Overexpression of the gene for Rpb7 subunit of yeast RNA polymerase II rescues the phenotypes associated with absence of the largest, nonessential subunit Rpb4

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Abstract

The easily dissociable subcomplex of Rpb4 and Rpb7 subunits of yeast RNA polymerase II has been considered, for long, to play a role in stabilizing Pol II under stress. On the basis of previous genetic and biochemical observations, it was proposed that within the subcomplex one of the functions of Rpb4p is to stabilize the interaction between Rpb7p and the rest of Pol II. We took a direct approach to test the latter possibility by overexpression and mutagenesis of *RPB7* in absence of Rpb4p. We report here the results, which support the latter hypothesis. While it has been previously reported that absence of Rpb4p results in reduction in overall transcription by Pol II, our comparative analysis of RNAs from *RPB4* and *rpb4*Δ cells suggests that there are indeed several genes differentially expressed between the two cells. We propose that the qualitative differences in overall transcription in presence and absence of Rpb4p imply a more active role for Rpb4p in transcription of at least a subset of genes.

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Introduction

The transcription machinery that is responsible for expression of protein coding genes is comprised of the core RNA polymerase II, the general transcription factors, the coactivator/mediator complexes, and a myriad of specific regulators (Hampsey 1998; Ishihama *et al.* 1998). The gene-specific regulators relaying the signals to the basal transcription machinery in response to changes in internal and external stimuli effect differential gene expression. The 12 core subunits of Pol II have been considered, until recently, to be responsible for carrying out the basic biochemical reactions involved in transcription, to confer stability on the core complex, and of course to provide surface for interaction with the general transcription factors (GTFs). The GTFs in turn mediate various promoter-specific interactions and help in recruitment of the basic transcription machinery to the promoter. Only recently has it been realized that one of the smaller Pol II core subunits, Rpb5, could differentially affect expression of a certain subset of genes and thus play a regulatory role (Miyao and Woychik 1998). The question that needs to be addressed now is whether other components of the core polymerase may also be able to cause differential gene expression through direct or indirect interactions with gene-specific regulators.

The subcomplex within the yeast RNA Pol II, comprised of Rpb4p and Rpb7p, has been implicated in stress response in yeast (Woychik and Young 1989; Choder 1993; Choder and Young 1993). It was originally observed that the *rpb4*Δ mutant showed several conditional phenotypes. These include high-temperature as well as low-temperature sensitivity and inability to grow in inositol-deficient media (Woychik and Young 1989). Deletion of *RPB4* is known to cause loss of resistance to various other stress conditions while over-expression of *RPB7* is known to enhance certain starvation-specific phenotypes (Woychik and Young 1989; Khazak *et al.* 1995). The Pol II purified from *rpb4*Δ cells as well as that from a mutant in the largest subunit of Pol II in yeast were shown to lack the two subunits Rpb4 and Rpb7 (Ruet *et al.* 1980; Edwards *et al.* 1991). This Rpb4p-deficient

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Pol II was shown to be defective in promoter-specific transcription initiation. These observations have led to various hypotheses explaining the role of the two subunits, and the one that suggests that Rpb4p somehow stabilizes Pol II under stress conditions has been favoured (Mckune *et al.* 1993). During preparation of this manuscript a report was published showing that multicopy expression of *RPB7* rescues the *rpb4*Δ-associated stress phenotypes (Sheffer *et al.* 1999).

Here we report that rather than stabilizing Pol II under stress, Rpb4 is required for stable interaction of the polymerase with Rpb7p even under moderate conditions. By genetic analyses, we have shown that overexpression of *RPB7* or relatively low-level expression of dominant mutants of *RPB7* rescue the *rpb4*Δ deletion phenotypes. We present the relevance of these observations in the light of earlier published reports, which suggested stress-response-specific role of Rpb4.

Materials and methods

General methods: Standard protocols were followed for mating of yeast (*Saccharomyces cerevisiae*) strains, sporulation tetrad dissection, plasmid segregation, etc. (Sherman *et al.* 1983; Ausubel *et al.* 1990). The yeast transformations were carried out by a modified lithium acetate method that does not involve heat shock of the yeast cells (Elble 1992). Denaturing polyacrylamide gels for analysis of the RNA arbitrary primed (RAP) PCR products were prepared and run as described in Ausubel *et al.* (1990). All the plasmids were transformed, amplified and manipulated in *Escherichia coli* DH5α. The manipulations of DNA were carried out as described in Ausubel *et al.* (1990).

