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Transcriptional Elements Involved in the Repression of Ribosomal Protein Synthesis

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The ribosomal proteins (RPs) of *Saccharomyces cerevisiae* are encoded by 137 genes that are among the most transcriptionally active in the genome. These genes are coordinately regulated: a shift up in temperature leads to a rapid, but temporary, decline in RP mRNA levels. A defect in any part of the secretory pathway leads to greatly reduced ribosome synthesis, including the rapid loss of RP mRNA. Here we demonstrate that the loss of RP mRNA is due to the rapid transcriptional silencing of the RP genes, coupled to the naturally short lifetime of their transcripts. The data suggest further that a global inhibition of polymerase II transcription leads to overestimates of the stability of individual mRNAs. The transcription of most RP genes is activated by two Rap1p binding sites, 250 to 400 bp upstream from the initiation of transcription. Rap1p is both an activator and a silencer of transcription. The swapping of promoters between *RPL30* and *ACT1* or *GAL1* demonstrated that the Rap1p binding sites of *RPL30* are sufficient to silence the transcription of *ACT1* in response to a defect in the secretory pathway. Sir3p and Sir4p, implicated in the Rap1p-mediated repression of silent mating type genes and of telomere-proximal genes, do not influence such silencing of RP genes. Sir2p, implicated in the silencing both of the silent mating type genes and of genes within the ribosomal DNA locus, does not influence the repression of either RP or rRNA genes. Surprisingly, the 180-bp sequence of *RPL30* that lies between the Rap1p sites and the transcription initiation site is also sufficient to silence the Gal4p-driven transcription in response to a defect in the secretory pathway, by a mechanism that requires the silencing region of Rap1p. We conclude that for Rap1p to activate the transcription of an RP gene it must bind to upstream sequences; yet for Rap1p to repress the transcription of an RP gene it need not bind to the gene directly. Thus, the cell has evolved a two-pronged approach to effect the rapid extinction of RP synthesis in response to the stress imposed by a heat shock or by a failure of the secretory pathway. Calculations based on recent transcriptome data and on the half-life of the RP mRNAs suggest that in a rapidly growing cell the transcription of RP mRNAs accounts for nearly 50% of the total transcriptional events initiated by RNA polymerase II. Thus, the sudden silencing of the RP genes must have a dramatic effect on the overall transcriptional economy of the cell.

The *Saccharomyces cerevisiae* cell invests enormous resources in the synthesis of ribosomes. In a rapidly growing cell, the 100 or more rRNA genes are responsible for at least 60% of total transcription (59). The 137 ribosomal protein (RP) genes are among the most active in the genome (57). As a result, the cell has evolved mechanisms that tightly control the synthesis of the components of the ribosome. The 78 RPs are synthesized in nearly equimolar amounts that are matched to the synthesis of rRNA. Furthermore, the synthesis of these components is coordinately responsive to many changes within or outside the cell, including heat shock, carbon source, and nutrient availability (reviewed in references 44 and 59).

The primary level of regulation is at transcription (7, 16, 24). Most RP genes have similar promoter motifs, consisting of two Rap1p binding sites in tandem arrangement 250 to 400 bp upstream of the transcription initiation site recently compiled by Lascaris et al. (26), followed by one or two T-rich regions, and then a region of about 180 bp that includes the putative TATA element. Serial promoter deletions of RP genes, including *RPS14A* (*CRY1*), *RPL25*, and *RPL28* (*CYH2*), have shown that the Rap1p sites are responsible for about 90% of promoter activity, with the rest due to the T-rich regions (41, 45, 47). Deletions downstream of the T-rich elements have little effect on the rate of transcription but can affect the initiation site (47). For a small minority of RP genes, including *RPL3*, *RPL4A*, *RPL4B*, and *RPS28A*, a single Abf1p site replaces the two Rap1p sites (8, 13). Nevertheless, such genes appear to be regulated coordinately with the others. (In this paper, we have used the new nomenclature for RPs and their genes that was recently adopted (32, 64a). Thus, L30 was previously known as L32, L3 was previously known as Tcm1p, and L8 was previously known as L4.)

Rap1p is an essential, abundant DNA-binding protein that has several functions in the cell. It is a context-dependent transcription regulator (50), responsible for activating the transcription of many of the most highly transcribed genes, encoding not only the RPs and other translation factors but also the very abundant glycolytic enzymes (reviewed in reference 49). Rap1p also acts as a transcriptional silencer in at least two contexts. At the silent mating type loci, Rap1p cooperates with Abf1p and the origin of replication complex (ORC) to repress transcription (1). At the telomeres, Rap1p binds to the $[C_{1\text{-}3}A]_n$ repeats (63), leading to the silencing of genes adjacent to telomeres. In both cases Rap1p recruits multiple copies of Sir3p and Sir4p that participate in the silencing, perhaps by interacting with the tails of histones H3 and H4 (38). A mutant

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TABLE 1. *S. cerevisiae* strains used in these experiments

Strain	Genotype	Reference or source
W303a	*MAT***a** *ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100 ssd1-1*	54
169ts	*MAT*α *ade2-1 leu2-3,112 ura3-1 his3-11,15 can1-100 ssd1-1 ypt6-1*	28
JV7-2a	As W303, *rpl30*Δ::*HIS3* (pYE: *CEN*, *URA3 GAL1-RPL30*)	58
BL17	*MAT***a**/*MAT*α *YPT6/ypt6-1 rpl30*Δ::*HIS3/RPL30 his3-11,15/his3-11,15 ura3-1/ura3-1 leu2-3,112/leu2-3,112 ade2-1/ade2-1 can1-100/cam1-100 ssd1-1/ssd1-1* (pRS316: *CEN*, *URA3 ACT1-RPL30*)	This study
BL174	*ypt6-1 rpl30*Δ::*HIS3* (pRS316: *CEN*, *URA3 ACT1-RPL30* [construct A])	This study
BL175	*ypt6-1 rpl30*Δ::*HIS3* (pRS316: *CEN*, *URA3 ACT1-RPL30* [construct B])	This study
BL176	*ypt6-1 rpl30*Δ::*HIS3* (pRS316: *CEN*, *URA3 ACT1-RPL30* [construct C])	This study
BL177	*ypt6-1 rpl30*Δ::*HIS3* (pRS316: *CEN*, *URA3 ACT1-RPL30* [construct D])	This study
Y260	*MAT***a** *ura3-52 leu2 rpb1-1*	42
JW1201	*MAT*α *ypt6-1 ade2-1 leu2-3,112 ura3-1 his3-11,15 can1-100 ssd1-1 act1*Δ::*URA3 RPL30-ACT1*	This study
YDS2	*MAT***a** *leu2 ade2 his3 trp1 ura3* (~W303)	D. Shore
YDS714	*MAT*α *leu2 ade2 his3 trp1 ura3 sir2*Δ::*HIS3*	D. Shore
JW1210	*MAT*α *leu2 ade2 his3 trp1 ura3 sly1-1 sir2*Δ::*URA3*	This study
JW1211	*MAT*α *leu2 ade2 his3 trp1 ura3 sly1-1 hst1*Δ::*URA3*	This study
YDS430	*MAT*α *leu2 ade2 his3 trp1 ura3 sir3*Δ::*LEU2*	D. Shore
BL180	*YPT6 SIR3*	This study
BL181	*YPT6 sir3*Δ::*LEU2*	This study
BL182	*ypt6-1 SIR3*	This study
BL183	*ypt6-1 sir3*Δ::*LEU2*	This study
YDV122	*MAT*α *leu2 ade2 his3 trp1 ura3 sir4*Δ::*LEU2 hmr*Δ::*TRP1*	D. Shore
BL185	*YPT6 SIR4*	This study
BL186	*YPT6 sir4*Δ::*LEU2*	This study
BL187	*ypt6-1 SIR4*	This study
BL188	*ypt6-1 sir4*Δ::*LEU2*	This study

