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Maf1 Is Involved in Coupling Carbon Metabolism to RNA Polymerase III Transcription▽†

Małgorzata Cieśla,[1] Joanna Towpik,[1] Damian Graczyk,[1] Danuta Oficjalska-Pham,[1,2]‡ Olivier Harismendy,[2]§ Audrey Suleau,[2] Karol Balicki,[1] Christine Conesa,[2] Olivier Lefebvre,[2] and Magdalena Boguta[1]*

Department of Genetics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland,[1] and CEA, iBiTecS, Gif Sur Yvette F-91191, France[2]

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RNA polymerase III (Pol III) produces essential components of the biosynthetic machinery, and therefore its activity is tightly coupled with cell growth and metabolism. In the yeast *Saccharomyces cerevisiae*, Maf1 is the only known global and direct Pol III transcription repressor which mediates numerous stress signals. Here we demonstrate that transcription regulation by Maf1 is not limited to stress but is important for the switch between fermentation and respiration. Under respiratory conditions, Maf1 is activated by dephosphorylation and imported into the nucleus. The transition from a nonfermentable carbon source to that of glucose induces Maf1 phosphorylation and its relocation to the cytoplasm. The absence of Maf1-mediated control of tRNA synthesis impairs cell viability in nonfermentable carbon sources. The respiratory phenotype of *maf1*-Δ allowed genetic suppression studies to dissect the mechanism of Maf1 action on the Pol III transcription apparatus. Moreover, in cells grown in a nonfermentable carbon source, Maf1 regulates the levels of different tRNAs to various extents. The differences in regulation may contribute to the physiological role of Maf1.

In its natural environment, the budding yeast *Saccharomyces cerevisiae* is confronted with variations in sugar availability. Fermentation is the pathway by which cells obtain energy in the presence of glucose, even under aerobic conditions. After consuming all the available glucose, yeast cells use ethanol or another nonfermentable carbon source and get energy from mitochondrial respiration. Gradual glucose exhaustion or abrupt withdrawal of fermentable carbon source results in widespread changes in gene expression at both the transcriptional and the translational levels (6, 16, 31).

Yeast growth is controlled by two global nutrient-sensing signal transduction cascades, the RAS and the target of rapamycin (TOR) cascades. In response to glucose, the RAS cascade stimulates the synthesis of cyclic AMP (cAMP), which subsequently activates protein kinase A (PKA), the main player in the pathway. Multiple PKA substrates are known to support growth in glucose and to negatively regulate the physiology of the stationary phase and nutrient starvation and stress (37). The central components of the TOR pathway consist of two TOR kinases and a phosphate switch composed of protein phosphatase 2A and its inhibitor Tap42 (9). TOR signaling controls all three RNA polymerase systems involved in ribosome biogenesis (22, 39, 42). Polymerase II (Pol II)-directed transcription of ribosomal protein genes is regulated by the proteins Fhl1, Ifh1, and Crf1; Fhl1 is controlled by the TOR and Crf1 by the RAS pathway (21, 34). The mode of coordination of the Pol II-dependent transcription of ribosomal protein genes with the activity of Pol I and Pol III is currently unknown. A recent study suggested that Pol I activity is a key factor for this coordination (17).

Pol III transcription is controlled by Maf1, a general negative regulator conserved among organisms from yeast to humans (12, 27, 28). Maf1 of *S. cerevisiae* specifically inhibits Pol III transcription (25), whereas in human glioblastoma cells, Maf1 is a negative regulator of both Pol I-dependent and Pol III-dependent transcription (14). In yeast, Pol III is repressed in response to diverse stresses, such as nutrient deprivation, rapamycin treatment, secretory defects, or DNA damage, and all these signals are mediated by Maf1 (7, 19, 40). Maf1 is activated by protein phosphatase 2A, a component of the TOR pathway. In response to rapamycin, dephosphorylated Maf1 is transferred into the nucleus and acts as a Pol III transcriptional "brake" (25, 30). Independent studies have identified yeast Maf1 as an in vitro substrate of PKA kinase (3, 23). Maf1-mediated repression of Pol III transcription by rapamycin was decreased by unregulated, high PKA activity (23). It was postulated that PKA inhibited nuclear import of Maf1 following rapamycin stress (23, 43). This indicated that besides its function in TOR signaling, Maf1 also plays a role in transmitting signals via the RAS pathway. However, the mechanism of Pol III transcription repression is more complex; nuclear localization of dephosphorylated Maf1 is not sufficient, and an additional, so-far-unknown step is required for Maf1 activity in the nucleus (23).

Since Maf1 is the only Pol III repressor known in yeast, it was of interest to establish its role under physiological condi-

* Corresponding author. Mailing address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland. Phone: 4822 592 1312. Fax: 4822 658 4636. E-mail: magda@ibb.waw.pl.

‡ Present address: Unit of Epigenetic Regulation, Avenir INSERM–FRE2850 CNRS, Fernbach Bldg., Institut Pasteur, 25 rue du Dr-Roux, 75724 Paris CEDEX 15, France.

§ Present address: Department of Neurobiology, Scripps Research Institute, 10550 North Torrey Pines Road, San Diego, CA 92037.

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tions that require transcription inhibition and that are not generally considered as a stress. Here we show that transcription regulation by Maf1 is important for some physiological changes between two types of carbon sources. The control of tRNA synthesis mediated by Maf1 becomes essential when yeast cells adapt to respiration. We show that Maf1 phosphorylation and intracellular distribution differ between cells growing in medium with glucose and cells growing in a nonfermentable carbon source but that these differences are PKA independent. Moreover, in cells grown in a nonfermentable carbon source, Maf1 regulates the levels of different tRNAs to various extents. The respiratory phenotype of the *maf1*-Δ mutant could be suppressed by several mutations in the Pol III apparatus, but apparently decreased transcription was not sufficient for this suppression. Presumably, in the absence of Maf1, a defective Pol III may undergo some unspecified rearrangement to become more transcriptionally active.

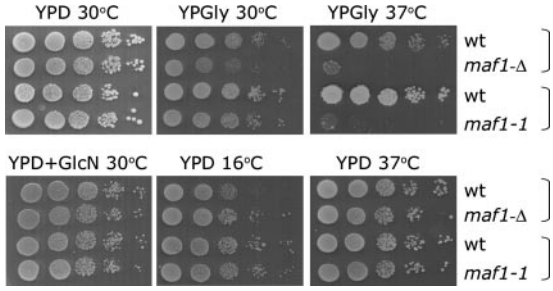

FIG. 1. Growth defect of *maf1* mutants grown on media with a nonfermentable carbon source. Tenfold serial dilutions of cells were plated on YPD (rich glucose medium), YPGly (rich glycerol medium), or YPD medium supplemented with glucosamine hydrochloride (GlcN, 10 mg/ml) and incubated at the indicated temperatures. The control wild type (wt) for *maf1*-Δ is YPH500, whereas the control for *maf1-1* is T8-1D.