Media: Yeast cells were grown in either YP (1% yeast extract, 2% peptone) with 2% glucose or galactose as the carbon source or in synthetic minimal medium (0.67% yeast nitrogen base without amino acids) with the desired supplements and either 2% glucose or 2% galactose as carbon source (Sherman *et al.* 1983).

Strains and plasmids: The yeast strains and plasmids used in this study are described in tables 1 and 2 respectively. The results presented here were originally observed in the SY5 strain and reproduced in the SY10 strain, which was derived from SY5 crossed with SY6210.

PCR mutagenesis: The basic mutagenic PCR was carried out as described by Sadhale and Woychik (1994). The *RPB7* gene fragment in the plasmid pPS25 was PCR-amplified in presence of $MnCl_2$ and various concentrations of $MgCl_2$ between 0.5 and 2.0 mM. The PCR products were pooled and introduced along with equimolar amounts of a gapped plasmid, pBP86, into the SY5 strain (tables 1 and 2). The transformants were screened at 34°C and 37°C and further characterized in the SY10 strain.

Assay for temperature sensitivity: All strains were first streaked on solid synthetic minimal medium containing either 2% glucose or 2% galactose + 1 % xylose as carbon source (Ausubel *et al.* 1990). Cells were harvested and suspended in water and spotted at comparable densities. In all experiments described below, the pre-inoculum was always in a medium containing glucose, and the cells were pelleted, washed and resuspended in the medium containing the respective sugar. To test temperature sensitivity the replica plates were incubated at 22°C, 34°C or 37°C for three days before photography.

Growth characteristics of the yeast strains: Five-ml pre-cultures were grown till mid-log phase at 22°C and then subcultured into 50 ml of appropriate media at approximately $A_{600} = 0.05$. They were maintained under shaking conditions (100 rpm) at 22°C, 34°C and 37°C. Cell density was monitored by measuring absorbance at 600 nm. The morphological alterations were monitored by microscopic observations using an Olympus BX-50 microscope with Nomarski optics at

Table 1. List of plasmids.

Plasmid	Description	Copy number per cell	Reference
pPS24	pJH359	~50	Scafe *et al.* 1990
pPS5	YEplac181	~50	Geitz and Sugino 1988
pPS2	YCplac111	1–2	Geitz and Sugino 1988
pPS12	pYES2	~50	Invitrogen, USA
pPS25	*RPB4* gene in pPS5	~50	Sadhale and Woychik 1994
pBP86	*RPB7* gene in which the open reading frame is replaced by *Hin*dIII restriction site	1–2	This work
pNS114	*Bam*HI–*Sal*I fragment from pPS25 subcloned into pPS2	~50	This work
pPS40	*RPB4* ORF cloned under the *GAL1* promoter in the pPS12 vector	~50	This work
pRA56	1.4-kb *Bam*HI–*Sal*I fragment containing *RPB7* gene in pPS2	1–2	This work
pPS26	1.4-kb *Bam*HI–*Sal*I fragment containing *RPB7* gene in pPS5	~50	Sadhale and Woychik 1994
pPS41	*RPB7* ORF cloned in the pPS12 vector under the *GAL1* promoter	~50	This work

Table 2. List of strains.

Name	Genotype/description	Reference/source
SY5	*MATa, ade2-1, leu2-3,112, ura3-52, rpb4Δ::HIS3*	Woychik and Young 1989
SY6210	*MATα, ade2-1, his3Δ200, leu2-3,112, lys2-801, trp1Δ901, ura3-52*	Vijayraghavan *et al.* 1989
SY5 × SY6210	*MATα/MATa, ade2-1/ade2-1, leu2-3,112/leu2-3,112, his3Δ200/his3Δ200, ura3-52/ura3-52, TRP1/trp1Δ901, LYS2/lys2-801, RPB4/rpb4Δ::HIS3*	This work
SY10	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3* (a segregant of the SY5 × SY6210 cross)	This work
SY101	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pPS5	This work
SY102	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pNS114	This work
SY103	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pPS25	This work
SY104	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pPS40	This work
SY105	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pRA56	This work
SY106	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pPS26	This work
SY107	*MATa, leu2-3,112, lys2, ura3-52, rpb4Δ::HIS3*/pPS41	This work
SY11	*MATα, leu2-3,112, lys2, trp1Δ901, ura3-52, rpb4Δ::HIS3* (a segregant of the SY5 × SY6210 cross)	This work