allele, *rap1*^{s}, relieves the repression at silent mating type loci. The overexpression of Sir3p or Sir4p can restore the repression (53).

When yeast cells growing at 23°C are shifted to 37°C, both growth and protein synthesis continue unabated; indeed, depending on strain background, they sometimes increase (11). Furthermore, mRNA synthesis, measured by the incorporation of [^{3}H]uracil into poly(A)$^+$ RNA, is unaffected (24). Yet, during the first 20 min after such a shift the level of RP mRNAs declines by about 80% and then recovers to the original level by about 60 min (11, 15). This down-regulation of RP mRNA has been attributed to a temporary transcriptional silencing of RP genes (24) and to an increased turnover of RP mRNAs (15). In neither case have the elements responsible been identified (15, 45).

Recently, we have shown that a defect at any of several points in the secretory pathway down-regulates the synthesis of ribosomes, through the repression of transcription of both rRNA and RP genes (28, 36). Inhibitors that disrupt the secretory pathway, such as brefeldin A and tunicamycin, also repress ribosome synthesis. A similar effect on rRNA transcription was observed in a *sec23* mutant (29). These results suggest that there exists an intracellular signal transduction pathway between the secretory apparatus and the master control of ribosome biosynthesis.

To identify the elements of an RP gene that makes it subject to repression by a temperature shock and by the failure of the secretory pathway, we have carried out experiments which demonstrate (i) that the rapid decline in mRNA levels is due entirely to the rapid repression of transcription, coupled to a normal, rapid turnover of the mRNA (this repression does not depend on the presence of Sir2p, Sir3p, or Sir4p) and (ii) that the repression of transcription is mediated by two *cis* elements, the Rap1p binding sites and a 180-bp sequence just upstream of the transcription initiation site. Either of these elements alone can mediate at least 75% of repression.

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. Unless otherwise stated, cultures were grown on 2% yeast extract–1% Bacto Peptone–2% glucose (YPD) at 23°C. Cultures were shifted from 23 to 37°C by pouring them into a large, prewarmed flask shaking in a water bath. Cultures were shifted from YPGal to YPD by filtration onto a Millipore filter which was immediately transferred to the new medium. The whole operation took less than 20 s.

Yeast total RNA isolation. Generally, 10-ml volumes of cultures in log phase were harvested by pouring them on crushed ice (46). Cells were collected by centrifugation, resuspended in 400 μl of AE buffer (50 mM NaAc [pH 5.3], 1 mM EDTA), and transferred to microcentrifuge tubes. Forty microliters of 10% sodium dodecyl sulfate was added, and the mixture was vortexed. An equal volume of fresh, AE buffer-saturated phenol was added, and the mixture was vortexed for 2 min. The samples were then incubated at 65°C for 4 min and chilled rapidly in dry ice-ethanol until phenol crystals appeared. The samples were centrifuged at maximum speed for 10 min, and the aqueous layer was transferred to another tube, extracted with phenol-chloroform at room temperature for 5 min, and then centrifuged for 5 min. Total RNA was precipitated with ethanol, washed with 70% ethanol, dried, and resuspended in 50 μl of sterile water.

RNA gel and Northern analysis. Ten micrograms of total RNA was fractionated on 1.5% agarose gels containing 6% formaldehyde. The RNA was transferred to Nytran (Schleicher & Schuell) nylon membranes, cross-linked with UV light, and then baked in a vacuum for 1 h at 80°C. Northern blot analysis was performed as described previously (10). ^{32}P-labelled antisense RNA probes and oligodeoxynucleotides were used to detect mRNAs (36). rRNA was labelled with [^{3}H$_3$]methionine (60) and analyzed by fluorography (36).

Construction of fused genes with site-directed mutagenesis. For the *ACT1* gene constructs, the A of ATG in the coding region is at position +1. For *RPL30*, the transcription initiation site is considered position +1 and is 58 nucleotides upstream of ATG. To fuse the *RPL30* promoter to the *ACT1* transcription unit, the *URA3* gene was first cloned into pMDJ8, which contains a 1.8-kb fragment with the complete *RPL30* sequence, from −443 to +1520. The new plasmid, containing divergently expressed *URA3* and *RPL30* genes, each from its own promoter, was then used as a template for PCR. The left-hand primer contained *ACT1* sequences from −410 to −364 and the reverse complement of *URA3* sequences from +915 to +890. (The *URA3* open reading frame [ORF] is 803 bp). The right-hand primer contained *ACT1* sequences from −96 to −141 and *RPL30* sequences from −1 to −26. PCR amplified a fragment in which the entire *URA3* gene and *RPL30* promoter sequences (to the transcription start site at +1) were bracketed by *ACT1* sequences. The PCR fragment was integrated into the chromosomal *ACT1* locus of strain 169ts or JW142 by homologous recombination, selecting for *URA3*. The integration reconstituted the *ACT1* transcription unit, under the control of the *RPL30* promoter (Fig. 4B). The correct integration