MATERIALS AND METHODS

Strains and plasmids. Yeast strains used in this study included *maf1*-Δ, a derivative of YPH500 (*MAT*α *ade2-101 his3*-Δ*200 leu2*-Δ*1 lys2-801 trp1*-Δ*63 ura3-52*), and the *maf1-1* mutant strain MT6-7, isogenic to T8-1D (*MAT*α *SUP11 mod5-1 ade2-1 ura3-1 lys2-1 leu2-3,112 his4-519*) (25, 27). Dominant allele $RAS2^{Val19}$ was constructed from YPH500 by introducing the YCp50/$RAS2^{Val19}$ plasmid, kindly provided by J. Thevelein. Under our experimental conditions, over 76% of cells saved the YCp50/$RAS2^{Val19}$ plasmid. The A364A strain (*MAT***a** *ade1 ade2 lys2-1 his7 tyr1 gal1 ura1*) and the isogenic *cdc25-1* mutant were obtained from the ATCC collection of yeast strains (Berkeley, CA). *rpc128-1007*, the second-site suppressor of *maf1*-Δ, was isolated from the MJ9-2B (*MAT*α *maf1*-Δ *SUP11 ade2-1 ura3-1 lys2-1 leu2-3,112 his3*) strain background. A cross to the wild-type strain and subsequent meiosis resulted in MJ15-9C (*MAT***a** *rpc128-1007 SUP11 ade2-1 ura3-1 lys2-1 leu2-3,112 his3*).

Pol III mutants *rpc31-236* (38), *rpc25-S100P* (45), *rpc160-112* (8, 33), *rpc160-750* (33), *rpc11*-Sp (4), and YMLF2 with regulated levels of the C17 subunit (11) were obtained from the Service de Biologie Intégrative et Génétique Moléculaire, CEA/Saclay (France). To delete *MAF1*, a kanamycin cassette surrounded on each side by ~250 bp of *MAF1*-flanking regions was synthesized by PCR, using specific primers and genomic DNA of YI3945 (*maf1*::*kanX*; Euroscarf). Each single mutant was transformed with the 2,079-bp DNA product, and transformants were selected on yeast extract-peptone-dextrose (YPD) medium supplemented with G418 sulfate (Geneticin). In each case, the replacement of *MAF1* with the kanamycin cassette was confirmed by PCR.

Preparation of RNA and real-time PCR quantification. Cultures (10 ml) were grown in yeast extract-peptone-glycerol (YPGly) medium at 30°C to log phase (A_{600} of about 0.8) and then shifted to the restrictive temperature (37°C) for 2.5 h. The cells were harvested by centrifugation and resuspended in 50 mM Na acetate, pH 5.3, 10 mM EDTA. Total RNA was isolated by heating and freezing the cells in the presence of sodium dodecyl sulfate (SDS) and phenol as described previously (35). RNA was treated with DNase I (Fermentas). First-strand cDNA for each sample was synthesized by reverse transcription of total RNA, using RevertAid H minus Moloney murine leukemia virus reverse transcriptase (Fermentas) according to the manufacturer's instructions. Real-time PCR was performed using a LightCycler model 1.5 instrument (Roche Molecular Biochemicals) according to the manufacturer's instructions. Reaction mixtures (10 μl, final volume) were assembled with the following components: 4.8 μl H_2O, 1.2 μl 4 mM $MgCl_2$, 1 μl cDNA, 1 μl 0.5 μM of each primer, and 1 μl of 10× LightCycler FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals). The 35S ribosomal DNA (rDNA), amplified with the primers TCGACC CTTTGGAAGAGATG and CTCCGGAATCGAACCCTTAT, served as the reference gene. Primers used to amplify tRNA genes (tDNA) were as follows: to amplify $tDNA^{met}$, GCTTCAGTAGCTCAGTAGGAA and TGCTCCAGGGG AGGTTC; to amplify $tDNA^{Leu}$, GGTTGTTTGGCCGAGCG and TGGTTGC TAAGAGATTCGAACTC; and to amplify $tDNA^{Gly}$, GGGCGGTTAGTGTA GTGGTT and TGAGCGGTACGAGAATCGAA. All sets of reactions were conducted in triplicate, and each set included a nontemplate control. The specificity of individual real-time PCR products was assessed by melting-curve analysis carried out immediately after PCR completion. The fit-point method, using LightCycler software version 4.05 (Roche Molecular Biochemicals), was applied for crossing-point determination. Relative tRNA expression levels were calculated using the Pfaffl model and the relative expression software tool (26).

Microarray hybridization. Total RNA (20 μg) supplemented with 200 pg or 20 pg of each control RNA (CAB, RCA, and RbCl from Stratagene) was preincubated for 10 min at 70°C with 1 μl of the primer mixture (2 pM for each specific primer) and 2 μg of poly(dT) oligonucleotide and completed to a final volume of 15.4 μl with RNase-free water. The specific primers were designed to hybridize to the 3′ end of all mature tRNAs analyzed. After 5 min on ice, 14.6 μl of reverse transcription mixture was added with amino-allyl dUTP as previously described (10), and the reaction mixture was incubated for 2 h at 42°C. Then, RNA was digested by adding 1.6 U RNase A and 2 U RNase H over 30 min at 37°C. After inactivation of the enzymes for 15 min at 65°C, amino-allylic cDNAs were purified in water with a YM30 ultrafiltration device (Microcon-Amicon). Subsequent labeling with Cy3 and Cy5 dyes (Amersham) and purification were performed as already described (10).

Other methods. Northern analysis, protein extraction, and immunoblotting used in this study are described in the supplemental material.

RESULTS

Upregulation of yeast RNA Pol III activity results in a growth defect in a nonfermentable carbon source. Despite its role as a global regulator of Pol III transcription, Maf1 is not essential in yeast. Inactivation of the *MAF1* gene (*maf1*-Δ) results in deregulated Pol III activity regardless of the environmental conditions (40). As previously reported, the absence of functional Maf1 in the *maf1*-Δ or *maf1-1* mutant results in elevated tRNA levels under some growth circumstances (27). This observation raises the question of whether the mutant cells can tolerate such an excess of tRNA and other Pol III transcripts under unfavorable growth conditions. One could expect the detrimental effect of high tRNA levels under poor growth conditions. However, at a low temperature (16°C) or in the presence of glucosamine, which inhibits glucose metabolism, the cell growth rate of the *maf1*-Δ and *maf1-1* mutants was indistinguishable from that of the respective wild-type isogenic controls (Fig. 1). In contrast, for cells grown in medium with a nonfermentable carbon source (YPGly medium, at 30°C), growth inhibition (compared to growth in medium with a fermentable source) was clearly stronger in cells lacking functional Maf1. This observation pointed to a defect in mitochondrial function, the most common cause of which is the degradation of mitochondrial DNA (mtDNA), resulting in the formation of petite colonies known as [*rho*$^-$]. It is known that inactivation of multiple nuclear genes in yeast leads to [*rho*$^-$]

TABLE 1. Inactivation of Maf1 does not affect [*rho*$^-$] induction[a]

Strain	% ± SD of [*rho*$^-$] formation
wt (T8-1D)	5.25 ± 2.15
maf1-1	4.35 ± 0.95
wt (YPH500)	6.50 ± 2.90
maf1-Δ	5.75 ± 2.33

[a] Over 100 single colonies were crossed with a [*rho*^{0}] strain, and diploids were selected and tested for respiratory competence. The experiment was done in triplicate. The control wild-type (wt) strain for *maf1*-Δ is YPH500, whereas the control for *maf1-1* is T8-1D. Values for [*rho*$^-$] formation are shown as percentages ± standard deviations (SD).

formation, often by an indirect mechanism. The frequency of [*rho*$^-$] formation, however, was unaffected in *maf1* mutant cells (Table 1). The *maf1*-Δ as well as the *maf1-1* cells stopped growing at 37°C on YPGly medium, while they grew on a fermentable carbon source, YPD medium. The growth deficiency of the *maf1* mutants at 37°C was also observed for media with other nonfermentable carbon sources, like ethanol or lactate (data not shown). This phenotype was not due to the loss of mitochondrial DNA (typical for the [*rho*$^-$] mutants) because cell growth could be restored by transferring the plates from 37°C to 30°C (data not shown).

