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A Growth Rate-Limiting Process in the Last Growth Phase of the Yeast Life Cycle Involves RPB4, a Subunit of RNA Polymerase II

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Saccharomyces cerevisiae **cells, grown on a fermentable carbon source, display two growth phases before they enter the stationary phase: a rapid phase (log phase) followed by a slow phase. It was previously shown that a subunit of the yeast RNA polymerase II, RPB4, positively affects the activity of the enzyme in post-log phases but has little or no effect on its activity in log phase. Here, I show that RPB4 level limits the growth rate during the slow growth phase. Thus, a small increase in RPB4 protein level, in cells carrying multiple copies of the *RPB4* gene, results in an almost twofold increase in the growth rate during this phase. Furthermore, *RPB4* expression is differentially regulated in the two growth phases. During the slow growth phase, a posttranscriptional process which controls the RPB4 level and thus can control growth rate becomes active. These results reveal a complex growth control mechanism, in which the transcriptional apparatus is probably a limiting element, operating in the last stages of the yeast growth.**

Growth control is a central topic in biological studies, partly because its perturbation can lead to cancer. The yeast *Saccharomyces cerevisiae* can serve as an excellent model system to study eukaryotic growth control because of its relative simplicity, advanced genetics, and ease of manipulation. Yeast cells cultured on a fermentable carbon source undergo a major growth control event in the last stages of growth. This event, termed the diauxic shift (11, 17, 19), is a shift from fast logarithmic growth (log phase) to a much slower growth phase. During the diauxic shift, which lasts 0 to several h, depending on the strain and growth conditions, cell metabolism changes from mainly fermentation to aerobic metabolism (11, 19), accompanied by additional morphological and biochemical changes. Following the diauxic shift, cells use the products of fermentation, mainly ethanol, as their carbon source and continue to grow for one to three generations before they stop dividing and enter the stationary phase (for more details, see reference 4 and references therein).

Recently it was shown that one of the key regulatory processes responding to the environmental changes during yeast growth is a global change in the pattern of RNA polymerase II transcription (4). Transcription of most genes that are transcribed in log phase is gradually and temporarily repressed as cells move through post-log phases (4). Some genes are transcriptionally induced during or following the diauxic shift (1, 2, 4, 4a, 7, 15, 16, 18, 19), and the transcription of a small group of genes is changed relatively little or not at all along the growth to stationary phase (4). It was suggested that at least some of these constitutively transcribed genes play a role in maintaining viability in stationary phase (4). Little is known about the posttranscriptional regulatory mechanisms operating during and following the diauxic shift, but a few studies on translational and posttranslational processes during these phases have been reported: (i) protein synthesis in stationary phase is largely arrested (3), correlating with the low level of most mRNAs (4); (ii) studies on BCY1, the regulatory subunit of cyclic AMP-dependent protein kinase, revealed that it is differentially modified in log phase and in post-log phases (20); and (iii) activity of PRB1, a vacuolar protease B, is regulated posttranscriptionally following the diauxic shift. Thus, although *PRB1* mRNA appears immediately following the log phase, protease B activity can be detected only at later stages of the growth cycle (14). The biological role of these posttranscriptional processes is still not clear.

The fourth-largest subunit of yeast RNA polymerase II is encoded by a single-copy *RPB4* gene. RPB4 has recently been shown to be a stress-specific subunit of the yeast RNA polymerase II, necessary for transcription during heat shock and in post-log phases. Cells lacking *RPB4* grow almost indistinguishably from wild-type cells in log phase, but in post-log phases they grow poorly and lose viability more rapidly than wild-type cells (4a).

Here I show that the level of RPB4 is limiting for growth during the slow growth phase. Thus, strains carrying multiple copies of the *RPB4* gene and expressing higher levels of RPB4 grow faster than the wild type during the slow growth phase. Moreover, different regulatory mechanisms control the constitutive expression of *RPB4* in the various growth phases. This additional level of regulation of *RPB4* expression, active following the diauxic shift, is consistent with a possible role of RPB4 as a growth-limiting factor.