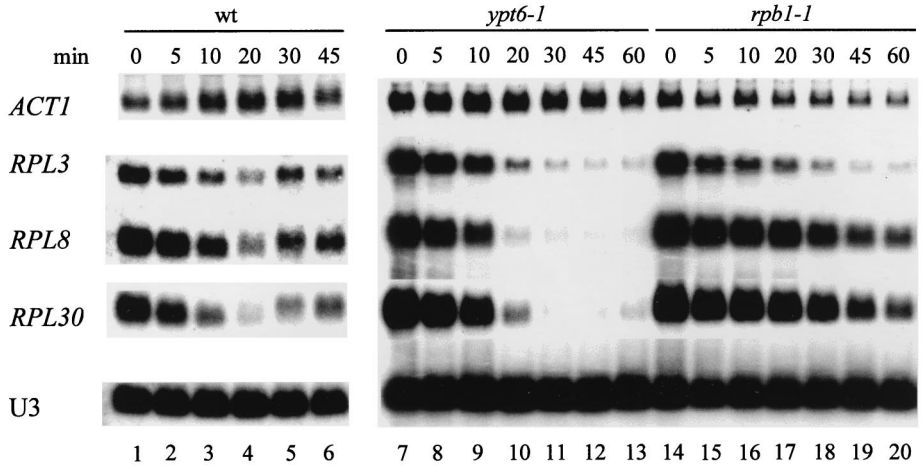

FIG. 1. The level of RP mRNAs is down-regulated in W303 (wild type), 169ts (*ypt6-1*), and Y260 (*rpb1-1*) strains. The indicated strains (Table 1) were grown to log phase in YPD at 23°C. An aliquot was harvested, the rest of the culture was shifted to 37°C, and aliquots were harvested as indicated. Total RNA was isolated, and 10 μg of RNA was analyzed by Northern blotting. Individual RNAs were detected by using either antisense RNA probes, for *RPL30* and *ACT1*, or oligonucleotide probes, for *RPL3*, *RPL8*, and U3 snoRNA, as described in Materials and Methods.

was verified by PCR. The correct initiation of transcription was verified by primer extension. The resulting strain was designated JW1201 (Table 1).

Constructs A to D (Fig. 4C) were made starting with a pUC19-based plasmid carrying a 2.2-kb *Eco*RI fragment from *RPL30*. The promoter region was replaced with a fragment containing the promoter of *ACT1* (construct A; Fig. 4B). From this construct, the upstream activating sequence (UAS) of *ACT1* was replaced with portions of the UAS of *RPL30* (constructs B, C, and D; Fig. 4C). For use in yeast, the constructs were subcloned into the *CEN*/*URA3* plasmid, pRS316 (51). Primer extension revealed that about 50% of the transcripts initiated at the normal site for RPL32, and 50% initiated nine nucleotides downstream.

Constructs E to H (Fig. 9A) were made, starting with the vector pRS316 carrying *RPL30*. A large portion of the ORF downstream of the intron was replaced by the ORF encoding the green fluorescent protein (GFP), generating construct E (see text for details). From construct E, different portions of the *RPL30* promoter were replaced with a 500-bp fragment containing the four Gal4p binding sites from *GAL1* (nucleotides −829 to −324; with the start codon numbered +1), generating constructs F to H.

Deletion mutations were constructed by PCR (18). A circular plasmid carrying the gene of interest was used as a template for PCR catalyzed by Tli DNA polymerase (Promega), which has proofreading ability. PCR products were purified from an agarose gel with a DNA extraction kit (Qiagen). The purified DNA was used directly for self-ligation with the standard protocol, except that 10 U of polynucleotide kinase was included in the ligation reaction. The deletion constructs were sequenced.

mRNA half-life measurement. The strains to be tested were grown overnight at 23°C to log phase. Cell cultures were concentrated fivefold and then shifted to 37°C (43). At the indicated time points 2-ml aliquots of the cultures were quickly collected and frozen in dry ice-ethanol. Total RNA was isolated and analyzed as described above. RNA levels were quantified relative to the U3 internal loading standard by using PhosphorImager (Molecular Dynamics) analysis.

RESULTS

Repression of RP gene expression. The mRNAs encoding RPs appear to be particularly sensitive to environmental changes. As shown in Fig. 1, a temperature shift from 23 to 37°C leads to a rapid decline in RP mRNA levels that is temporary in wild-type (wt) cells (lanes 1 to 6) but permanent in cells with a temperature-sensitive (ts) mutation in the secretory pathway, exemplified by *ypt6-1* (lanes 7 to 13). This is not true of most non-RP genes, e.g., *ACT1*, or of the stable small nucleolar RNA (snoRNA) U3. These results could be explained by the hypothesis that the transcription of RP genes is extinguished after the temperature shift, temporarily in wt cells and permanently in cells with a defect in the secretory pathway. However, it has also been suggested that the loss of RP mRNA after a heat shock is due instead to an accelerated turnover of RP mRNA (15).

In an attempt to distinguish between these alternatives we have determined the half-life ($t_{1/2}$) of an RP mRNA when its transcription is halted in two distinct ways. In one case we employed strain Y260, which carries *rpb1-1*, a ts allele of the largest subunit of RNA polymerase II (Pol II); in such cells, RNA Pol II-dependent transcription is reduced to less than 10% within 2 min after a shift to 37°C (42, 43). When Y260 is transferred to the nonpermissive temperature, the mRNA derived from most RP genes declines with a $t_{1/2}$ of about 20 min (Fig. 1, lanes 14 to 20; Fig. 2; Table 2). The contrast between the effects of a secretory mutant (Fig. 1, lanes 7 to 13) and a polymerase mutant (lanes 14 to 20) (Fig. 2) supports the hypothesis that the turnover of RP mRNAs is accelerated due to heat shock in wt or *sec* cells.

To halt transcription in a way that does not depend on a temperature shift we employed modified *RPL30* in which the UAS was replaced with that of *GAL1*. When this gene is used as the sole source of L30, cells will grow only in the presence of galactose. In that case substantial *RPL30* mRNA is present (Fig. 3A). Once the galactose is replaced with glucose, transcription is immediately repressed (20, 30); the mRNA derived both from *GAL1-RPL30* (Fig. 2) and from the *GAL1* and *GAL10* genes themselves declines rapidly (Fig. 3A), with a $t_{1/2}$ of 5 to 7 min for the transcript encoding L30 and 3 to 5 min for the *GAL* genes (Fig. 3A and Table 2), as observed previously (3). *ACT1* mRNA and U3 RNA are unaffected. This result suggests that the $t_{1/2}$ for the *RPL30* mRNA is artificially extended in the *rpb1-1* strain. Indeed, when the *rpb1-1* strain is grown on galactose and shifted to the nonpermissive temperature, the $t_{1/2}$ of the *GAL1* and *GAL10* mRNAs is increased greatly, independent of whether the galactose has been replaced with glucose (Fig. 3B). Thus, the influence of *rpb1-1* on the measured $t_{1/2}$ of mRNAs is not limited to the transcripts of the RP genes.