We monitored the effect of growth inhibition in a nonfermentable carbon source on Pol III transcription activity. Total RNA was isolated from yeast grown in glucose or glycerol medium to exponential phase, followed with a shift to a nonpermissive temperature for 2.5 h. RNAs were resolved by electrophoresis in agarose gel and stained with ethidium bromide. To estimate the quantities of tRNA and rRNA, total RNA isolated from equal amounts of cells (0.125 optical density [OD] unit) was loaded onto each lane of the gel. In comparison to growth by fermentation in glucose, the glycerol-based respiratory growth resulted in a dramatic decrease in 28S and 18S rRNA levels, but this decrease was the same as that in the wild-type cells and the *maf1*-Δ mutant (Fig. 2A). Clearly, the rRNA levels were not affected in the *maf1*-Δ cells. In contrast, tRNA levels were decreased in the wild-type but not in the *maf1*-Δ cells grown in a nonfermentable carbon source. For a more precise quantification, RNAs isolated from equal amounts of cells (0.25 OD unit) from the *maf1*-Δ and the parental strains grown either in glucose or in glycerol medium with a shift to the nonpermissive temperature were resolved by urea-polyacrylamide gel electrophoresis (PAGE) and analyzed by Northern blotting with probes specific to individual tRNAs (Fig. 2B). In the control strain grown in glycerol, the $tRNA^{Leu}$ level decreased by 33%, but the $tRNA^{Gly}$ level did not change. Cells with inactive Maf1 grown in glycerol medium with a shift to 37°C had markedly higher levels of each tested tRNA, although this increase was different for individual tRNA species. Less pronounced differences in tRNA expression were observed for the *maf1*-Δ cultures grown in glycerol medium without a shift to the nonpermissive temperature (results not shown). The same cells grown in glucose exhibited only minor or no increase in tRNA levels compared to that of the wild type. As observed previously (27), the 5S rRNA level was not significantly affected by the absence of Maf1. U3 RNA, synthesized by Pol II, may serve as an internal control since its level was not changed in the *maf1*-Δ cells.

Thus, we conclude that under respiratory conditions, the *maf1*-Δ mutant accumulates relatively high levels of tRNA which may be toxic to the cells at high temperatures, especially as the deregulation results in unbalanced levels of different Pol III transcripts.

Maf1 regulation is increased in a nonfermentable carbon source, but the effect varies among different tRNAs. To quantify the Maf1-directed repression, we used a Pol III-specific microarray (unpublished data) harboring all the different tRNA genes (one for each of the 52 different tRNA families described in the *Saccharomyces* Genome Database) as well as all the other genes transcribed by Pol III. Total RNA was isolated from yeast cells grown in glucose or glycerol medium to exponential phase, followed with a shift to the nonpermissive temperature for 2.5 h. Because of the small number of

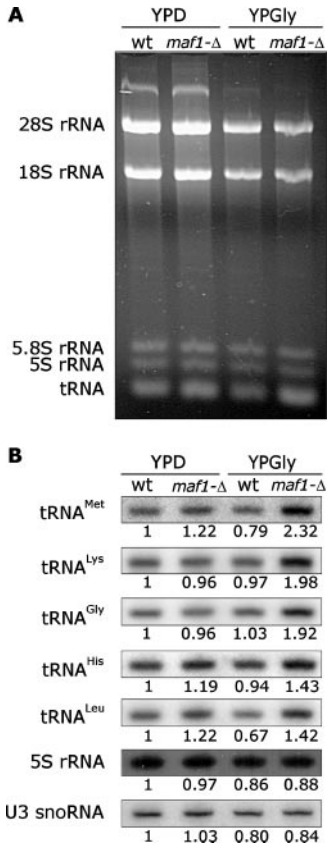

FIG. 2. Inactivation of *MAF1* leads to tRNA accumulation that is increased when cells grow on nonfermentable carbon sources. Total RNA was isolated from the *maf1*-Δ and wild-type (wt) control strain (YPH500) cells grown in glucose medium (YPD) or glycerol medium (YPGly) after a shift to a nonpermissive temperature (2.5 h at 37°C). (A) RNA from cells at OD 0.125 were separated on 2.8% agarose gels and stained with ethidium bromide. (B) Northern analysis. RNA from 0.25 OD unit of cells were separated on 8 M urea-8% polyacrylamide gels, followed by hybridization using labeled oligonucleotide probes complementary to various tRNAs, 5S rRNA, and U3 snoRNA (described in the supplemental material). Bands were quantified as described in Materials and Methods. In order to determine relative levels, the amount of each RNA present in wt cells grown in YPD medium was set to 1.

probes coming from the same functional family (i.e., Pol III transcripts), we assisted the normalization by using control probes (not shown) corresponding to three exogenous gene products, CAB, RCA, and RbCl, from *Arabidopsis thaliana* (spike RNA; Stratagene). Since the RNA yield per cell was different under glucose and glycerol growth conditions (Fig. 2A), all our data were normalized with the *SNR7* gene (encoding U5 RNA transcribed by RNA Pol II) as the reference gene. The gene arrays in Fig. 3A (and Table S1 in the supplemental material) display normalized ratios for each gene according to the red-green color scale.

Class III gene expression levels were mostly similar in the wild-type and the *maf1*-Δ strains grown in YPD medium (Fig. 3A, lane 1, average of the ratios of 0.92). Expression levels were consistent with previous data, indicating that Maf1 phosphorylation and cytoplasmic localization prevented Pol III repression during exponential growth in glucose (25, 30). As shown in Fig. 2 (compare the first two lanes), the temperature shift at 37°C did not induce Maf1-mediated repression of class III genes. In contrast, a change to respiratory growth conditions in the presence of Maf1 induced a decrease in class III gene expression (Fig. 3A, lane 2, average of the ratios of 0.78) that correlated well with that shown in Fig. 2 (compare lane 3 with lane 1). A similar change in the growth medium in the absence of Maf1 (Fig. 3A, lane 3, average of the ratios of 2.70) impaired the repression of class III gene expression, as also shown in Fig. 2 (compare lane 4 with lane 2). A direct comparison of the *maf1*-Δ and the wild-type cells grown in YPGly medium after a shift to the restrictive temperature (Fig. 3A, lane 4, average of the ratios of 4.06) showed that the levels of class III RNAs were substantially higher in the mutant cells.