Cells carrying multiple copies of the *RPB4* gene grow faster than wild-type cells during the slow post-log growth phase. Recently, it has been shown that cells lacking *RPB4* can grow at a nearly wild-type rate during log phase. However, in post-log phases, *rpb4*$^-$ cells grow more slowly and lose viability more rapidly than wild-type cells (4a). In order to investigate the effect of *RPB4* overexpression on growth, the *RPB4* gene, under the control of its own promoter, was placed on a high-copy-number plasmid (see Fig. 1A for plasmid map) and introduced into a wild-type strain, Z480 (13), by the lithium acetate transformation (10). Figure 1B shows growth curves of Z480 and an isogenic strain carrying

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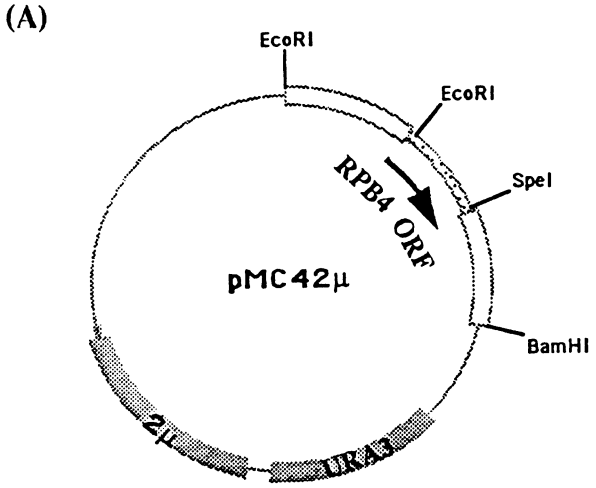