Integrating the data of Fig. 1, 2, and 3 and Table 2 suggests the following scenario. A temperature shift from 23 to 37°C leads to an immediate, but temporary, repression of the transcription of RP genes. In wt cells, the transcription of RP genes resumes after about 20 min. In a *sec* mutant, however, the transcription of RP genes does not resume, and RP mRNA is reduced to a very low level. These are likely to be two separate phenomena because either the ablation of the protein kinase C

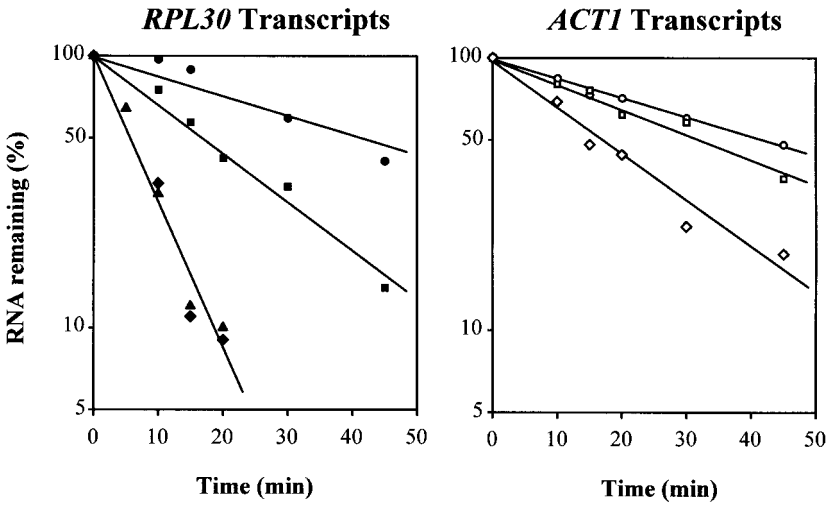

FIG. 2. A portion of the data used to generate Table 2. Cultures were handled as described in the text. The values represent the levels of the indicated RNA measured by PhosphorImager analysis, normalized to the amount of U3 RNA in that lane of the gel, and further normalized to 100% at the start of the experiment. Note that the repression of the *GAL1-L30* transcripts by glucose is indistinguishable from the repression of the *RPL30* transcripts by the secretory defect. The transcripts shown are as follows, with the method of repression in parentheses: ACT1-L30 (*rpb1-1*) (●), RPL30 (*rpb1-1*) (■), RPL30 (*ypt6-1*) (◆), GAL1-L30 (glucose) (▲), ACT1 (*rpb1-1*) (○), RPL30-ACT1 (*rpb1-1*) (□), and RPL30-ACT1 (*ypt6-1*) (◇).

pathway (40) or mutation of the silencing domain of Rap1p (35) relieves the repression of transcription due to a secretory defect without affecting the repression due to heat shock. The immediate kinetics of the repression due to a defect in the secretory pathway are obscured by the cell's response to heat shock. Nevertheless, we have found that the inhibition of the endoplasmic reticulum-Golgi communication with brefeldin A appears to repress the transcription of RP genes within 15 min (36), while direct stress on the plasma membrane, with the intercalating drug chlorpromazine, leads to repression almost immediately (40).

These data suggest that there is no need to invoke accelerated turnover (15) to explain the response of RP mRNA to a temperature shift. The $t_{1/2}$ of RP mRNAs observed in response to a temperature shift in either a wt or a *sec* cell is the same as that observed at 37°C when transcription is halted due to glucose repression of the *GAL1* promoter (Fig. 3A). However, the extended $t_{1/2}$ of both the RP and the GAL mRNAs when transcription is extinguished by the inactivation of RNA polymerase II suggests that this experimental approach may be having broader physiological effects on the RNA metabolism of the cell.

TABLE 2. $t_{1/2}$ of mRNA in strains of various genotypes at 37°C[a]

Genotype	$t_{1/2}$ of mRNA (min)		
	wt[b]	*ypt6-1*	*rpb1-1*
RPL3[c]	9.0	9.5	11
RPL8	8.5	7.5	20
RPS6	9.0	8.0	20
RPS28[c]	8.5	8.0	20
RPL30	7.5	7.0	20
GAL1-L30[d]	5.5		
GAL1-L30[d] (23°C)	11		
GAL1[d]	3.0		>20
GAL10[d]	2.5		>20
ACT1	23[e]		40
RPL30-ACT1		16	34

[a] The data are the averages of at least two but in most cases four experiments. They are reproducible within about ±10% except for the very short $t_{1/2}$ mRNAs, for which the determinations are sensitive to small variations in the way the experiment is carried out. These are reproducible to about ±1 min.
[b] $t_{1/2}$ in the first 30 min following a shift from 23 to 37°C (see the text).
[c] These promoters have Abf1p-binding sites (see the text).
[d] After shifting from YPGal to YPD (4% dextrose).
[e] From reference 14, carried out at 30°C; it would be shorter at 37°C.

RP promoter mediates repression. The experiments whose results are shown in Fig. 1 and 2 demonstrate an almost instantaneous and complete repression of RP gene transcription. What sequence elements of the RP genes are responsible for this repression? The promoter of *RPL30* (Fig. 4A) resembles the promoters of most RP genes, with two Rap1p binding sites as the major UAS, and one or two T-rich regions which have some promoter activity (41, 45, 47), followed by a less well defined region that contains the putative TATA box. Previous work has implicated the Rap1p binding sites as the elements mediating the regulation of transcription, in the response to a carbon source shift (7, 16), to amino acid starvation (37), and to cyclic AMP (cAMP) (25, 39), but not in the response to a temperature shift (41, 45).