As noted earlier (Fig. 2B), all tRNAs were not affected by *mat1*-Δ to the same extent (Fig. 3A, Table 2, and Table S1 in the supplemental material). For example, 14 of the 52 tRNA gene families were repressed by more than twofold in cells lacking Maf1 grown in YPD medium with a shift to 37°C (Fig. 3A, lane 1). This effect occurred irrespective of tRNA gene redundancy, but all genes that were repressed in the absence of Maf1 in YPD medium were repressed to similar levels in the presence of Maf1 in YPGly medium (Fig. 3A, compare lane 1 with lane 2). Furthermore, the ratio of tRNA gene levels varied from 1 to 5 upon transference to respiratory growth in cells lacking Maf1 (Fig. 3A, lane 3). This may be the result of differential tRNA stability under respiratory growth conditions or a potential additional Maf1-independent mechanism for regulating tRNA gene transcription by Pol III.

An especially high variation in tRNA levels was noted in a comparison of *maf1*-Δ cells and wild-type cells grown in YPGly medium after a shift to the restrictive temperature (Fig. 3A, lane 4). The particularly important initiator $tRNA^{Met}_{CAU}$ was affected to a minor extent (2.65- ± 1.23-fold increase in *maf1*-Δ), whereas the elongator $tRNA^{Met}_{CAU}$ level was affected more (7.78- ± 3.21-fold increase). The tRNA species most sensitive to Maf1 were two $tRNA^{Phe}_{GAA}$ species, representing different gene families (with 11.16- ± 2.22-fold and 11.46- ± 2.62-fold increases), $tRNA^{Thr}_{AGU}$ (8.53- ± 2.15-fold increase), and $tRNA^{Trp}_{CCA}$ (10.42- ± 4.12-fold increase). Other isoacceptor $tRNAs^{Thr}$ levels were less affected. Interestingly, $tRNA^{Lys}_{CUU}$, known to be imported to mitochondria (15), was sensitive to Maf1 when the cells were grown in glycerol medium (6.03- ± 2.44-fold

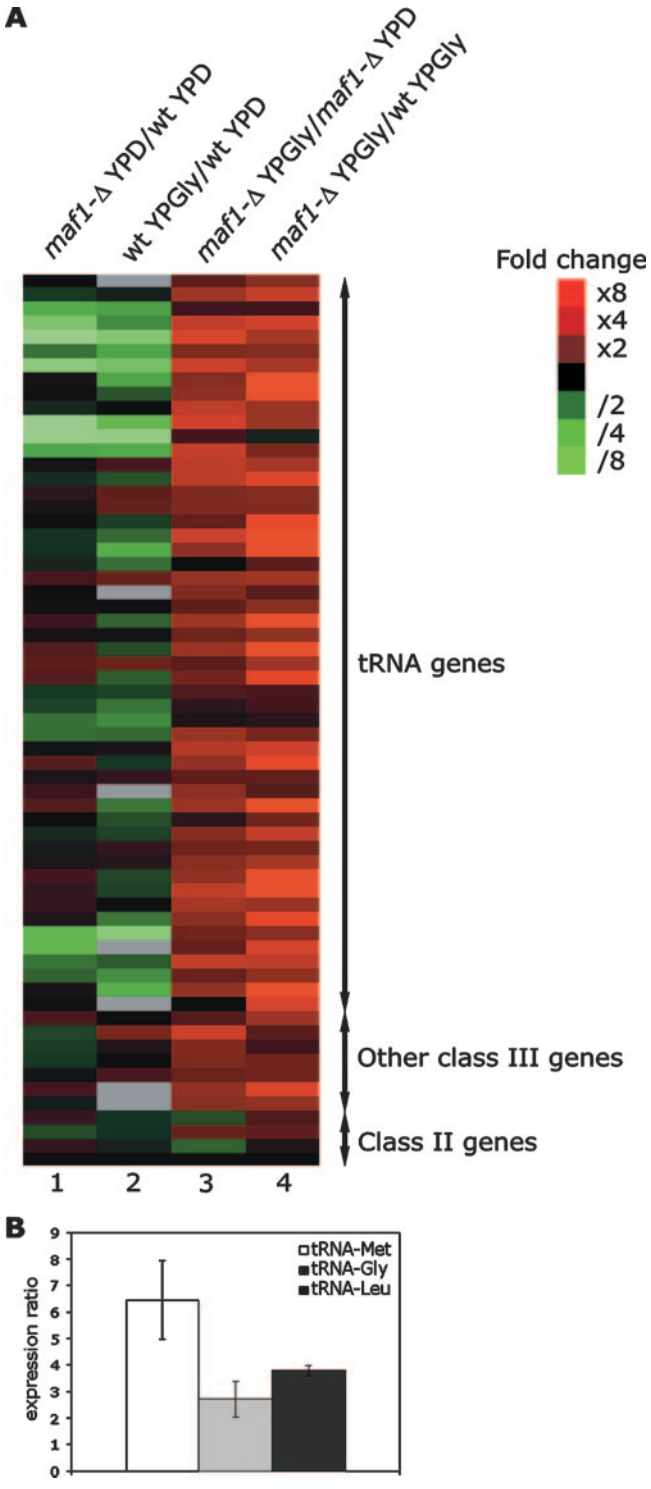

FIG. 3. Pol III genes are not equally regulated by Maf1. (A) Microarray hybridization. Expression ratios of Pol III-transcribed genes in the *maf1*-Δ mutant and the wild-type (wt) control strain (YPH500) grown in glucose medium (YPD) or glycerol medium (YPGly) with a shift to 37°C are shown. Results are presented according to the red-green color scale. Gray bar, not determined. (B) Comparison of expression of selected tDNA genes, $tDNA^{Met}$ (tM[CAU]J1), $tDNA^{Gly}$ (tG[UCC]G), and $tDNA^{Leu}$ (tL[CAA]A), in the *maf1*-Δ and the wild-type YPH500 cells, determined by real-time PCR. Expression levels were normalized to those of the control 35S rDNA. The relative expression ratios and standard deviations were calculated using the Relative Expression Software Tool.

TABLE 2. Quantification of microarray hybridization for selected tRNA genes[a]

tRNA species	Gene name[b]	Fold increase ± SD of *maf1*-Δ/wt expression ratio
$tRNA^{Phe}$	tF(GAA)B	11.16 ± 2.22
$tRNA^{Phe}$	tF(GAA)N	11.46 ± 2.62
$tRNA^{Thr}$	tT(AGU)B	8.53 ± 2.15
$tRNA^{Trp}$	tW(CCA)G1	10.42 ± 4.12
$tRNA^{Lys}$	tK(CUU)D1	6.03 ± 2.44
$tRNA^{Gln}$	tQ(UUG)C	1.57 ± 0.43
$tRNA^{Gln}$	tQ(UUG)B	1.49 ± 0.58
$tRNA^{Gln}$	tQ(UUG)E2	1.26 ± 0.32
$tRNA^{Gln}$	tQ(CUG)M	2.23 ± 1.15
$tRNA^{Met}$	tM(CAU)E	2.65 ± 1.23
$tRNA^{Met}$	tM(CAU)J1	7.78 ± 3.21
$tRNA^{Gly}$	tG(UCC)G	2.99 ± 0.37
$tRNA^{Leu}$	tL(CAA)A	8.98 ± 2.06

[a] Comparison of the expression ratio between the *maf1*-Δ and the control wild-type (wt) (YPH500) strains grown in glycerol medium (YPGly) with a shift to the restrictive temperature.
[b] Gene names are derived from the *Saccharomyces* Genome Database.

increase in *maf1*-Δ cells). In contrast, other tRNAs reported as mitochondrially imported, $tRNA^{Gln}_{UUG}$ and $tRNA^{Gln}_{CUG}$ (29), were not affected (Table 2).