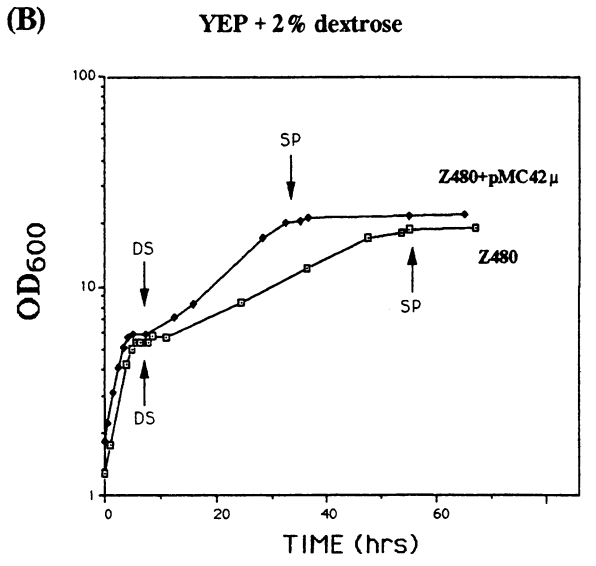

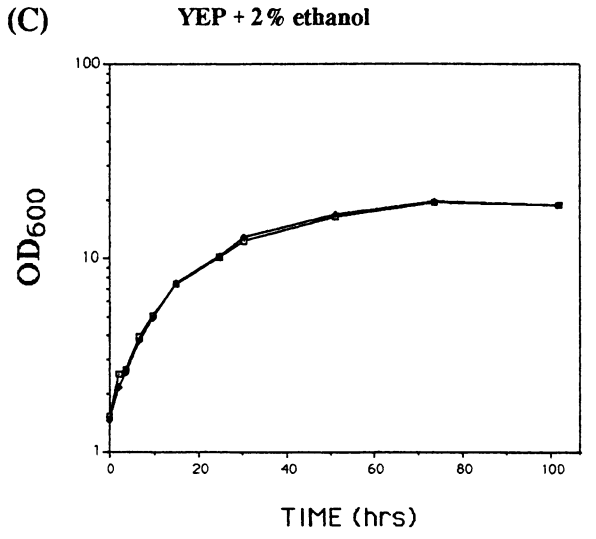

the plasmid Z480+pMC42μ, which were cultured on YPD (2% Bacto Peptone, 1% yeast extract [Difco Laboratories], 2% glucose). Both strains show a typical biphasic growth, divided clearly by the diauxic shift (marked by an arrow). In the first rapid phase (log phase), both isogenic strains grow at comparable rates. Surprisingly, following the diauxic shift, cells carrying the plasmid grow faster than the wild-type cells. Whereas wild-type Z480 cells divide every 21 h, Z480+pMC42μ cells divide every 12 h. This phenomenon was observed in three different strain backgrounds. Interestingly, whereas these three wild-type strains differ in their growth rate during the slow phase (19 to 24 h per generation), their derivatives, carrying pMC42μ, grow at a similar rate (~12 h per generation) in this phase (results not shown). A control plasmid, pMC4Δ12μ, in which *RPB4* was disrupted by replacing the *Eco*RI-*Spe*I fragment of its coding sequence (Fig. 1A) with *HIS3* (by using fragment shuffling between pRP42 [21] and pMC42μ), was constructed. This plasmid is otherwise identical to pMC42μ. Cells carrying this control plasmid grow indistinguishably from the wild type (results not shown).

Following the diauxic shift, cell metabolism changes from mainly fermentation to aerobic metabolism, and cells use the product of fermentation, ethanol, as the primary carbon source (11, 19). It was therefore important to determine whether the effect of multiple copies of *RPB4* on growth, observed following the diauxic shift, is due to the change in the availability of the carbon source. Figure 1C demonstrates that Z480+pMC42μ grows indistinguishably from Z480 on rich medium containing ethanol as the primary carbon source. The comparable growth of the two strains indicates that the change in carbon source alone cannot account for the different post-log growth rates observed when these strains are cultured on YPD. This result suggests that during the diauxic shift that occurs when cells are cultured on a fermentable carbon source, cells undergo a complex differentiation event which cannot be mimicked by changing the carbon source.

Other possible reasons that Z480+pMC42μ grows faster than the wild type during the slow growth phase are the different efficiencies with which both strains use up the nutrients during growth in the log phase and differences in the secretion of metabolic by-products to the medium during growth in the log phase. The indistinguishable growth rates

FIG. 1. Growth curves of cells carrying multiple copies of *RPB4* and wild-type cells carrying a single copy of *RPB4*, inoculated in rich medium containing dextrose or ethanol as the primary carbon source. (A) Plasmid map. pMC42μ was constructed by ligating an ~2.3-kbp *Eco*RI-*Bam*HI fragment from pRP41 (21), containing *RPB4* promoter, coding sequences, and flanking sequences, with the *Eco*RI-*Bam*HI fragment of pRS426 (5). The *RPB4* open reading frame (ORF) is marked by an arrow. Only yeast sequences are specified. (B) Growth on dextrose-containing medium. Wild-type cells, carrying a single copy of *RPB4* (Z480) and cells carrying *RPB4* on a multicopy plasmid (Z480+pMC42μ) were grown in a selective medium and then transferred to 250-ml flasks, each containing 75 ml of YPD medium at an initial OD_{600} of 0.01. Cells were further grown at 27°C with constant shaking at 280 rpm. OD_{600} was measured during growth and plotted as a function of time. Arrows indicate transition points. DS, diauxic shift; SP, stationary phase. Similar results were obtained by counting cells under the microscope instead of measuring OD_{600} (not shown). (C) Growth on ethanol-containing medium. The same strains were treated as for panel B, except that cells were inoculated into YEP plus 2% ethanol instead of YPD.

of these two strains in log phase and the similar optical densities at 600 nm (OD_{600}) at which they enter the diauxic shift argue against these possibilities. To address these possibilities experimentally, equal volumes of cells in the beginning of the slow growth phase were spun and the supernatant of Z480 was added to the pellet of Z480+pMC42μ cells and vice versa. Cells were resuspended and allowed to grow further till stationary phase. It was found that Z480+pMC42μ cells, which continued to grow in the medium previously used by Z480, still grew faster than Z480 cells, which continued to grow in the medium previously used by Z480+pMC42μ (results not shown). This result shows that the differential growth of these two strains is not due to differences in the constituents of the media. These results, combined with the growth characteristic of cells lacking *RPB4* (see above), indicate that changes in RPB4 protein level have the most pronounced effect in the slow growth phase. Furthermore, these combined results indicate that the natural level of RPB4 protein in wild-type cells limits the cell growth rate during the slow growth phase.