To determine which of the elements in the promoter of RP genes mediate the repression of transcription in response to a defect in the secretory pathway we made two promoter swap constructs, in which the *RPL30* promoter drives the *ACT1* transcript (Fig. 4B) and the *ACT1* promoter drives the *RPL30* transcript (Fig. 4C). Primer extension demonstrated that the resulting *ACT1* transcripts were initiated at the same site as that for the endogenous *ACT1*; about half of the *RPL30* transcripts were initiated at the correct site, with the rest being initiated nine nucleotides downstream (data not shown). The *ACT1-RPL30* construct is not responsive to the *ypt6-1* mutation (Fig. 5, lanes 1 to 5). The *RPL30-ACT1* construct is responsive to the *ypt6-1* mutation, with the level of its transcript decreasing monotonically from the time of the temperature shift (Fig. 5, lanes 18 to 23, and Fig. 2). The rate of decline in *RPL30-ACT1* mRNA is lower than that of *RPL30*, with a $t_{1/2}$ of about 16 min, presumably because the intrinsic stability of the *ACT1* transcript is greater. Once again, the $t_{1/2}$ measured here is substantially shorter than that measured with the *rpb1-1*

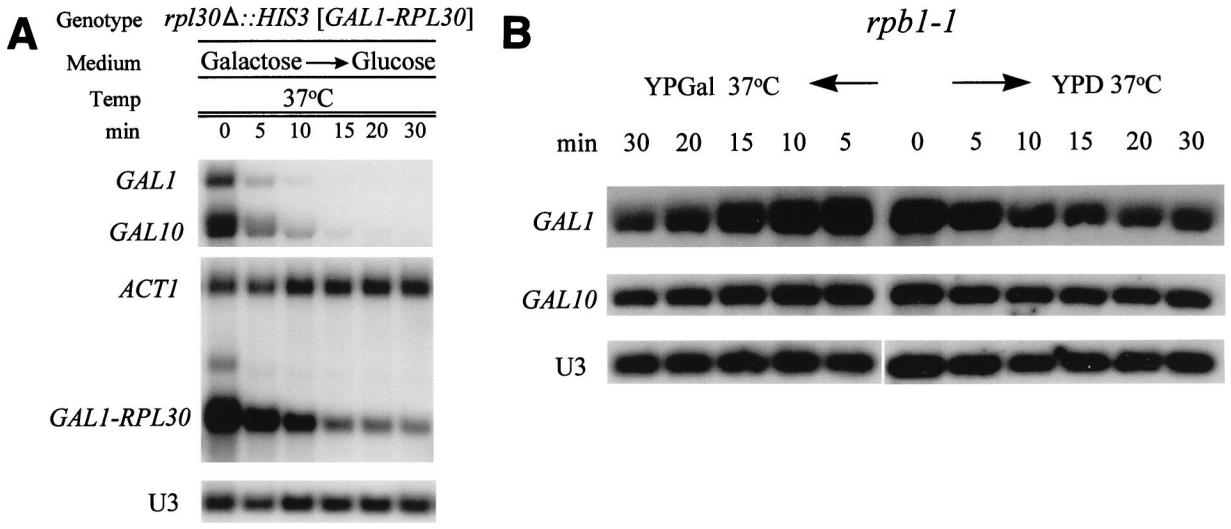

FIG. 3. (A) Direct measurement of the $t_{1/2}$ of the mRNA encoding L30. Strain JV7-2a (*rpl30*Δ::*HIS3* [pYE: *CEN, URA3 GAL1-RPL30*]) was grown at 37°C in YPGal medium and shifted to YPD (4% dextrose) as described in Materials and Methods. Cells were harvested just before and at intervals after the shift, and RNA was prepared and analyzed by Northern blotting as described previously. After PhosphorImager analysis, the $t_{1/2}$ of the *GAL1*, *GAL10*, and *GAL-RPL30* mRNAs was determined by using the stable U3 RNA as a loading control (Table 2). Note that the same experiment was carried out at 23°C, in which case the $t_{1/2}$ of the mRNAs was about twice as long. (B) Extended $t_{1/2}$ of the *GAL1* and *GAL10* mRNAs in an *rpb1-1* strain. Strain Y260 (*rpb1-1*) was grown in YPGal medium at 23°C. At zero time, one sample was taken; half the remaining culture was shifted to 37°C, and the other half was filtered and resuspended in YPD (4% dextrose), prewarmed, and maintained at 37°C. Samples were taken at the indicated times, and RNA was prepared and analyzed as for panel A.

mutation (Fig. 2 and Table 2), reinforcing the suggestion that *rpb1-1* has a broad effect on mRNA stability.

Rap1p binding sites mediate RP gene repression. If the *RPL30-ACT1* construct is repressed while the *ACT1-RPL30* transcript is not, the influence of the secretory pathway on *RPL30* must depend solely on sequences upstream of the transcription initiation site. In an attempt to identify which sequences are involved, the UAS of the *ACT1* promoter of construct A was replaced with fragments of the *RPL30* promoter (constructs B, C, and D shown in Fig. 4C). Plasmids carrying these genes were used to transform BL17, a diploid strain with the genotype *RPL30*/*rpl30*Δ::*HIS3 YPT6*/*ypt6-1*. Following sporulation and dissection of tetrads, strains carrying *ypt6-1* and with constructs A to D as the only source of L30 were identified (strains BL174 to BL177 in Table 1).

At the permissive temperature, the level of mRNA derived from each of the constructs was about the same (Fig. 5, zero time), suggesting that the two Rap1p sites (construct D) are sufficient to substitute for the UAS of *ACT1*. Upon a shift to the nonpermissive temperature, there is a rapid decline in the levels of mRNA derived from constructs B, C, and D. Clearly the presence of Rap1p binding sites, either with (construct B) or without (construct D) T-rich regions, makes the test gene responsive to the secretion defect. These results suggest that in this context the 40-bp sequence containing the two Rap1p binding sites is a sufficient *cis* element to effect repression in response to a defect in the secretory pathway.

It has been reported that Rap1p is degraded in cells depleted of cAMP (39), resulting in the reduced transcription of RP genes. To determine if limiting Rap1p lies behind the repression of RP transcription in response to a defect in the secretory pathway, we overexpressed Rap1p in both W303 and 169ts, by transforming each strain with a 2μm plasmid carrying wt *RAP1*. The shift of the transformants from 23 to 37°C led to a loss of RP mRNA that was indistinguishable from that shown in Fig. 1 and 5 (data not shown), suggesting that the repression of RP gene transcription is not due to limiting Rap1p.

The SIR complex is not involved in the repression induced by secretion defects. While Rap1p is a major transcriptional activator, it is also a major transcriptional silencer, at the silent mating type loci and at telomeric regions (reviewed in reference 49). In both cases, Rap1p recruits Sir3p and Sir4p to form a complex that inhibits the transcription of the adjacent genes (38). Since our results implicate Rap1p in mediating the repression of RP gene transcription, we asked if Sir3p and Sir4p are involved in the repression. The effects of tunicamycin on the mRNA levels of RP genes and others were determined in strains lacking components of the SIR complex (Fig. 6). It is apparent that the presence of tunicamycin leads to a substantial repression of the transcripts of both *RPS6* and *RPL30*, with little effect on *ACT1* or *PYK1*. The deletion of *SIR2*, *SIR3*, or *SIR4* does not alter the repression of the RP genes. The induction of *KAR2* is an expression of the "unfolded protein response" demonstrating that the tunicamycin is active on these cells (4).