two-hour intervals for 36 hours. Photographs were taken at time points that showed significant alterations.

RNA isolation and analysis of transcriptional pattern: Total yeast RNA was isolated from *rpb4Δ* strains containing different numbers of copies of *RPB7* as described (Vijayraghavan *et al.* 1989). The analysis of the transcriptional pattern was carried out by performing RAP-PCR on total RNA isolated from different yeast strains. The RAP-PCR was carried out using the RAP-PCR kit (Cat. # 200440) from Stratagene according to manufacturer's instructions.

Results

Overexpression of RPB7 rescues the rpb4Δ defect in a dosage-dependent manner

We proposed that in wild-type yeast interaction between Rpb7p and the rest of the polymerase, which is inherently weak, is stabilized by Rpb4p. We have schematically depicted this in figure 1 (top panel). The second panel depicts the corollary that in the *rpb4Δ* mutant Rpb7p tends to dissociate from the rest of the polymerase and this leads to significant reduction in transcription. Thus the conditional phenotypes observed in absence of Rpb4p can be the result of the transcriptional defects due to this unstable interaction (Mckune *et al.* 1993). One can extrapolate from the above that under these conditions if we overexpressed *RPB7* the need for Rpb4p to stabilize the interaction could be eliminated. This possibility is depicted in the third panel. To test this model, we overexpressed *RPB7* to various levels in a haploid *rpb4Δ* strain and tested if temperature sensitivity (ts) and slow-growth phenotypes associated with *rpb4Δ* are rescued. We also expressed *RPB4* from the corresponding vectors as positive control and observed complete rescue of the two phenotypes as expected (data not shown). Figure 2