Using real-time PCR (Fig. 3B), we confirmed and quantified the effects of *maf1*-Δ on the expression of selected tRNA gene species. RNA was isolated from *maf1*-Δ mutant cells and control wild-type cells grown in glycerol medium with a shift to 37°C. cDNAs synthesized by reverse transcription were used as templates for amplification of $tDNA^{Met}$ (tM[CAU]J1), $tDNA^{Gly}$ (tG[UCC]G), and $tDNA^{Leu}$ (tL[CAA]A). The 35S rDNA gene served as a reference gene, based on the assumption that *maf1*-Δ does not affect rRNA synthesis. The steady-state levels of $tDNA^{Met}$, $tDNA^{Leu}$, and $tDNA^{Gly}$ were found to be increased, respectively, 6.44 (±1.47)-fold, 3.79 (±0.20)-fold, and 2.70 (±0.69)-fold in the *maf1*-Δ cells with respect to those of the control strain (values were consistent with microarray data shown in Table 2). Thus, the real-time PCR analysis confirmed our observation that individual Pol III genes are repressed by Maf1 to various extents.

Maf1 phosphorylation and intracellular distribution in cells grown in glucose medium differ from those in cells grown in a nonfermentable carbon source. A recurring physiological transition in growing yeast cultures involves a progressive shift from fermentation to respiration. We mimicked this metabolic change by transferring yeast cells from a glucose to a glycerol liquid medium. Wild-type cells in the exponential growth phase in a glucose culture were rapidly pelleted, washed, and suspended in prewarmed medium containing glycerol as the sole carbon source and incubated at 30°C with shaking. According to previous results (25), a slowly migrating phosphorylated form of Maf1 was observed in cells collected in the exponential phase from glucose medium. Transfer of the cells to a glycerol-containing medium caused a rapid dephosphorylation of Maf1 (Fig. 4A). Only the fast-migrating dephosphorylated form of Maf1 was observed after 10 min. Maf1 appears to be a substrate of the cAMP-dependent kinase PKA, and it has been shown recently that increased levels of PKA activity diminish the dephosphorylation of Maf1 under particular stress conditions (23). Thus, one might predict that an increased PKA activity would limit Maf1 dephosphorylation following the transition to a glycerol medium. PKA is under the control of the Ras1 and Ras2 proteins, which stimulate adenylate cyclase to produce cAMP (2). Constitutive activation of PKA can be achieved by the dominant allele $RAS2^{Val19}$ expressed from a plasmid. Expression of the $RAS2^{Val19}$ allele, however, did not counteract Maf1 dephosphorylation when cells grew in a glycerol medium (Fig. 4A). Similarly, no effect on Maf1 dephosphorylation following the transfer from a glucose- to a glycerol-containing medium was observed in the *cdc25-1* mutant, with a decreased adenylate cyclase activity (Fig. 4A) (32).

Subsequently, we investigated whether the intracellular distribution of Maf1 was affected by a shift to respiratory metabolism. Maf1-specific antibodies were used to localize Maf1 in yeast cells by immunofluorescence. As previously reported (25), under favorable growth conditions in glucose, the fluorescence signal was uniformly distributed throughout the cells. Remarkably, in cells grown under respiratory conditions, the Maf1 signal was concentrated in the nuclear compartment and overlapped with the 4′,6′-diamidino-2-phenylindole (DAPI)-stained areas of the wild-type as well as the $RAS2^{Val19}$ and *cdc25-1* mutant cells (Fig. 4B). Therefore, nonfermentative metabolism stimulates Maf1 activity by dephosphorylation and subsequent relocation of Maf1 into the nucleus.

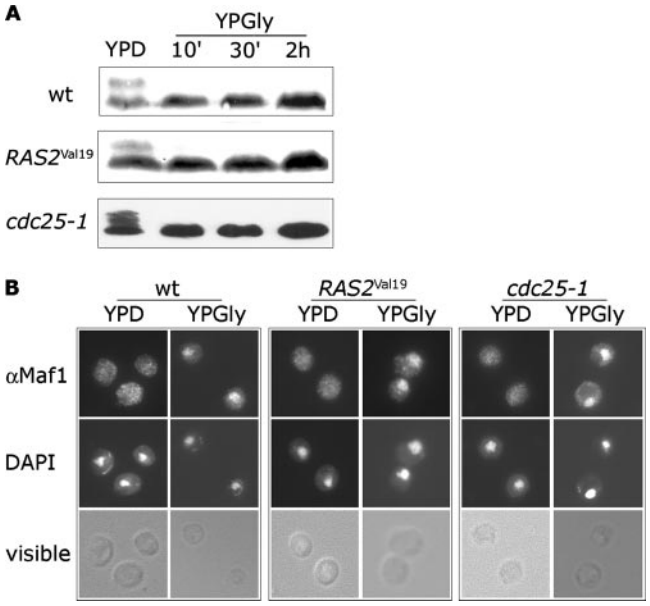

FIG. 4. Dephosphorylation and nuclear localization of Maf1 under respiratory conditions. (A) The wild-type strain YPH500 and the $RAS2^{Val19}$ and *cdc25-1* mutants were grown to exponential phase in rich glucose medium (YPD), then transferred to prewarmed rich glycerol medium (YPGly), and further incubated at 30°C. Crude extracts from trichloroacetic acid-precipitated cells were analyzed by SDS-PAGE with a modified acrylamide-bisacrylamide ratio, followed by immunoblotting using polyclonal anti-Maf1 antibodies. The slower-migrating diffuse band corresponds to phosphorylated forms of Maf1. The phosphorylation pattern of Maf1 in the *cdc25-1* mutant was identical to that observed for the isogenic A364A control strain (not shown). (B) Maf1 localization was analyzed by immunofluorescence microscopy using polyclonal anti-Maf1 antibodies. Cells were grown in glucose medium (YPD) to exponential phase, then transferred to glycerol medium (YPGly), and incubated for 2 h. Nuclei were stained with DAPI.

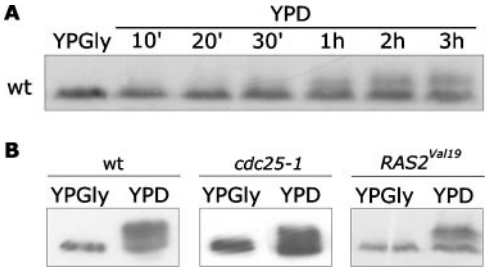

FIG. 5. Maf1 phosphorylation after cells were transferred from respiratory conditions to glucose. Crude extracts from trichloroacetic acid-precipitated cells cultivated in glycerol medium (YPGly) and transferred to glucose medium (YPD) were analyzed by SDS-PAGE with a modified acrylamide-bisacrylamide ratio, followed by immunoblotting using polyclonal anti-Maf1 antibodies. The slower-migrating diffuse band corresponds to phosphorylated forms of Maf1. (A) Kinetics of Maf1 phosphorylation in wild-type cells (YPH500) harvested at the indicated time after transfer to YPD medium. (B) Maf1 phosphorylation in mutants with altered PKA activity. Wild-type (A364A), *RAS2*^{Val19}, and *cdc25-1* cells were grown in YPGly medium and harvested 2 h after transfer to YPD medium.