As a more direct test, strains containing null alleles of either *SIR3* (YDS430) or *SIR4* (YDV122) were crossed with 169ts, the strain carrying *ypt6-1*. From each diploid, we selected a tetrad that provided the four combinations of the *YPT6* and *SIR3* alleles (strains BL180 to BL183; Table 1) or the *SIR4* allele (strains BL185 to BL188; Table 1). If either Sir3p or Sir4p were essential for the repression, its absence would eliminate the repression of RP mRNAs in cells carrying the *ypt6-1* allele. As shown in Fig. 7, the repression of the transcription of RP genes, induced either in *YPT6* cells by heat shock or in *ypt6-1* cells by a failure in the secretory pathway, depends neither on Sir3p nor on Sir4p.

Sir2p is not necessary for repression of either rRNA or RP genes. Sir2p is another participant in the repression of the silent mating type loci, although its relationship to Rap1p is less clear than that of Sir3p and Sir4p. Surprisingly, Sir2p has

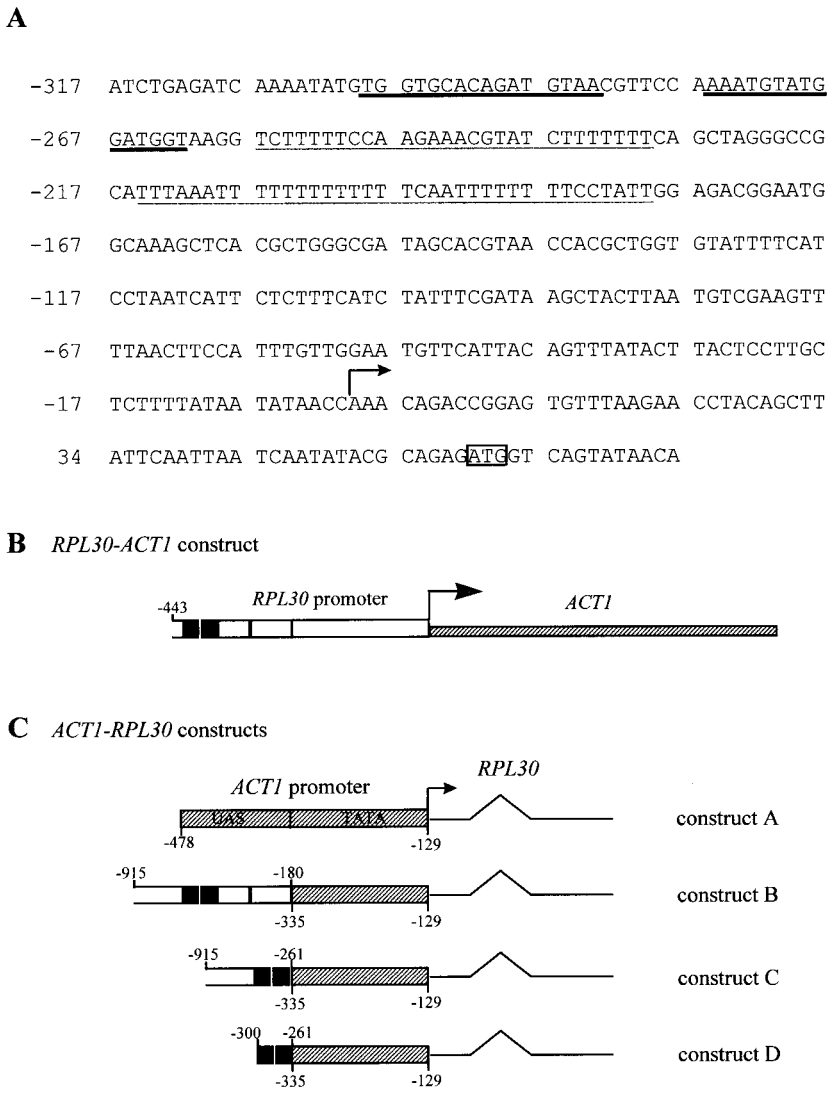

FIG. 4. (A) The promoter of *RPL30*. The start codon is boxed. The two Rap1p binding sites are indicated by a heavy underline, and the two T-rich regions are indicated by a light underline. The initiation of transcription, termed +1 and marked with an arrow, is 58 nucleotides upstream of ATG. (B) The *RPL30-ACT1* fusion gene. See Materials and Methods for details. (C) Constructing the fused gene with the *ACT1* promoter driving the *RPL30* transcript (constructs A to D); see Materials and Methods. The stippled area represents sequences from *ACT1*. Nucleotides from the *RPL30* promoter were fused to the *ACT1* TATA region to form constructs B, C, and D. The hatched boxes are *ACT1* sequences; the open boxes are *RPL30* sequences; the black boxes represent the *RPL30* Rap1p binding sites. The line represents the L30 transcript. The nucleotide boundaries of the *RPL30* sequences are shown above the constructs, and those of the *ACT1* sequences are shown below the constructs. Because the *ACT1* gene has multiple sites of transcription initiation, the numbering is in reference to ATG of the coding region.

recently been shown to participate in the silencing of a Pol II-transcribed gene within the ribosomal DNA locus (52), although it has not been implicated directly in the control of rRNA transcription. To determine whether Sir2p plays a role in the repression of ribosome synthesis, we deleted *SIR2* or its close relative *HST1*, which can partially substitute for *SIR2* in silencing (5), from a strain carrying a ts mutation in *sly1*, an essential component of the secretory pathway (36), yielding strains JW1210 and JW1211. The results of shifting these two strains to the nonpermissive temperature are shown in Fig. 8. In this case, the cells were grown in a medium lacking methionine and pulsed for 3 min with [C^3H_3]methionine as a measure of rRNA transcription. RNA prepared from the cells was separated on an acrylamide gel, the upper portion was subjected to fluorography, and the lower portion was subjected to Northern analysis. From the latter it is apparent that the mRNA for L30 disappears rapidly, just as in the *ypt6-1* strain shown in Fig. 1. C^3H_3 groups are incorporated predominantly into 35S rRNA, which is then processed through the intermediate 27S and 20S species to the mature 25S and 18S rRNAs (56). In a 3-min pulse (Fig. 8, 0-min lanes), most of the radioactivity has already passed into the 27S and 20S intermediates, and some is in mature 18S rRNA. We have previously shown that the transfer to the nonpermissive temperature causes a rapid repression of rRNA transcription in *sly1-1* cells, with little effect on wt cells (36). Similarly, the transfer of the *sly1-1 sir2*Δ or the *sly1-1 hst1*Δ double mutant strains to the nonpermissive temperature leads to a strong inhibition of the incorporation of C^3H_3 into rRNA (Fig. 8, 20- and 60-min lanes), just as for the cells carrying *sly1-1* alone. What little 35S RNA is formed is processed slowly if at all, presumably due to a lack of RPs. We conclude that neither Sir2p nor Hst1p is involved in