During log phase, RPB4 protein level is directly proportional to *RPB4* gene copy number. The effect of *RPB4* copy number on growth raised the possibility that the regulation of its expression is involved in a growth rate control mechanism during the slow growth phase. In order to study how *RPB4* expression is regulated, a careful quantitation of *RPB4* gene copy number, mRNA level, and protein level was carried out in cells carrying pMC42μ and in wild-type cells (carrying a single copy of *RPB4* [21]). Quantitation analyses were done by densitometric analysis of autoradiograms or by using a Betascope 603 blot analyzer (Betagene) to directly determine radioactivity of appropriate bands. However, both of these methods yield linear and reliable results only within a relatively narrow range. For this reason, a series of appropriate dilutions were carried out, when needed, and the final results were calculated on the bases of both the appropriate band intensities and the appropriate dilution factor.

To determine the plasmid copy number per cell, the cellular DNA, extracted as previously described (8), was cut with *Eco*RI. This enzyme releases the same 900-bp fragment from both the chromosomal locus and the plasmid (see plasmid map in Fig. 1A). *Eco*RI-digested DNA, from the wild type and from Z480+pMC42μ, was analyzed by Southern blot hybridization (6) with the same 900-bp *Eco*RI fragment as the probe. The relative copy number was estimated by comparing the band intensity of the wild type with those resulting from a series of dilutions of the Z480+pMC42μ DNA. Figure 2A shows the *RPB4* copy number analysis in log-phase cells. Visual inspection of the results demonstrates that Z480+pMC42μ cells have 20- to 60-fold more copies of the *RPB4* gene than do wild-type cells. Quantitation obtained by the Betascope analyzer indicated that cells carrying the plasmid have 24-fold more *RPB4* genes (Table 1). Since wild-type cells carry one copy of the *RPB4* gene (21), it is concluded that during log phase, Z480+pMC42μ cells carry 23 copies of the *RPB4* gene on the episomal plasmid and 24 copies of the *RPB4* gene altogether.

RPB4 mRNA level per cell was determined by Northern (RNA) blot hybridization as previously described (4), with the *RPB4* coding sequence as the probe (Fig. 1A). *RPB4* mRNA levels were normalized to the total RNA (mainly rRNA and tRNA), which was demonstrated to be a reliable means of normalization during growth to stationary phase, since it is changed relatively little during growth to stationary phase (4) (Table 1, footnote *b*). Application of comparable amounts of RNA was verified by an ethidium bromide