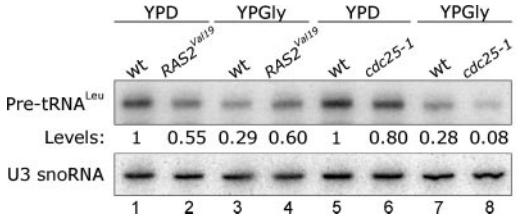

FIG. 6. PKA is involved in Pol III regulation, coupled to carbon metabolism. Total RNA isolated from the *RAS2*^{Val19} and *cdc25-1* mutants and isogenic wild-type control cells (YPH500 and A364A, respectively), grown in glucose medium to late exponential phase (YPD medium) or transferred to glycerol medium and grown for 2 h at the permissive temperature (YPGly medium), was hybridized with intron-specific tRNA^{Leu} and U3 snoRNA probes. Bands were quantified as described in Materials and Methods. In order to determine relative levels, the amount of tRNA^{Leu} present in wild-type (wt) cells grown in YPD medium was set to 1. Lanes are numbered to facilitate explanation in the text.

To further investigate the effect of carbon metabolism on Maf1 regulation, in a reciprocal experiment, cells grown in glycerol were transferred to a glucose medium. Such a transition from respiration to fermentation metabolism resulted in Maf1 phosphorylation. Compared to the rapid dephosphorylation process, which was completed in less than 10 min (Fig. 4A), the phosphorylation was relatively slow and took nearly 2 h (Fig. 5A). Similar Maf1 phosphorylation upon transition from a nonfermentable carbon source to a glucose source was also observed in both mutants with altered PKA activity (Fig. 5B).

In parallel with its slow phosphorylation, Maf1 also gradually relocated out of the nucleus. At 1 h after cells were transferred to glucose medium, almost all cells had Maf1 in the nucleus. After the second hour, the Maf1 signal became distributed uniformly throughout the cell. The kinetics of Maf1 relocation to the cytoplasm was much slower than its nuclear importation in response to rapamycin (25). The rate of phosphorylation and relocation of Maf1 to the cytoplasm in the *RAS2*^{Val19} mutant cells upon transfer from a nonfermentable carbon source to a glucose source was similar to that in the wild type (data not shown).

Next, we assessed if mutants with altered PKA activity affected the decrease in Pol III transcription upon the transition from fermentation to respiratory conditions. The *RAS2*^{Val19} and *cdc25-1* mutants and the isogenic wild-type control cells were grown in glucose medium to exponential phase, transferred to prewarmed glycerol medium, grown for 2 h at the permissive temperature, and used for the isolation of RNA, which was then analyzed by Northern blotting. An intron probe was used to determine the synthesis of the tRNA^{Leu} precursor (Fig. 6). The transition to respiratory conditions caused a threefold decrease in pre-tRNA^{Leu} synthesis in wild-type cells (Fig. 6, compare lanes 3 and 7 with lanes 1 and 5) but a 10-fold decrease in the *cdc25-1* mutant cells (Fig. 6, compare lanes 8 and 6). In contrast, there was no decrease of tRNA^{Leu} precursor in the *RAS2*^{Val19} mutant with enhanced PKA activity when cells were transferred to glycerol (Fig. 6, compare lanes 4 and 2). Clearly, PKA affects Pol III regulation coupled to a transfer from fermentation to respiration. However, the molecular mechanism probably involves a PKA target protein other than Maf1, since the changes in Maf1 phosphorylation and intracellular distribution in response to a carbon source are independent of the levels of PKA activity.

Genetic interaction of Maf1 with Pol III subunits. As an inhibitor of Pol III transcription, the yeast Maf1 performs a significant role in nonfermentative metabolism. The increased transcription in the *maf1* mutants caused a toxic effect under physiological conditions of respiration. According to our previous data, this toxicity can be overcome by a second site mutation in the gene encoding the largest Pol III subunit, C160 (1, 27). This mutation decreased the level of tRNA, enabling genetic suppression of the *maf1* phenotype (27). In order to find mutants in other Pol III subunits that overcome the *maf1*-Δ phenotype, we further applied a genetic screen for second-site suppressors that bypass the growth defect in a nonfermentable carbon source. One of the spontaneous suppressors (Fig. 7A) was recessive, not allelic, to *rpc160* mutants and gave a cold-sensitive phenotype. When a yeast wild-type centromeric library was introduced into the suppressor strain, a genomic fragment was selected which complemented the cold-sensitive phenotype and was genetically linked to the suppressor locus. Subcloning revealed the *RPC128* gene encoding the second largest Pol III subunit. The mutant allele, called *rpc128-1007*, was isolated from the suppressor strain. It had a single nucleotide change resulting in a substitution of the conserved Gly with Ala, localized close to the nucleotide binding motif in the C terminus of C128 (Fig. 7B) (36). The *rpc128-1007* mutant had 1.6-fold-reduced tRNA levels compared to that of 18S rRNA (Fig. 7C). This led to the conclusion that the toxic effect of *maf1*-Δ in a nonfermentable carbon source, resulting from enhanced tRNA levels, could be overcome by decreasing transcription by altering C160 or other Pol III components.

To better understand the biological meaning of *maf1* suppression, we decreased Pol III transcription by partial inactivation of the C17 Pol III subunit. We were able to change the C17 level by modulating the expression of the *RPC17* gene cloned under the regulated *tetO* promoter. The addition of

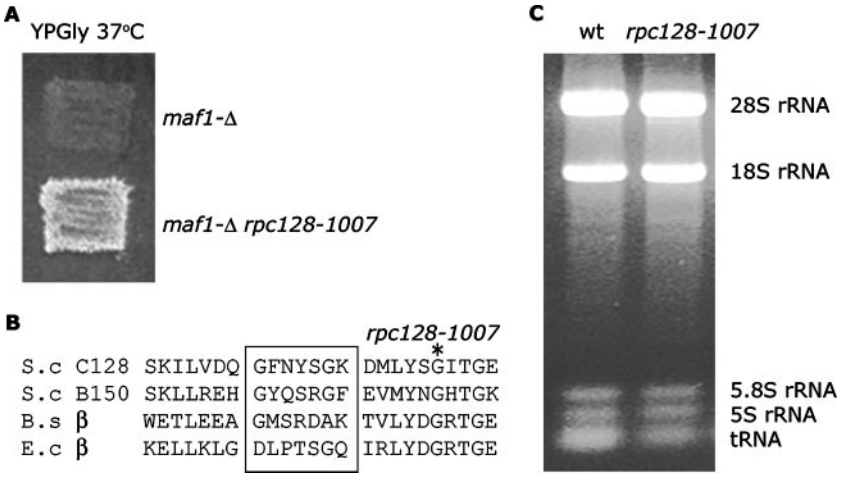

FIG. 7. The mutation in the conserved region of the C128 Pol III subunit is a second-site suppressor of *maf1*-Δ. (A) Growth phenotype. MJ9-2B *maf1*-Δ and MJ9-2B *maf1*-Δ *rpc128-1007* cells were grown on YPD medium overnight, replica plated on YPGly medium, and incubated for 3 days at 37°C. (B) Localization of the *rpc128-1007* mutation close to the nucleotide binding motif $G_{995}X_4GK_{1001}$. The sequence surrounding the mutation is conserved in the C128 and B150 subunits, the second largest subunits of yeast Pol III and Pol II, respectively, as well as in their prokaryotic homologues, β subunits from *Escherichia coli* and *Bacillus subtilis.* (C) Decreased tRNA level in the *rpc128-1007* mutant. Total RNA was isolated from the MJ15-9C *rpc128-1007* strain and the control wild-type strain grown in glucose medium after a shift to the nonpermissive temperature (2.5 h at 16°C) and analyzed on 2.4% agarose gel stained with ethidium bromide.