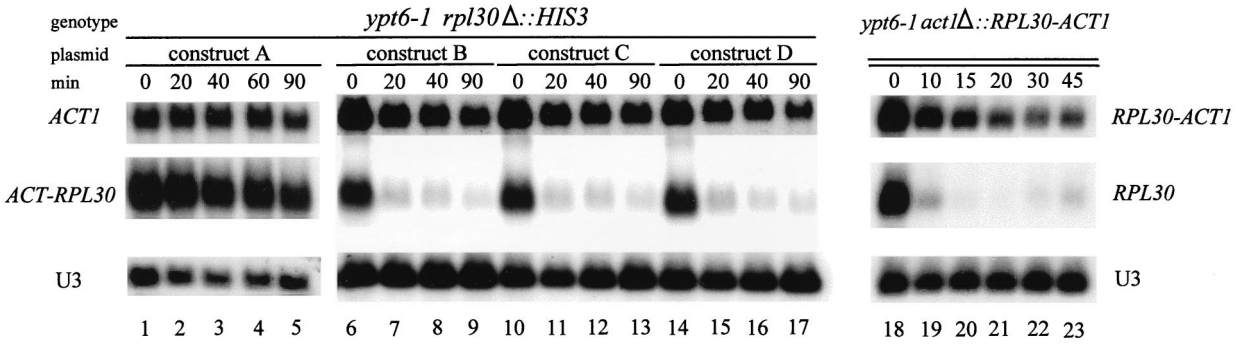

FIG. 5. Rap1p binding sites mediate the repression of RP mRNAs. *ypt6-1 rpl30*Δ::*HIS3* strains containing constructs A to D (strains BL174 to BL177; Table 1) and strain JW1201, in which *RPL30-ACT1* is the only source of actin sequences, were grown to log phase at 23°C and then shifted to 37°C. Aliquots were harvested at the indicated times, and RNA was prepared and analyzed as described previously.

the repression of ribosome synthesis in response to a defect in the secretory pathway.

Non-Rap1p binding sites also mediate RP gene repression. Although Rap1p binding sites contribute most of the transcriptional activation of RP genes, some activity remains after the deletion of the Rap1p binding site, due to the T-rich elements (41, 45, 47). This residual activity of the RP genes still responds to heat shock, implicating sequences other than the Rap1p sites in the regulation of transcription of these genes. To identify such sequences and to determine if they are also involved in the response to a secretion defect, we developed a series of constructs based on the reporter gene *RPL30-GFP*, in which the ORF of GFP replaced the ORF of L30, starting with amino acid 4 in the second exon of *RPL30* (Fig. 9A, construct E). The spliced transcripts contain the 5′ untranslated region of *RPL30*, seven nucleotides from the *RPL30* ORF, the GFP ORF, and the 3′ untranslated region of *RPL30*. Note that the repression of *RPL30* is independent of sequences downstream of the transcription initiation site (*ACT-RPL30*; Fig. 5, lanes 1 to 5). Increasing portions of the *RPL30* promoter were replaced with four Gal4p binding sites derived from the *GAL1* promoter, generating constructs F, G, and H (Fig. 9A). The reporter gene constructs, on *CEN*-based plasmids, were transformed into cells carrying the *ypt6-1* allele. The necessary L30 protein is supplied by the genomic *RPL30*. Since the GFP ORF is 400 nucleotides longer, the *RPL30* and *RPL30-GFP* mRNAs can be compared directly on Northern blots by using a probe complementary to the first exon of *RPL30*.

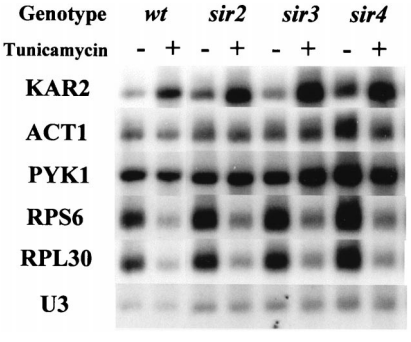

FIG. 6. The SIR complex does not mediate the repression of RP genes in response to a defect in the secretory pathway. Cultures of strains YDS2 (wt), YDS714 (*sir2*Δ), YDS430 (*sir3*Δ), and YDV122 (*sir4*Δ) were grown in YPD at 30°C. One half of each culture was treated with 1 μg of tunicamycin per ml for 2 h. All eight cultures were harvested on crushed ice, and RNA was prepared and subjected to Northern analysis, by using several probes.

For the first set of experiments the cells were grown in glucose to repress transcription from the *GAL1* region, thereby revealing the transcriptional contribution of the residual *RPL30* sequences (Fig. 9B). In a *ypt6-1* background, following a temperature shift, construct E (*RPL30-GFP*) is repressed as severely as endogenous *RPL30*, confirming that the promoter of the RP genes mediates the repression caused by a failure in the secretory pathway. Deletion of the two Rap1p binding sites, leaving the T-rich domains (construct F), leads to the loss of 90% of the transcriptional activity. The 10% of the activity remaining is repressed as severely as endogenous *RPL30*. The presence of a single T-rich domain (construct G) has only 5% of normal transcription, also repressible. Deletion of both Rap1 sites and both T-rich domains (construct H) leads to a loss of any detectable transcription. These results confirm that the T-rich region can activate some transcription of *RPL30*, consistent with the observations for the other RP genes *RPL28*, *RPS14*, and *RPL25* (41, 45, 47). Nevertheless, upon a temperature shift whatever residual transcription that remains is repressed in the *ypt6-1* mutant (Fig. 9B). This result, in the absence of Rap1p sites, implicates the remaining downstream elements of the *RPL30* promoter in the repression of transcription.