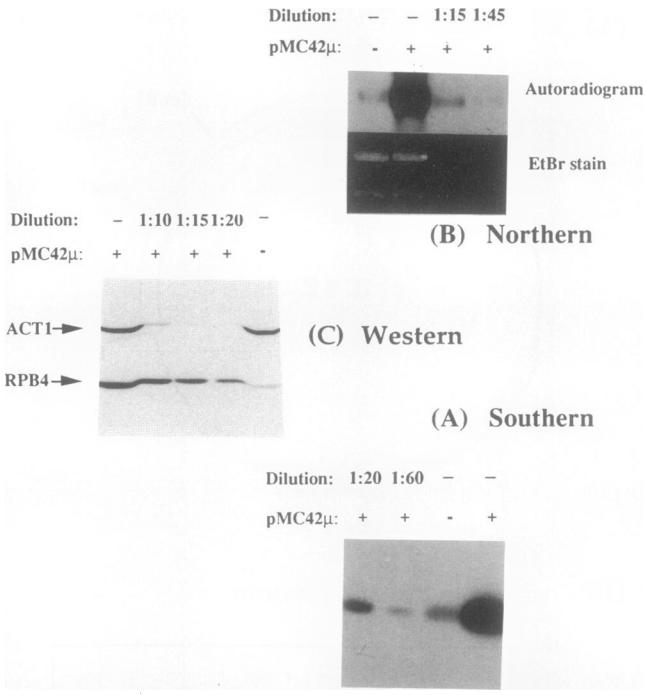

FIG. 2. A direct correlation between *RPB4* copy numbers (A), *RPB4* mRNA levels (B), and RPB4 protein levels (C) during log phase. (A) Relative levels of *RPB4* copy numbers in Z480 and Z480+pMC42μ. Cellular DNA samples were digested with *Eco*RI, and equal amounts of DNA digests, or the same volume of the indicated dilutions, were analyzed by Southern blot hybridization with an *Eco*RI 900-bp fragment (see plasmid map in Fig. 1A) as the probe. (B) Relative levels of *RPB4* mRNA in Z480 and Z480+pMC42μ. Equal amounts of total RNA, or the same volume of the indicated dilutions, were analyzed by Northern blot hybridization (see text), with the *Eco*RI-*Spe*I fragment of the *RPB4* open reading frame (see plasmid map in Fig. 1A) as the probe. EtBr staining of the rRNAs is shown below the autoradiogram. (C) Relative protein levels in Z480 and Z480+pMC42μ. Proteins from equal amounts of cells or equal volumes of the indicated dilutions were analyzed by Western blot. The filter was sequentially reacted with antibodies against the indicated proteins. Affinity-purified anti-RPB4 antibodies were obtained from A. Sentenac (9). Monoclonal anti-actin (mouse) antibodies (clone C4), which recognize the yeast actin (ACT1), were purchased from ICN.

(EtBr) staining of the rRNA (Fig. 2B, EtBr stain). Figure 2B shows the analysis of log-phase mRNA. Quantitation obtained by Betascope analyzer indicated that a Z480+pMC42μ cell has 22-fold more *RPB4* mRNA molecules than does a wild-type cell (Table 1).

RPB4 protein level per cell was obtained by Western (immunoblot) analysis. Whole-cell protein extraction was done essentially as previously described (12). Protein samples (15 to 50 μg per lane) were electrophoresed in 5 to 15% gradient polyacrylamide gel (1.5 mm by 16 cm by 20 cm). Following electrophoresis, proteins were electrotransferred onto a nitrocellulose filter (BA85; Schleicher & Schuell) by using 10 mM CAPS buffer (Sigma) (pH 11)–10% methanol at 0.5 A for 95 min. The filter was stained with Ponsau S to ensure a good transfer and then blocked with 5% lowfat dry milk (Stop & Shop) in TBS-T (10 mM Tris-HCl, [pH 8.0], 150 mM NaCl, 0.05% Tween 20) at room temperature and reacted with the appropriate antibodies in the blocking buffer at room temperature. The enhanced chemiluminescence

TABLE 1. *RPB4* expression is differentially regulated in the two growth phases: quantitative summary of the results presented in Fig. 2 and 3

Strain	Log phase			Slow growth phase			Stationary phase[a]		
	No. of gene copies	mRNAs[b]	Proteins[b]	No. of gene copies	mRNAs[b]	Proteins[b]	No. of gene copies	mRNAs[b]	Proteins[b]
Z480	1	1	1	1	0.45	0.7	1	0.25	0.7
Z480+pMC42μ	24	22	25	24	20	3	24	12	1.3

[a] Beginning of stationary phase.

[b] Levels of mRNAs and proteins are given per cell. In Fig. 2 and 3, mRNA levels are normalized to the total RNA. Because small changes in the total RNA levels per cell are observed when cells progress to the stationary phase, these values were corrected accordingly. The corrections are based on the following estimations of total RNA per cell (Z480 and Z80+pMC42μ alike): 380 fg per cell during log phase, 370 fg per cell undergoes the diauxic shift, 350 per cell towards the end of the slow growth phase, and 320 fg per cell during stationary phase. Values obtained for wild-type cells during log phase are arbitrarily defined as 1.

method (Amersham) was used for detection by the manufacturer's procedure. Figure 2C shows the analysis of log-phase cells. Densitometric analysis of the results shown in Fig. 2C indicated that RPB4 protein level in a log-phase Z480+pMC42μ cell is 25-fold greater than that of a wild-type cell (Table 1).

Results obtained so far demonstrate a direct correlation between *RPB4* copy number, mRNA level, and protein level in log-phase cells.