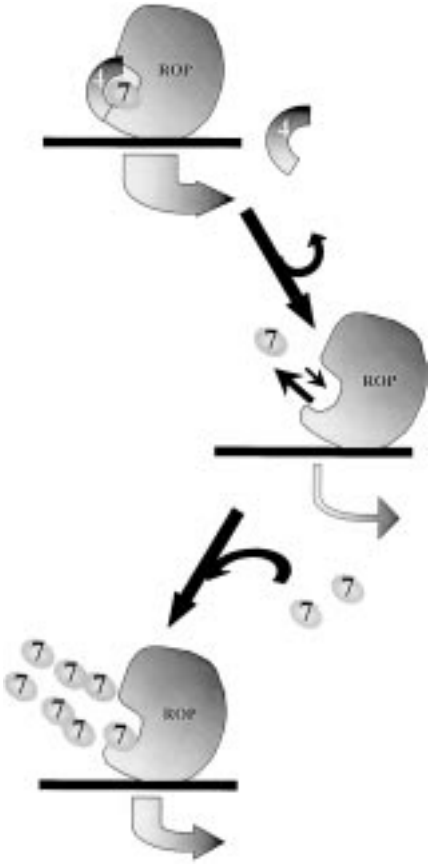
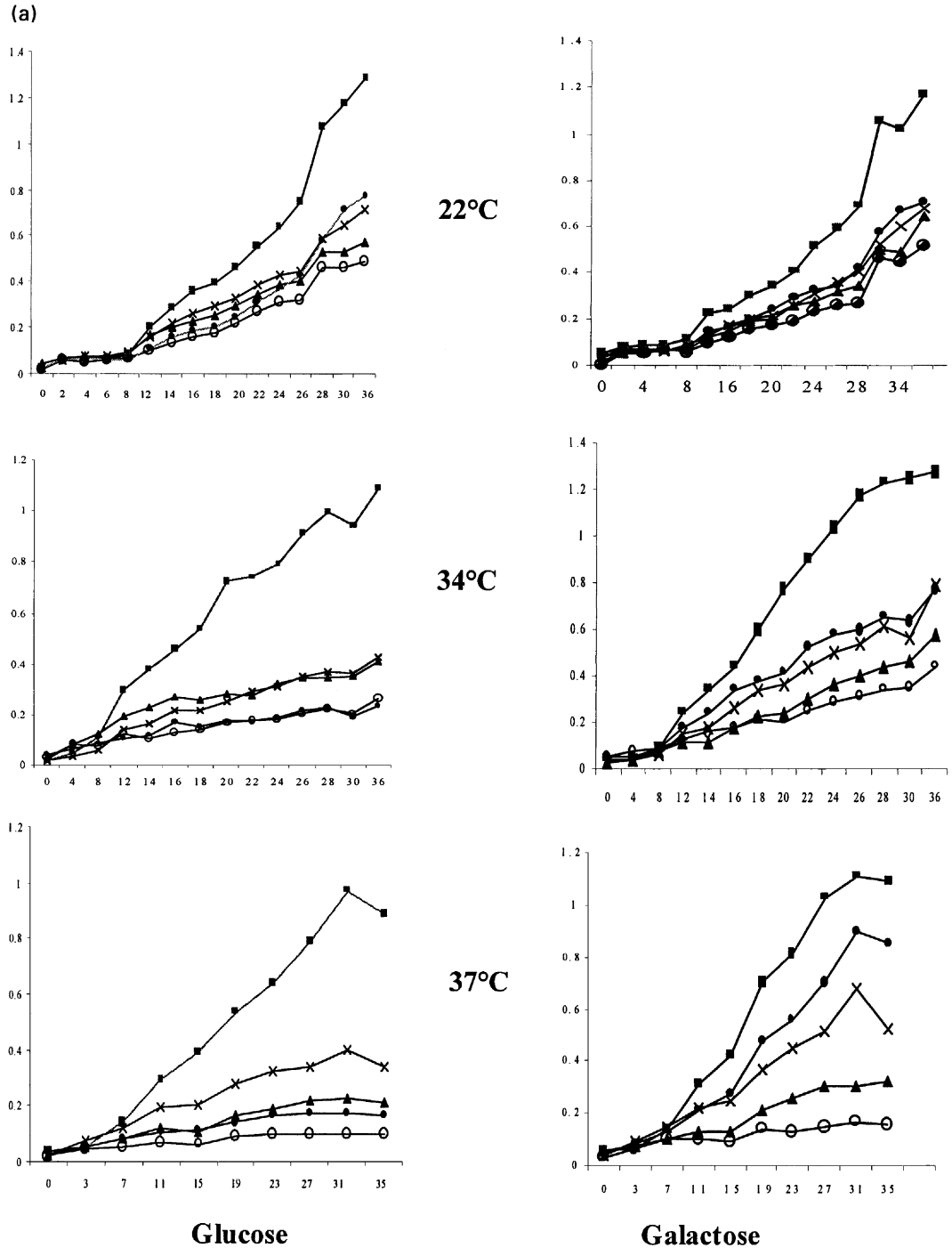


Figure 1. The model representing the Rpb4p-stabilized interaction between Rpb7p and the rest of the Pol II (ROP) and its effect on transcription. The top part shows the situation in the wild type while the second and third parts represent the *rpb4Δ* strain. In all the parts the core polymerase consisting of 12 subunits is shown above the horizontal line, which represents the DNA, while the right-pointing arrow under the horizontal line represents the level of transcription (thickness of arrow). For clarity, the Rpb4/7 subcomplex has been shown much larger than its size in relation to ROP. See text for explanation.