doxycycline to the growth medium resulted in a decreased level of C17 protein and a lower basal Pol III activity, whereas in the absence of the antibiotic, Pol III was not affected (11). To create a strain with nonfunctional Maf1 and to monitor Pol III activity, we deleted *MAF1* in the strain expressing *RPC17* from the *tetO* promoter. The *maf1*-Δ glycerol-negative phenotype was observed when C17 expression was high. The growth defect of *maf1*-Δ was compensated for in the presence of doxycycline when C17 expression and Pol III activity levels were low (Fig. 8). The suppression of the *maf1*-Δ phenotype appeared as a consequence of the restored lower level of tRNA.

However, this conclusion raised another question: would any Pol III defect that lowers tRNA levels allow a bypass of *maf1*-Δ toxicity under respiratory conditions? To answer it, we deleted

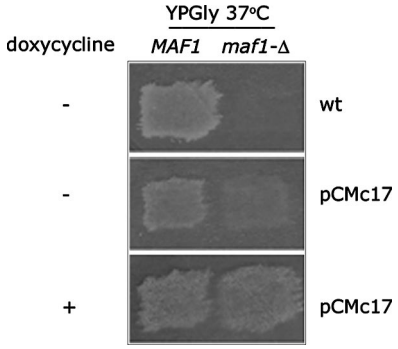

FIG. 8. Suppression of the *maf1*-Δ phenotype on a nonfermentable carbon source upon decreased expression of the C17 Pol III subunit. Growth on glycerol medium was compared for the wild-type (wt) control strain YPH500 and the strain YMLF2 with regulated levels of C17 Pol III subunit (pCMc17) carrying the active *MAF1* chromosomal gene or the *maf1*-Δ deletion. Cells were grown on YPD medium overnight, replica plated on YPGly medium, and incubated for 3 days at 37°C. YPGly medium plates were supplemented with doxycycline, as indicated.

MAF1 in several Pol III mutants in which the mutations were known to affect the enzyme activity in various ways (Table 3). To test the suppression, these *maf1*-Δ derivatives of Pol III mutants were tested for growth on a nonfermentable carbon source (YPGly medium, at 37°C). Two termination mutants, *rpc160-750* and *rpc11*-Sp (4, 18, 33), and one initiation mutant, *rpc31-236* (38), were able to compensate for the toxic defect of the *maf1*-Δ mutant. Other mutants, *rpc25-S100P*, affecting initiation (45), and *rpc160-112*, affecting elongation (8, 33), did not suppress the *maf1*-Δ phenotype (Table 3). The decrease of tRNA levels in individual mutants was quantified by analyzing RNA extracted from cells grown in YPD medium at 30°C, which were then shifted to 37°C for 2.5 h (Table 3). Interestingly, the suppression seems to be correlated not with an overall decrease (*n*-fold change) in tRNA levels but rather with specific defects in Pol III mutants. Moreover, no significant differences in tRNA levels were measured in mutants versus those in wild-type cells grown in glycerol medium with a shift

TABLE 3. Suppression of the *maf1*-Δ phenotype by various Pol III mutations[a]

Pol III mutant	Defect in Pol III transcription	Mutated Pol III subunit	Suppression of *maf1*-Δ	Fold decrease in tRNA levels
rpc31-236	Initiation	C31	+	2.5
rpc25-S100P	Initiation	C25	−	2.6
rpc160-112	Elongation	C160	−	1.2
rpc160-750	Termination	C160	+	1.4
rpc11-Sp	Termination	C11	+	1.3
rpc128-1007	Not studied	C128	+	1.6

[a] To determine suppression of the *maf1*-Δ phenotype, Pol III mutants with a deleted chromosomal *MAF1* gene and respective control strains were grown on YPD medium overnight, replica plated on YPGly medium, and incubated for 3 days at 37°C. To estimate the decrease in tRNA levels, total RNA was separated by electrophoresis. Intensities of tRNA bands stained with ethidium bromide were quantified using Image Quant 6.2 software.

to 37°C (ratio between 1.1 and 1.4; data not shown). Thus, mutations in genes encoding Pol III subunits that were ultimately rate limiting for transcription in cells grown in glucose media appeared to be not limiting for transcription under respiratory growth conditions at a high temperature.

DISCUSSION

Investigation of the impact of Maf1 on yeast physiology indicates that Maf1 performs a significant inhibitory role in normally growing cells. This new function of Maf1 couples Pol III transcription with metabolic processes and/or energy production that is dependent on the carbon source. It is now obvious that not only the Pol II transcriptome but also the Pol III genes are regulated during the transition from fermentative to glycerol-based respiratory growth and that Maf1 is essential for this regulation. Previous work indicates that dephosphorylation is the way by which nuclear importation and Maf1 activity are regulated in response to rapamycin. Here we show that Maf1 is phosphorylated and localized in a manner that is dependent on the carbon source, which determines yeast metabolism. Glucose depletion and transfer to a nonfermentable carbon source result in Maf1 dephosphorylation and importation into the nucleus. An opposite transition, from a nonfermentable carbon source to a glucose medium, is followed by Maf1 phosphorylation and relocation from the nucleus to the cytoplasm. It is not clear if the phosphorylation occurs before or after Maf1 export to the cytoplasm. Our kinetics studies suggest that relocalization to the cytoplasm is coincident with Maf1 phosphorylation. The mechanism of Maf1 nuclear exportation remains to be determined.

Maf1 of *S. cerevisiae* is a serine-rich protein (15.7% of serines) and, according to a Swiss-Prot PROSITE search, contains 30 potential phosphorylation sites. The kinase involved in Maf1 phosphorylation following a transfer to glucose is currently unknown. The cAMP-dependent PKA kinase was a potential candidate since Maf1 had already been experimentally confirmed as its substrate in vitro (3, 23). We therefore tested strains carrying the mutations $RAS2^{Val19}$ and *cdc25-1*, each of which affected the synthesis of cAMP differently. We found that neither mutation altered the pattern of Maf1 phosphorylation following transfer to a different carbon source. No effect of *cdc25-1* on the kinetics of Maf1 phosphorylation upon transfer to glucose medium was observed (data not shown). Moreover, the high PKA activity in the $RAS2^{Val19}$ mutant did not limit the dephosphorylation and nuclear relocation of Maf1 in response to glucose depletion. In conclusion, we suggest that another, so-far-unknown kinase participates in the regulation of Maf1 activity toward the coupling of Pol III transcription with carbon metabolism.