Differential regulation of RPB4 protein level during growth to stationary phase. Because *RPB4* copy number seems to have a greater effect on growth following the diauxic shift than it has before cells reach this point (Fig. 1B), I investigated the expression of *RPB4* during the slow growth phase and compared it with that in log phase. Southern analysis revealed that the plasmid copy numbers remained comparable in all growth phases (Fig. 3A; Table 1). This phenomenon was not unique to *RPB4* sequence or to this particular

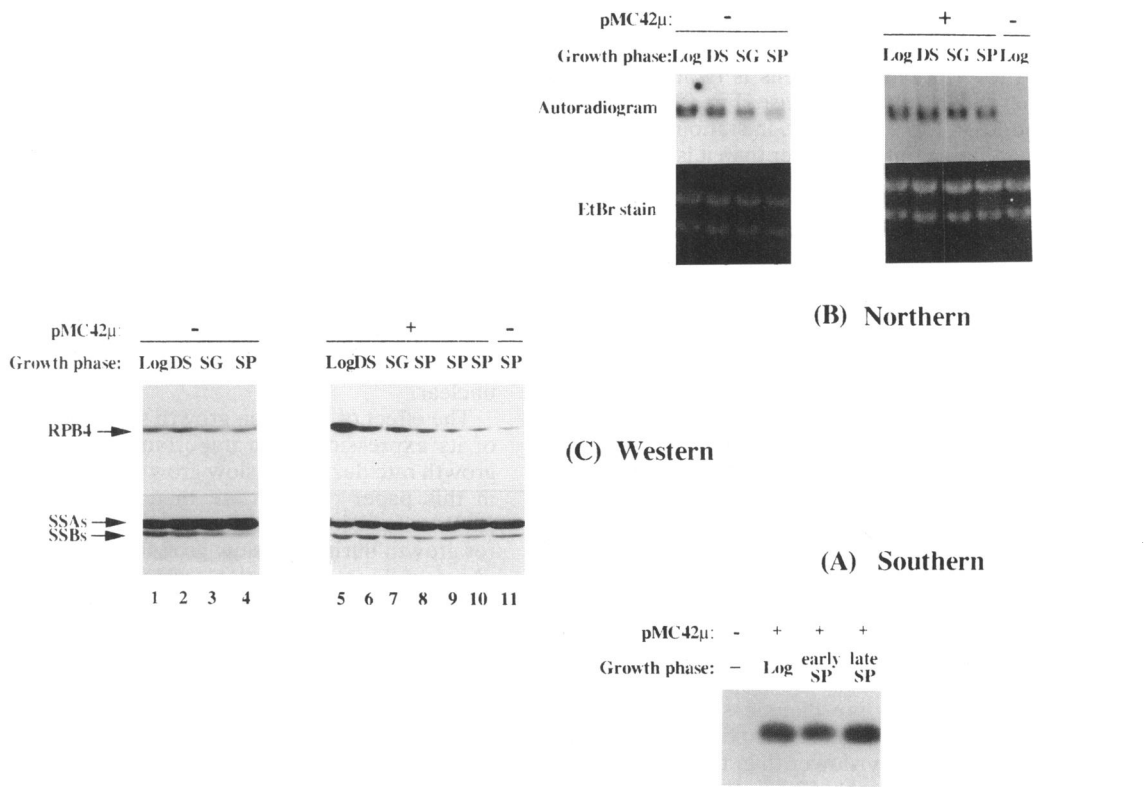

FIG. 3. *RPB4* copy number (A), mRNA levels (B), and protein levels (C) as a function of growth to stationary phase. (A) Relative levels of *RPB4* copy number in Z480+pMC42μ as a function of growth phase. Copy number was determined as for Fig. 2A. Lane –, wild-type control lane (growth phase is irrelevant). early SP, beginning of stationary phase; late SP, 2 weeks after cells enter the stationary phase. (B) Relative levels of *RPB4* mRNA in Z480 and in Z480+pMC42μ as a function of growth to stationary phase. *RPB4* mRNA from cells at the indicated growth phase was analyzed as for Fig. 2B. Log, log phase; DS, diauxic shift; SG, slow growth phase. SP, 4 days in stationary phase. The autoradiogram shown in the left panel was derived from ~20-fold-longer exposure than that in the right panel. EtBr staining of rRNA is shown below autoradiograms. (C) Relative protein levels in Z480 and in Z480+pMC42μ as a function of growth to stationary phase. Proteins from equal amounts of cells at the indicated growth phase were analyzed as for Fig. 2C. Growth phases are as designated for panel B. Lane 4, 4 days in stationary phase; lane 8, beginning of stationary phase; lane 9, 4 days in stationary phase; lane 10, 2 weeks in stationary phase; lane 11, beginning of stationary phase. SSAs (containing SSA proteins, which comigrate) and SSBs (containing SSB proteins, which comigrate) are members of the HSP70 family.