(a)
22°C
34°C
37°C
Glucose
Galactose
(b)
22°
34°
37°
G
D
1
2
3
4
5

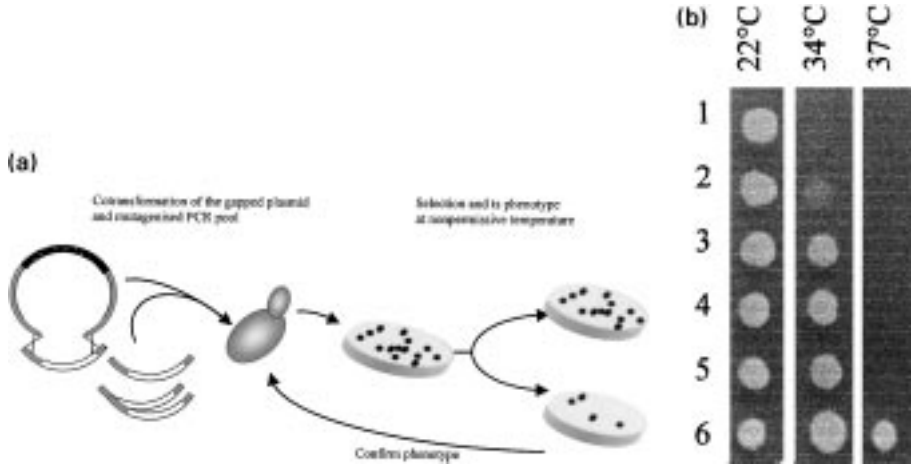


Figure 3. Dominant mutants of *RPB7* allow growth at higher temperature in absence of Rpb4p. (a) Screen for mutants: The *rpb4*Δ strain was transformed with a pool of PCR-mutagenized *RPB7* gene fragment along with gapped plasmid pBP86 containing the 5′ and 3′ untranslated regions of the gene. Only *in vivo* recombination between the PCR product and the gapped plasmid will generate a complete plasmid, which will give rise to transformants. For further details, see the Materials and methods section. (b) Rescue of temperature sensitivity of *rpb4*Δ strain in *RPB7* mutants. The mutants and control strains were spotted on SD media and incubated at the permissive and nonpermissive temperatures. Numbers 1 to 6 are *rpb4*Δ, *rpb4*Δ+ pRA56, *rpb4*Δ+ mut #3, *rpb4*Δ+ mut #22, *rpb4*Δ+ pPS26 and *rpb4*Δ+ pNS114.

shows the rescue of both the slow growth and the temperature sensitivity of *rpb4*Δ cells harbouring various plasmids carrying the *RPB7* open reading frame under its own or *GAL1* promoter control. At a nonpermissive temperature, e.g. 34°C, *RPB7* on a multicopy plasmid (in SY106) appears to rescue both the phenotypes to significant extent. At the higher nonpermissive temperature, 37°C, the difference between the strains overproducing Rpb7p under *GAL1* control (SY107) and the parent *rpb4*Δ strain (SY101) is very pronounced. The growth curves show that in glucose, when *RPB7* expression under *GAL1* promoter is not induced, leaky expression from this promoter allows rescue of temperature sensitivity to some extent (figure 2a). Figure 2b also shows that the *rpb4*Δ strain does not survive at 34°C but the same strain carrying the *RPB7* gene on a 2μ plasmid does and so does the strain carrying *RPB7* under the *GAL1* promoter control in noninducing condition. We tested the levels of transcript corresponding to the *RPB7* open reading frame (ORF) in all the above cases. It was found that as the level of *RPB7* transcript increased, greater extent of rescue of the ts and slow-growth phenotypes was achieved. The observation that *RPB7* overexpression rescues the *rpb4*Δ phenotype in a dose-dependent manner suggests that, at the least, one of the roles of Rpb4p is to stabilize the interaction of Rpb7p with the core polymerase, as proposed in the model above (figure 1).

Dominant mutants of RPB7 eliminate the need for Rpb4p at nonpermissive temperature

On the basis of the above results we proposed that it should be possible to obtain dominant mutants of *RPB7* that are presumably improved in their interaction with RNA Pol II and therefore can dispense with the requirement for Rpb4p even when the mutant *RPB7* is expressed at relatively low levels. As seen above *RPB7* expressed on a single-copy plasmid does not rescue the ts phenotype associated with *rpb4*Δ. We reasoned that if a mutant *RPB7* ORF in the context of its own untranslated regions on the same plasmid could rescue the phenotype, this could be most likely due to better interaction of the mutant Rpb7p with core Pol II, a situation similar to the wild-type gene expressed at higher levels. We achieved this by transforming the *rpb4*Δ strain with a pool of PCR-mutagenized *RPB7* along with a gapped CEN plasmid vector (pBP86) containing the untranslated upstream and downstream regions of *RPB7* (figure 3a).

