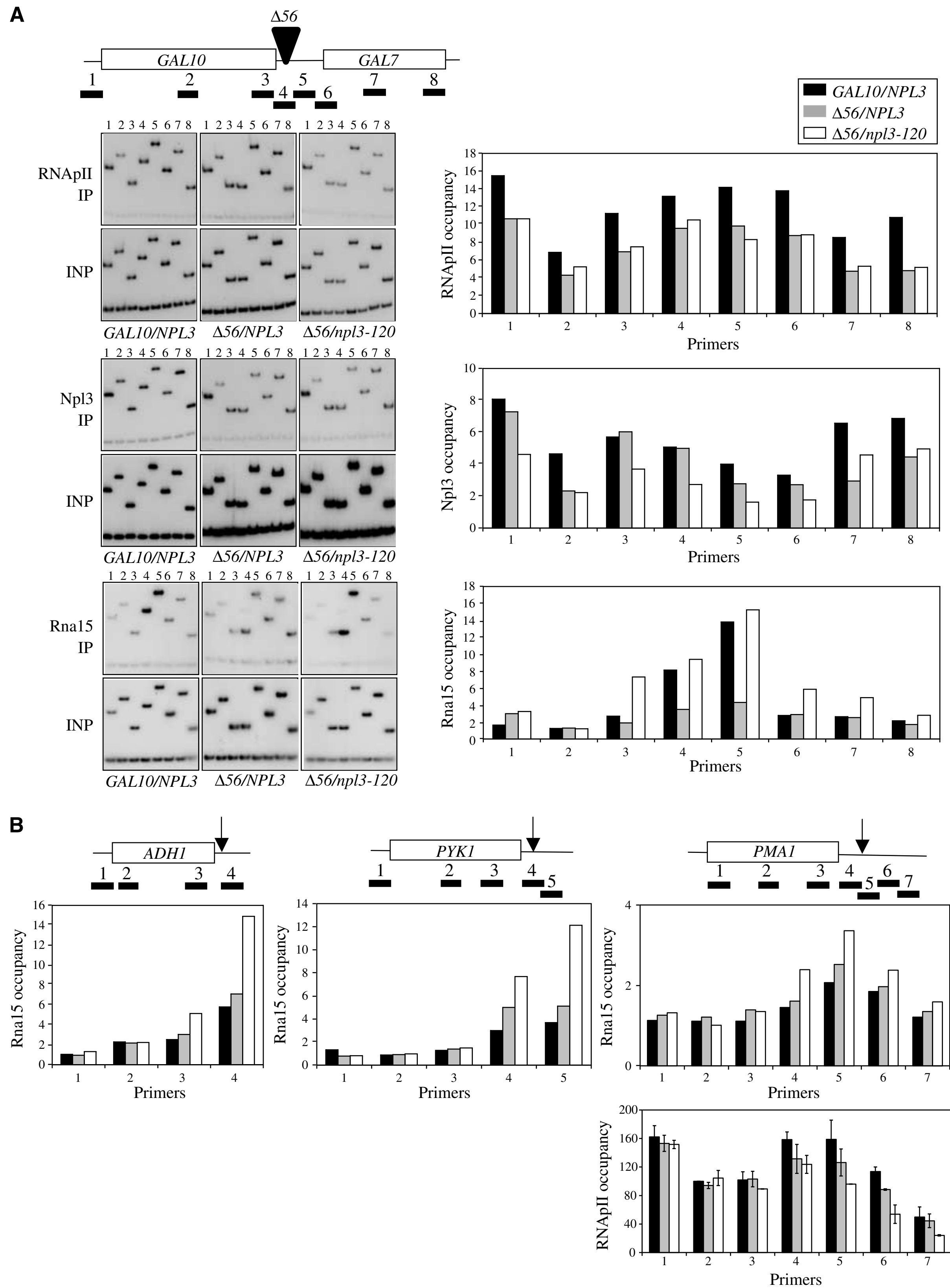

Figure 7 Increased recruitment of polyadenylation/termination factor Rna15 at the 3′ end of genes in the *npl3-120* strain. (**A**) ChIP was performed in wild-type (FY268), *gal10Δ56* (YSB1768), and *gal10Δ56, npl3-120* (YSB1777) strains. The same batch of chromatin from each strain was immunoprecipitated with antibodies against RNApII (8WG16), Npl3, or Rna15. PCR products are shown of the immunoprecipitates (IP) and input chromatin (INP) using primers as shown at top. Quantitations of results are shown to the right. (**B**) Results from ChIP of Rna15 at the *ADH1*, *PYK1*, and *PMA1* genes are shown in graphs. Positions of PCR primers are shown schematically above each graph; polyadenylation sites are indicated by arrows. ChIP of RNApII for *PMA1* is shown below the corresponding Rna15 ChIP. Note that for the RNApII quantitation, values were normalized to primer set 2 in the wild-type strain, which was arbitrarily set at 100.