Having established that an altered level of PKA activity did not affect Maf1 phosphorylation or localization upon transition from glucose to a nonfermentable carbon source, we also expected no effect on the regulation of Pol III activity during such a transition. However, a direct study of pre-tRNA synthesis in the PKA mutants resulted in an opposite conclusion. The high PKA activity in the $RAS2^{Val19}$ cells grown in glucose medium and transferred to respiratory conditions prevented a decrease in Pol III transcription. Consistently, in the *cdc25-1* mutant with a low PKA activity, Pol III activity was more repressed than in the corresponding wild type. A similar effect of altered PKA activity in mutants was previously observed by Moir et al. (23) on the repression of Pol III transcription upon rapamycin stress, but the authors reported an accompanying change in the pattern of Maf1 phosphorylation. Here, we clearly show that the same mutants which do not affect the phosphorylation and cellular localization of Maf1 do affect, under the same conditions, the regulation of Pol III activity. As suggested previously (23), there is another protein involved in Pol III transcription regulation. According to our hypothesis, this unknown protein might be directly regulated by PKA.

Our current results show that not all Pol III genes are regulated by Maf1 to the same extent when cells are grown in a nonfermentable carbon source. This conclusion is confirmed by quantification of a Pol III-specific microarray and by quantitative reverse transcription-PCR with selected tRNA genes. The mechanism and physiological basis of the variable Maf1 regulation of individual tRNAs remain unclear. Assuming a direct influence of Maf1 on the transcription rate, the variable effect of Maf1 on different tRNA genes could be dependent on the potential transcription efficiency of a given tRNA gene. tRNA genes with internal Pol III promoters are homologous, but the flanking sequences are different. This might be the reason for different occupancy of TFIIIB and Pol III (13). It has also been shown that the RSC chromatin remodeling complex is specific to Pol III genes, but not all tRNA genes bind RSC (24). The relative efficiency of transcription of individual tRNA genes is probably not the same, although this problem has not yet been solved. Therefore, we were not able to establish the relationship between the effect of Maf1 and the transcription efficiency of a particular tRNA gene.

The extent of Maf1 regulation does not correlate with the gene copy number for a given tRNA (see Table S1 in the supplemental material). The expression of single-copy tRNA genes is usually not much affected in *maf1*-Δ cells. However, the levels of $tRNA^{Val}$ (tV[AAC]E1, 13 copies) or $tRNA^{Asn}$ (tN[GUU]C, 10 copies) were also affected to a minor extent, whereas those of $tRNA^{Phe}$ (tF[GAA]N, 2 copies) and $tRNA^{Arg}$ (tR[ACG]J, 1 copy) were significantly increased in *maf1*-Δ cells grown under respiratory conditions. We also found no correlation between the extent of Maf1 regulation and codon usage corresponding to a given tRNA.

Unbalanced tRNA levels seem a likely reason for the *maf1*-Δ growth defect in glycerol medium. In contrast to some other tRNAs, initiator $tRNA^{Met}$ was not significantly affected by Maf1 since its expression ratio in a nonfermentable carbon source was 2.65 ± 1.23. It is known that depletion of initiator $tRNA^{Met}$ triggers reprogramming of genome expression in several Pol III mutants (5). Assuming that the relatively low level of initiator $tRNA^{Met}$ could be the reason for growth inhibition, its expression was increased by introducing a multicopy plasmid with the *IMT1* gene into the cells. However, no effect of the increased dose of initiator $tRNA^{Met}$ on the *maf1*-Δ phenotype was observed (data not shown). In *S. cerevisiae*, two Pol III-synthesized tRNAs have been reported as mitochondrially targeted, namely $tRNA^{Lys}$ and $tRNA^{Gln}$ (15, 29). The mitochondrial functions of these tRNAs are not fully clear, although there is indirect evidence for their role in mitochondrial translation. Our data show that $tRNA^{Lys}$ is 6.03- ± 2.44-fold increased in *maf1*-Δ, whereas $tRNA^{Gln}$ is not affected. Assuming that the

imported tRNAs function in mitochondria in a concerted fashion, their unbalanced levels could be disadvantageous for mitochondrial translation. However, no increased [*rho*$^-$] accumulation, typical for yeast mutants with mitochondrial translation defects, was observed in *maf1*-Δ strains.

Although nuclear Maf1 is known to function as a Pol III transcription repressor, the role of Maf1 in the cytoplasm remains unknown. One possibility, involving the mitochondrial scenario, is a function of cytoplasmic Maf1 in posttranscriptional tRNA control. At least two of the four subunits of the yeast tRNA endonuclease, Sen2 and Sen54, are located on the outer mitochondrial membrane, and this location is important for functional tRNA splicing (44). Although no mitochondrial phenotype of mutants affecting tRNA splicing has been found, one could assume that cytoplasmic Maf1 could somehow be involved. Interestingly, we identified the Sen54-encoding gene in a screen for putative activators of tRNA biosynthesis (M. Cieśla, unpublished). Nonetheless, inactivation of *MAF1* is not the first example of a mutation that alters tRNA biogenesis and the ability of yeast cells to grow in respiratory substrates. A partial deletion of the N-terminal domain of τ55, a TFIIIC subunit required for tRNA transcription, impairs its function and also cell growth at an elevated temperature in nonfermentable carbon sources (20).

The lethality of the *maf1*-Δ cells under restrictive conditions is caused by increased or unbalanced levels of some Pol III products because it can be overcome by decreased transcription in Pol III mutants. The *maf1*-Δ growth defect could be suppressed by selected mutations affecting Pol III transcription initiation or termination. Suppression of the temperature-sensitive phenotype of *maf1*-Δ in Pol III mutants may reflect simple compensation of the amount of active transcription complexes. Since not all Pol III mutants with similar decreases (*n*-fold) in tRNA levels suppressed *maf1*-Δ, we propose that only some mutations allow formation of functional Pol III complexes in the absence of Maf1. Interestingly, we found a reciprocal genetic interaction of Maf1 with the C31 Pol III subunit. Truncation of the C31 subunit in the *rpc31-236* mutant caused a temperature-sensitive phenotype (38). *rpc31-236* counteracted the *maf1*-Δ defect in a nonfermentable carbon source. Interestingly, *maf1*-Δ *rpc31-236* was no longer thermosensitive, indicating that in the absence of a negative regulator, the C31 truncation was not detrimental to Pol III transcription activity at an elevated temperature. C31 is part of a subcomplex of three Pol III-specific subunits (C31, C34, and C82) that is thought to interact with TFIIIB (41). The genetic interaction of Maf1 and C31 supports the model in which Maf1 affects the recruitment of Pol III by hampering its interaction with TFIIIB. Moreover, the gene encoding the Ded1 helicase was previously found to be a suppressor of *rpc31-236* (38). Ded1 is another putative link since it was immunopurified with Maf1 (25). These preliminary genetic data encourage us to study the mechanism of action of Maf1 on the Pol III transcription apparatus.

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