Figure 2. Temperature sensitivity and slow growth rate of *rpb4*Δ strain is rescued by overexpression of *RPB7*. (a) Growth curves show differences in growth rate of *rpb4*Δ cells with different levels of *RPB7* expression. The *rpb4*Δ strain SY101 (-○-) grows slowly at moderate (22°C) temperature and does not grow at elevated (34°C or 37°C) temperatures in comparison with the *RPB4* strain SY102 (-■-). Overexpression of *RPB7* in the strains SY105 (-▲-), SY106 (-×-) and SY107 (-●-) rescues the *rpb4*Δ slow-growth phenotype. (b) The following cultures were spotted in duplicate on the appropriate synthetic minimal media and incubated at the indicated temperatures. The carbon source in the medium is indicated above each panel (G = galactose, D = glucose). In each panel, numbers 1 to 5 are strains SY102, SY107 (*RPB7* ORF under *GAL1* promoter control), SY106 (*RPB7* gene on a multicopy plasmid), SY105 (*RPB7* gene on a single-copy plasmid), and SY101.

Recombination *in vivo* between the PCR product and the gapped plasmid generates a complete circular plasmid with mutagenized *RPB7* ORF in the context of wild-type upstream and downstream regions on a single-copy plasmid. Out of the 500 transformants that were tested for their ability to grow at the nonpermissive temperature of 34°C about 100 were reproducibly shown to have the expected phenotype. Plasmids from transformants showing the best rescue were recovered and reintroduced into the parent strain. These transformants, which reproducibly showed the expected phenotypes, were considered to harbour the mutants of interest. Figure 3b shows a typical temperature-sensitivity test at this stage in the screen. Two mutant plasmids (numbers 3 and 22) introduced into the *rpb4*Δ strain confer on the *rpb4*Δ cells as good an ability to survive at the nonpermissive temperature of 34°C as the *RPB7* wild-type gene on a multicopy plasmid (pPS26); this is in contrast to the growth ability conferred by the single-copy pRA56 carrying the *RPB7* wild-type gene. The plasmids carrying these mutant *RPB7* were sequenced. This revealed that both the mutants cause an identical, Thr→Ala change at amino acid position 28. We are in the process of confirming the phenotypes of the two mutants by recreating mutations at the specific residue by site-directed mutagenesis.

*Morphology of haploid S. cerevisiae is altered significantly on overexpression of RPB7 in rpb4*Δ *background*

One of the stress-related phenotypes of *S. cerevisiae* that has been characterized in great detail is the pseudohyphal growth exhibited by diploid cells in response to nitrogen starvation. The pseudohyphal growth is typified by cell elongation along with unipolar budding. The buds remain attached to the mother cells forming long columns of cells. Several mutations that enhance the sensing of severity of nitrogen starvation and several components of signal transduction pathways as well as transcriptional regulators have been described (Banuett 1998). Contrary to earlier suggestions that the phenotype is specific to diploids, it was observed that even haploids are able to show similar phenotypes with somewhat elongated cells that remain attached to each other. To distinguish this haploid phenotype the pattern has been referred to as agar-invasive growth (Gimeno *et al.* 1993).

It had been observed earlier that overexpression of *RPB7* and its human homologue in diploid cells predisposed to form pseudohyphae caused a highly exaggerated pseudohyphal phenotype in response to low-nitrogen conditions (Khazak *et al.* 1995). We monitored the morphology of haploid *rpb4*Δ strains overexpressing *RPB7* to various extents (described above). Both *rpb4*Δ and *RPB4* wild type exhibit axial budding pattern with normal yeast cell morphology. Even the *rpb4*Δ derivatives containing the *RPB7* gene on either the CEN or 2μ plasmid or the *RPB7* ORF under *GAL1* promoter (grown under noninducing conditions) showed similar morphology (data not shown). Interestingly, the *rpb4*Δ strain overexpressing *RPB7* under the highly inducible *GAL1* promoter showed the pseudohyphal phenotype (figure 4). The photographs in figure 4 show the morphology of *rpb4*Δ cells carrying either *RPB4* on a CEN plasmid or *RPB7* ORF under *GAL1* promoter control. The cells overexpressing *RPB7* in galactose show markedly elongated cells. One of us (P. P. S.) had previously shown that the wild type (*RPB4*) overexpressing *RPB7* under *GAL1* promoter exhibits normal yeast morphology (Khazak *et al.* 1995). We also expressed *RPB4* in the *rpb4*Δ strain already overexpressing *RPB7* and found that the morphology returns to normal yeast type (data not shown).