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suppress recognition of cryptic polyadenylation sequences, a function likely to be necessary given the weak consensus sequences for 3′ end formation in yeast. Since Npl3 appears antagonistic to termination, mutations in polyadenylation factors such as *RNA15* or *HRP1* could restore the balance between these opposing activities, explaining the observed suppression patterns (Henry *et al*, 1996).

Proper cotranscriptional packaging of mRNAs appears to be essential for proper recognition of polyadenylation/termination sequences. The RNA-binding protein Hrp1 is recruited cotranscriptionally (Komarnitsky *et al*, 2000) and promotes proper choice of polyadenylation sites (Kessler *et al*, 1997; Minvielle-Sebastia *et al*, 1998; Gross and Moore, 2001). Another hnRNP, the polypyrimidine tract-binding protein (PTB), was recently reported to play a role in RNA metabolism by decreasing formation of mRNA 3′ ends in mammalian cells (Castelo-Branco *et al*, 2004). Therefore, the interplay between different RNA-binding proteins appears to be critical for proper mRNA 3′ end recognition formation.

Materials and methods

Strains

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

Mutant isolation

Overnight cultures of *MAT*a (CKY185) and *MAT*α (YSB1768) cells, containing the Δ*56* deletion downstream of *GAL10*, were grown in YPD (yeast extract/peptone/dextrose) and 500 μl was inoculated into 2 ml of YPD and allowed to grow for 2 h (early log phase). Cells were then pelleted, washed, and plated (5×10^6 cells/plate) onto YPGal (2% galactose) + antimycin A (1 μg/ml) plates. Antimycin A is included to inhibit the respiration necessary for nonfermentative growth. Plates were mutagenized with ultraviolet light (50 J/m^2) and Gal$^+$ colonies were allowed to grow at 30°C. Colonies from mating types a and α were isolated from the irradiated plates. In addition, several colonies that grew spontaneously from a nonirradiated plate were isolated. Suppressor candidate strains were tested for dominance/recessive tests by mating to parent strains of the opposite mating type followed by replica plating onto YPGal + antimycin A plates. A total of 19 dominant suppressors, likely *cis*-acting, were identified and were not characterized further.

Recessive mutants were assayed for additional phenotypes, including heat sensitivity (37°C), cold sensitivity (16°C), and caffeine sensitivity (YPD plus 15 mM caffeine). In addition, recessive mutants were screened for the Spt$^-$ phenotype, which can be caused by mutations in certain elongation factors, by plating on lys$^-$ and his$^-$ plates (Winston and Carlson, 1992). A total of 58 recessive mutants were assigned to four complementation groups designated *sog1–4*. Members of each group were transformed with plasmids containing *SPT4*, *SPT5*, or *SPT6* (kindly provided by F Winston, Harvard University). Suppressors *sog2* and *sog3* were complemented with plasmids containing *SPT4* and *SPT6*, respectively. Identities of the mutant genes were confirmed by segregation of crosses to an *spt4*Δ::*URA3* (YSB1775) strain and an *spt6* (CKY122) strain.

The *sog1* gene was cloned by complementation using a genomic DNA library (Rose *et al*, 1987). Approximately 3000 transformants of a *sog1* mutant strain were selected using the library's *URA3* marker. These were screened by replica plating for complementation of the gal$^-$ and heat-sensitive (37°C) phenotypes of the *sog1* mutant. A total of 14 plasmids were recovered and rescreened for complementation. After restriction analysis, four plasmids were selected for sequencing. The sequences from four of the plasmids mapped to chromosome IV with an overlapping region 3463 bp upstream and 1654 bp downstream of the *NPL3* gene. The *sog1* suppressor was confirmed to be the *NPL3* gene through a linkage test using an *npl3*Δ::*KANMX* strain (Research Genetics) and by complementation with an *NPL3*-containing plasmid (kindly provided by P Silver, Harvard University; Henry *et al*, 1996). Three suppressor alleles of *sog1/npl3* were sequenced and were found to encode either amino-acid substitution L225S or E244K.

RNA preparation and analyses

Cells were induced with 2% galactose overnight. Total RNA was prepared as described previously (Ausubel *et al*, 1988). Northern blot transfer and hybridization were performed as described previously (Swanson *et al*, 1991). ^{32}P-labeled probes were generated by random hexamer labeling (Ausubel *et al*, 1988).