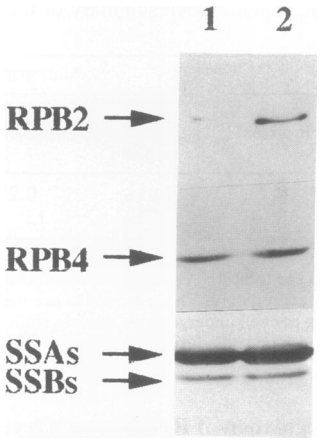

FIG. 4. Stationary-phase level of RPB2 protein in wild-type cells and in cells carrying multiple copies of *RPB2*. Z480 (lane 1) and Z480 carrying *RPB2* on a multicopy plasmid, pRY2161 (see text) (lane 2), were grown to the beginning of stationary phase under the same conditions as described for Fig. 1 to 3. Proteins from equal amounts of cells were subjected to Western analysis as for Fig. 2C. The filter was sequentially reacted with antibodies against the indicated proteins. Affinity-purified anti-RPB2 antibodies were obtained from A. Sentenac (9).

vector, as it was observed in a different plasmid carrying the 2μm sequence (results not shown).

RPB4 mRNA level in wild-type cells is twofold lower in slow growth phase than it is in log phase (Fig. 3B, left panel, cf. lanes SG and Log; Table 1). Later, in stationary phase, it is reduced further, being fourfold lower than it is in log-phase cells (Fig. 3B, left panel, cf. lanes SP and Log; Table 1). In cells carrying 24 copies of *RPB4*, *RPB4* mRNA level is high in all growth phases and is reduced up to approximately twofold in post-log phases (Fig. 3B, right panel; Table 1).

RPB4 protein level per wild-type cell is almost comparable in all growth phases (Fig. 3C, lanes 1 to 4). Its level is reduced by 30% during the diauxic shift and remains at this value at later stages of the life cycle (Table 1). In a striking contrast to the wild-type cells, RPB4 protein level in Z480+pMC42μ is dramatically different in the different growth phases. RPB4 protein level is high in the log phase (Fig. 2C and 3C, lanes 5), but during the diauxic shift it is reduced sixfold (Fig. 3C, lane 6). In the slow growth phase it is only approximately fourfold higher than it is in wild-type cells (Fig. 3C, lane 7; Table 1). In stationary phase, RPB4 protein level is further reduced to a nearly wild-type level (Fig. 3C, lanes 8 to 10; Table 1). This phenomenon was observed in three different genetic backgrounds (results not shown). The pattern of HSP70 proteins serves as a marker for the growth phase. Whereas SSBs band intensity is gradually reduced following the diauxic shift, SSAs band intensity is increased following the diauxic shift. In some gels, a band migrating slightly slower than the bulk SSA can be observed. This band is probably due to SSA3, which is induced following the diauxic shift (18).

As a control experiment, the gene encoding the second-largest subunit of RNA polymerase II, *RPB2*, was placed on a multicopy plasmid, pRY2161, and introduced in Z480 cells. pRY2161 was constructed by ligating an *Aat*II-*Bam*HI fragment containing the *RPB2* gene with an *Aat*II-*Bam*HI fragment containing the 2μm replication origin and *URA3* from YEp24 (a gift of M. Nonet and R. A. Young). To test whether RPB2 can be overproduced during stationary phase by placing its gene on a multicopy plasmid, I analyzed its protein level in Z480+pRY2161 compared with its level in Z480. Figure 4 shows that RPB2 protein level in stationary-phase Z480+pRY2161 cells is indeed substantially higher than it is in wild-type cells at the same phase. The pattern of HSP70 proteins shown in Fig. 4 is typical for stationary-phase cells (compare the HSP70 pattern in Fig. 3C, lanes 8 to 11, with that of Fig. 4). This differential stationary-phase level of RPB2 protein demonstrates that other genes can be overexpressed in stationary-phase cells by the same strategy used for *RPB4*.