*Comparison of transcripts from rpb4*Δ *and RPB4 strains by RAP-PCR*

Since the phenotypes observed in the absence of *RPB4* were so different from those observed in its presence, it was obvious that the two strains should have significant qualitative and/or quantitative differences in their patterns of gene expression. Since this difference is expected to be at the transcriptional level, we used the RAP-PCR technique to compare the transcripts expressed in *rpb4*Δ cells with the transcripts from *RPB4* cells grown at 22°C. The analysis was repeated several times to confirm reproducibility of the pattern observed. One autoradiograph showing a typical pattern is presented in figure 5. The RNAs were analysed using five different arbitrary primers and the products generated were compared. All the five primers used show differences in the transcriptional pattern between the two strains. It was interesting to see that there were products representing many specific transcripts upregulated in either *RPB4* or *rpb4*Δ cells. The observation thus indicates that presence of *RPB4* may regulate the expression of certain genes positively and that of others negatively and affects the overall transcriptional pattern in cells. Analysis of these

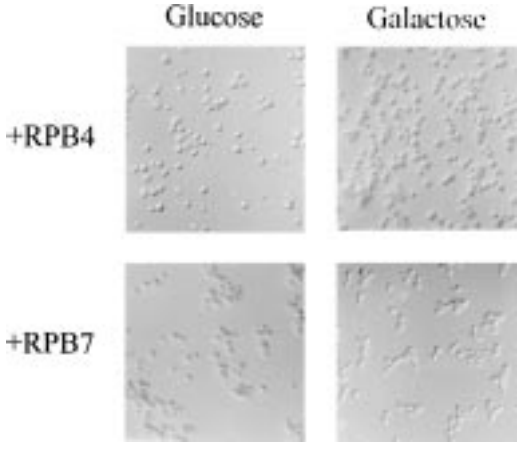


Figure 4. Overexpression of *RPB7* affects cell morphology in *rpb4*Δ haploids. The cells were observed at 400 × using Nomarski optics during growth at 22°C (represented in figure 2a). The sugar present in the medium and the gene introduced on plasmid are indicated. The photographs show the cells at about 22 hours of growth.