Reporter assay for termination readthrough

Wild type (YSB1799) and *npl3-120* (YSB1800) were transformed with plasmids pL101, pL201, or pHZ18Δ2Sma (kindly provided by C Moore, Tufts University; Hyman *et al*, 1991). These plasmids contain the *GAL10 UAS-CYC* promoter driving a transcript containing the *rp51* intron upstream of *lacZ*. Either the *ADH2-3′*end or the *CYC1-3′*end sequences are inserted into the *rp51* intron in pL101 or pL201, respectively (Hyman *et al*, 1991). Transformants were processed as described previously (Runner and Brewster, 2003) with the following modifications. After overnight growth in synthetic complete media with galactose but lacking uracil (SC-gal-URA$^-$) at 30°C, cells were pelleted and resuspended in 200 μl of SC-gal-URA$^-$ media. Four serial dilutions were spotted onto SC-gal-URA$^-$ plates. These were replica plated onto Whatman 1A filter paper on YPD or SC-gal-URA$^-$ plates and allowed to grow overnight at 30°C. Filters were assayed for *lacZ* activity as described previously (Burns *et al*, 1994).

For liquid cultures, wild-type (YSB1799) and *npl3-120* (YSB1800) strains transformed with plasmids pL101, pL201, or pHZ18Δ2Sma were grown in SC-raf-URA$^-$ overnight. Cells were supplied with new SC-raf-URA$^-$ media and induced with 2% galactose for 6 h. Cells (2×10^8) were pelleted and lysed and the resulting extracts were used to measure β-galactosidase activity as described previously (Ausubel *et al*, 1988). For quantitation, β-galactosidase units were normalized to protein concentrations.

Chromatin immunoprecipitations

ChIPs were performed essentially as described previously (Komarnitsky *et al*, 2000). Anti-Npl3 serum was a gift from D Tollervey and anti-Rna15 serum was a gift from Claire Moore. Oligonucleotides used for PCR are listed in Supplementary Table 2. For α-TBP immunoprecipitations, antibody was preincubated for 60 min at room temperature with protein A-agarose or protein G-Sepharose CL-4B (Amersham) beads as indicated and beads were washed once with TE pH 8.0. Chromatin solution was then added and reactions incubated overnight at 4°C. Immunoprecipitates were washed, protease treated, and decrosslinked. Conditions for PCR were as described previously (Komarnitsky *et al*, 2000). PCR products were quantified by PhosphoImager as described previously (Kim *et al*, 2004b). Briefly, the efficiency of amplification for each region relative to a chromosome V nontranscribed control region was calculated from the input sample. This value was then used to normalize the specific signal obtained from each immunoprecipitation. Division of this normalized value by the background amplification of the nontranscribed control gives a relative value (x-fold compared to the nontranscribed internal control) that allows trend comparison across samples to be performed. Note that a value of 1.0 means that there is no crosslinking above background.

In vitro transcription assays

Cell pellets were crushed in liquid nitrogen as described previously (Schultz *et al*, 1991) and then resuspended in 1 ml of transcription buffer A (200 mM Tris pH 7.9, 390 mM NH_4SO_4, 10 mM $MgSO_4$, 1 mM EDTA, 20% glycerol, 2 mM DTT, plus protein inhibitors (1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml benzamidine) per gram wet weight. These suspensions were then further subjected to glass bead disruption. Lysates were collected and treated as described previously (Keogh *et al*, 2002). Aliquots of whole-cell extract (13–45 mg/ml) were frozen in liquid nitrogen and stored at −80°C.

RNApII transcription reactions were carried out as described by Keogh *et al* (2002). Templates used were the plasmids pCYCds or pCYCpAmax (kindly provided by E Steinmetz and D Brow, University of Wisconsin Medical School; Steinmetz and Brow, 2003). RNA was resolved on a denaturing urea–acrylamide gel and quantified by PhosphoImager as described previously (Steinmetz and Brow, 2003). For each strain, the ratio of total counts of the

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downstream G-less cassette to total counts of upstream G-less cassette was normalized for the ratio of labeled U residues contained in each cassette (G-less1 = 102, G-less2 = 40 U residues) as described previously (Steinmetz and Brow, 2003).

Recombinant Npl3 protein

Escherichia coli strain BL21(DE3) was transformed with pSBEThis7-Npl3 (details available upon request). A 1 l culture (LB + kanamycin 25 μg/ml) grown at 25°C to $OD_{600} \approx 0.5$ was induced by the addition of 1 mM IPTG. All steps from this point on were performed at 4°C and all buffers contained protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 μg/ml benzamidine). Cells were harvested and lysates were prepared by sonication in binding buffer (10 mM Tris–HCl pH 7.9, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF). The soluble extract was incubated at 4°C with gentle rolling for 2 h with 2 ml of Ni-NTA resin (Qiagen). The resin was spun down gently, washed twice with 20 ml of binding buffer plus 10 mM imidazole plus 1 mM PMSF, twice with 20 ml of binding buffer plus 20 mM imidazole plus 1 mM PMSF, and resuspended in 5 ml of binding buffer plus 20 mM imidazole plus 1 mM PMSF. Resin was applied to a column and eluted in ten 500 μl fractions with binding buffer plus 200 mM imidazole. Recombinant Npl3 was assayed by Coomassie staining and by Western blotting with anti-Npl3 (kindly provided by D Tollervey, University of Edinburg; Venema and Tollervey, 1995).

Supplementary data

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

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