RPB4 has been shown recently to be a stress-specific subunit of RNA polymerase II, being assembled more efficiently with RNA polymerase II following the diauxic shift. Cells lacking *RPB4* can grow almost indistinguishably from wild-type cells under optimal growth conditions, but in post-log phases they grow poorly, their RNA polymerase II transcribes poorly, and they lose viability rapidly (4a). Here it is shown that cells carrying multiple copies of the *RPB4* gene grow similarly to the wild type during log phase but faster than the wild type during the slow growth phase. Strikingly, a fourfold increase in RPB4 protein level has an almost twofold effect on the growth rate during the slow growth phase. Therefore, I suggest that RNA polymerase II activity limits cell growth rate during the slow growth phase and that RPB4 has evolved to be a mediator in this process. The faster growth rate of cells overproducing RPB4 demonstrates that, during the slow growth phase, the medium can support faster growth but wild-type yeasts do not fully utilize the medium potential. Why, then, has *RPB4* been evolved as a single-copy gene and why is *RPB4* expression lower than needed for faster growth in the slow growth phase? One possibility is that, in nature, there are selectable advantages for growing slowly in the post-log growth phase. Cells undergo only one to three cell divisions in the slow growth phase, which may be important for the gradual physiological and biochemical modifications necessary for maintaining viability in the stationary phase (e.g., accumulation of storage carbohydrates). However, Z480+pMC42μ cells remain viable during at least 2 weeks in stationary phase (results not shown). Therefore, the answer to this question remains unclear.

The effect of *RPB4* on growth suggests that the regulation of its expression is an important parameter in controlling growth rate during the slow growth phase. Results presented in this paper demonstrate that, indeed, the regulation of *RPB4* expression reflects its potential role as a limiting factor for growth during the slow growth phase. During log growth phase, a direct correlation exists between *RPB4* gene copy number, mRNA level, and protein level. This indicates that, in this physiological state, *RPB4* expression is determined primarily by the transcriptional efficiency of its single-copy gene. This mode of expression is characteristic of many yeast genes. In post-log phases, *RPB4* expression is regulated differently from how it is in log phase. Whereas there is still an overall good correlation between *RPB4* gene copy number and its mRNA level, the protein level is little affected by the mRNA level. These results indicate that, following the log phase, a posttranscriptional regulatory mechanism is active, controlling RPB4 protein level. Unlike most genes that are transcriptionally repressed in post-log phases, with mRNA levels in stationary phases that are >10-fold lower than those in log phase (4), *RPB4* mRNA level remains relatively high even after 2 weeks in stationary phase. This suggests that a high level of mRNA is an important determinant of *RPB4* expression in post-log

phases. However, despite its relatively high level, *RPB4* mRNA level is slowly but significantly reduced following the diauxic shift (Table 1). Nevertheless, RPB4 protein level remains nearly constant. Taken together, these results indicate that the RPB4 protein level in post-log phases is not greatly affected by the level of its transcript. Results presented in Table 1 (under the heading Stationary phase) can best illustrate this notion; whereas the *RPB4* mRNA level in Z480+pMC42μ is 36-fold higher than that in the wild type, the ratio between the protein levels in the two strains is only 1.85. Since a relatively small increase in RPB4 protein level has a significant effect on cell growth rate, I propose that the mechanism that controls RPB4 protein level in the post-log phases plays a role in determining the growth rate during the slow growth phase. Whether the control of RPB4 protein level is at the level of translation or protein turnover remains to be determined.

The different modes of regulation of *RPB4* expression demonstrate the changes in gene expression that occur following the log phase. Within a relatively short period, during the diauxic shift, the regulation of *RPB4* expression is modified. Transcriptional induction of several genes during and following the diauxic shift has been reported previously (1, 2, 4, 4a, 7, 15, 16, 18, 19). The expression of *RPB4*, studied here, reveals a novel type of regulation in which a constitutive expression of a gene is differently regulated in the two growth phases of the yeast life cycle.

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