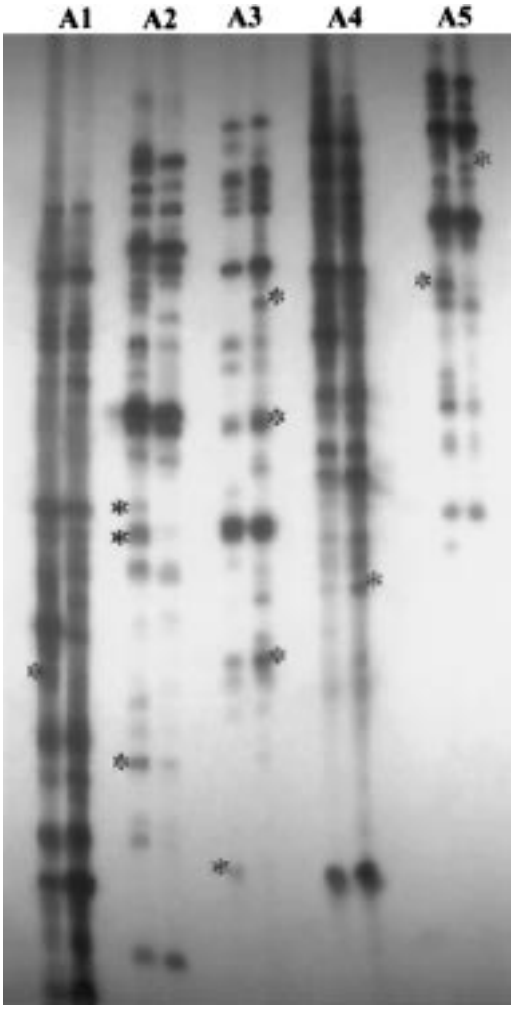


Figure 5. Differences in transcriptional pattern in *rpb4*Δ and *RPB4* cells are evident in RAP-PCR analysis of total RNA. A typical result of RAP-PCR analysis of RNAs from *RPB4* and *rpb4*Δ strains is shown. In each pair of lanes the left lane is *rpb4*Δ and the right lane *RPB4*. The label above each pair (A1 to A5) indicates the arbitrary primer used for RAP-PCR. The bands corresponding to differentially expressed transcripts are marked with an asterisk next to the lane showing increased level of transcript. See Materials and methods for details.

differentially regulated genes is currently in progress. It will enable us to identify the mediators of this regulation.

Discussion

We started our investigations by proposing the hypothesis that one of the two nonessential subunits, Rpb4, of yeast RNA Pol II could play a role in stabilizing the interaction between the essential subunit Rpb7 and the rest of Pol II even under non-stress conditions (Sadhale *et al.* 1998).

Here we have shown by two different approaches, namely (a) overexpression studies and (b) dominant-mutant analyses, that at least one of the roles of Rpb4 is indeed to stabilize the interaction between Rpb7p and the rest of the polymerase. The fact that Rpb7p rescues defects associated with lack of its interacting partner Rpb4p in a dose-dependent manner also supports our model shown in figure 1. Our observations that dominant mutants of *RPB7* rescue temperature sensitivity better than the wild-type subunit, at equivalent levels, corroborate the above results. Although this is not the only explanation one of the testable mechanisms by which these dominant mutants could act is by interacting more strongly with the rest of the polymerase.

It has been reported that Rpb4 has a role in stabilizing the polymerase during stress implying that in the absence of Rpb4p RNA Pol II becomes mechanically unstable under stress conditions (Mckune *et al.* 1993). Overexpression of *RPB7* has been recently shown to rescue certain phenotypes of *rpb4*Δ but the possibility of the role of Rpb4p in stabilizing the interaction between Rpb7p and the rest of the polymerase under moderate conditions has been overlooked (Rosenhech and Choder 1998; Sheffer *et al.* 1999). Our results suggest that even under moderate conditions Rpb7 requires Rpb4 to interact effectively with the rest of the polymerase.

The overall transcription pattern analyses using RAP-PCR indicate that lack of Rpb4p does not lead to a mere ubiquitous lowering of transcription but causes more qualitative changes as well. This implies that there is more to the function of the two proteins Rpb4p and Rpb7p in the subcomplex of Pol II. We suggest that Rpb4p not only plays a role in stabilizing the interaction between Rpb7 and the rest of the polymerase but also a more direct role in mediating interactions with specific transcriptional regulators, which in turn drive expression of specific genes under moderate conditions. We suspect that Rpb7p is involved in some interactions that are masked in the presence of Rpb4p. On the other hand, in combination with Rpb4p, alternative protein–protein interactions are possible. Thus by altering the levels of these two subunit polypeptides functionally, the cell may actually be able to regulate the above interactions and in turn produce different phenotypes.

Our results now allow us to present a different perspective on the role played by the Rpb4/7 subcomplex in affecting stress responses in yeast. On the basis of our results we predict that *rpb4*Δ cells show defects in transcription of several genes owing to altered protein–protein interactions in absence of Rpb4p. The associated stress response defects are probably an indirect effect on the expression of stress-response genes. This suggestion is in agreement with other observations reported in the literature but differs from most interpretations in that it confers on the Rpb4 subunit a more active role in transcription of stress-related genes (Maillet *et al.* 1999). Our results support the notion that the smaller subunits of core RNA polymerase II can actually play a regulatory role rather than a passive role as originally thought (Woychik and Young 1989). We propose that modulation of the relative levels of the two subunits could be one way to regulate gene expression in yeast.

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