Growth of these strains in a medium containing galactose permits Gal4p binding to the sites present in constructs F, G, and H, activating transcription, at the permissive temperature, to slightly higher levels than with the *RPL30* promoter itself (constructs F, G, and H; Fig. 9C). Nevertheless, when the cells are shifted from 23 to 37°C, the transcription of constructs F, G, and H is strongly repressed. The level of *GAL1* transcripts is not dramatically affected, at least at 20 and 40 min after the temperature shift (Fig. 9C). Since the $t_{1/2}$ of *GAL1* transcripts is <5 min (3) (Fig. 3A), Gal4p-promoted transcription must be continuing. Comparison of construct E with constructs F, G, and H suggests that a defect in the secretory pathway can largely, though not completely, repress the transcription of an RP gene that is driven by a novel activator, in this case Gal4p. As the results for constructs F and H barely differ, we again conclude that the T-rich regions play little role in the repression of transcription. Thus, the repression of construct H suggests that the 180 bp that lie between the T-rich elements and the origin of transcription of *RPL30* contain sequence elements that are sufficient for a major proportion of the *sec*-dependent repression of transcription of an RP gene. Comparing the data from Fig. 5 and 9 leads us to conclude that either the Rap1p sites or the 180-bp region will respond to the repression effected by a failure in the secretory pathway. In a different context, Neuman-Silberberg et al. (39) also concluded

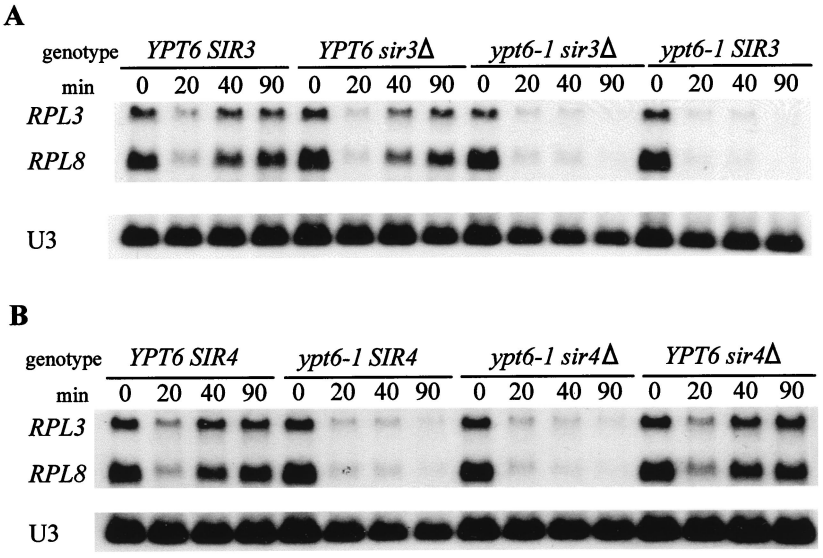

FIG. 7. Neither Sir3p (A) nor Sir4p (B) is involved in the repression of RP gene transcription in a *sec* mutant or during heat shock. Strains of the indicated genotypes (Table 1) were grown to log phase in YPD at 23°C. An aliquot was harvested, the cultures were shifted to 37°C, and aliquots were harvested at the indicated times. RNA was prepared and analyzed as described previously, except that all RNAs were detected with ^{32}P-labelled oligonucleotide probes.

that Rap1p-binding sites and downstream sequences could play separate roles in the regulation of RP transcription.

Is TAF_{145} responsible for the repression of RP gene transcription? A recent report suggests that the transcription of many of the RP genes is particularly vulnerable to a mutation in the transcription factor $TAF_{II}145$ (48). Perhaps $TAF_{II}145$ is responsible for the effects of the 180-nucleotide region implicated by construct H (Fig. 9B), since this region contains the TATA box with which $TAF_{II}145$ is presumably associated. However, while both *IPP1* (48) and *ACT1* (our unpublished data) are severely repressed by ts mutants of $TAF_{II}145$, they are not repressed by a ts mutant in the secretory pathway (*IPP1*; data not shown). Finally, a genome-wide analysis of the transcriptional effects of a ts allele of $TAF_{II}145$ suggests little specificity for RP genes (17). Thus, it appears that $TAF_{II}145$ is not the agent responsible for the repression of transcription of the RP genes under these conditions.

DISCUSSION

Turnover of RP mRNA. The determination of the intrinsic $t_{1/2}$ of an mRNA presents several experimental uncertainties. The most direct but most demanding method is the approach to equilibrium, which requires not only sensitive hybridization methods but also the determination of the approach to equilibrium of the nucleotide pools (12, 23). Alternate methods require turning off the transcription of all mRNA, using either inhibitors or the ts allele of *rpb1* (reviewed in reference 2). The former have proved inconsistent in yeast. The latter involves three perturbations of the cell: the raising of the temperature, which brings the heat shock response into play, the inhibition of any mRNA that might play a direct role in the $t_{1/2}$ of a specific mRNA, and the gradual loss of all the cell's mRNAs, which can change the dynamic of translation and, consequently, of turnover. A more specific approach is the use of a repressible promoter, such as *GAL1*, with which one can turn off the expression of a limited number of genes.

It is clear from Fig. 1, 2, and 3, as well as from Table 2, that the decline of RP mRNA after a temperature shift, in either wt or *sec* cells, is similar to that observed when the RP mRNA is under the control of the *GAL1* promoter that is suddenly repressed. This result has two major implications. It suggests that a heat shock temporarily represses and a *sec* mutant permanently represses RP transcription to nearly the same degree as glucose does the Gal4p-driven genes. It also suggests that the intrinsic $t_{1/2}$ of RP mRNAs can be estimated from the decline of the mRNA after a heat shock; for most RP mRNAs that value would be <10 min at 37°C (9, 61). For some genes we have made an independent determination of the $t_{1/2}$, based on approach-to-equilibrium labelling; for *RPL3* it is 13 min, and for *RPL30* it is 16.6 min (23). (Note that those two genes were known as Rp1 and Rp73, respectively, at that time.) This

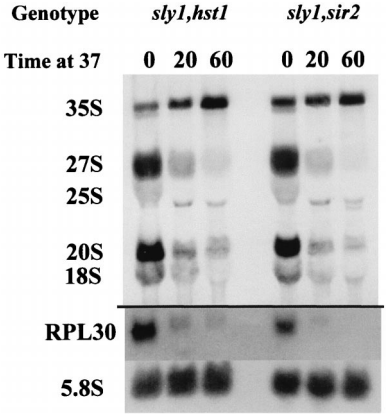

FIG. 8. Neither Sir2p nor Hst1p is involved in the repression of ribosome synthesis in response to a defect in the secretory pathway. Cultures of strains JW1210 and JW1211 were grown in methionine-free dropout medium at the permissive temperature of 23°C. At zero time an aliquot was labelled with 60 μCi [C^3H_3]-methionine for three minutes and poured onto crushed ice. The remainder of the culture was shifted to 37°C, and aliquots were similarly pulsed with [C^3H_3]-methionine after 20 and 60 min. RNA was prepared, and equal amounts were subjected to polyacrylamide gel electrophoresis. The upper portion of the gel was impregnated with En^{3}Hance and subjected to fluorography to show the incorporation of C^3H_3 groups into rRNA. The lower portion was used for Northern analysis with probes against *RPL30* and against 5.8S rRNA as a loading control (5.8S rRNA has no methyl groups).

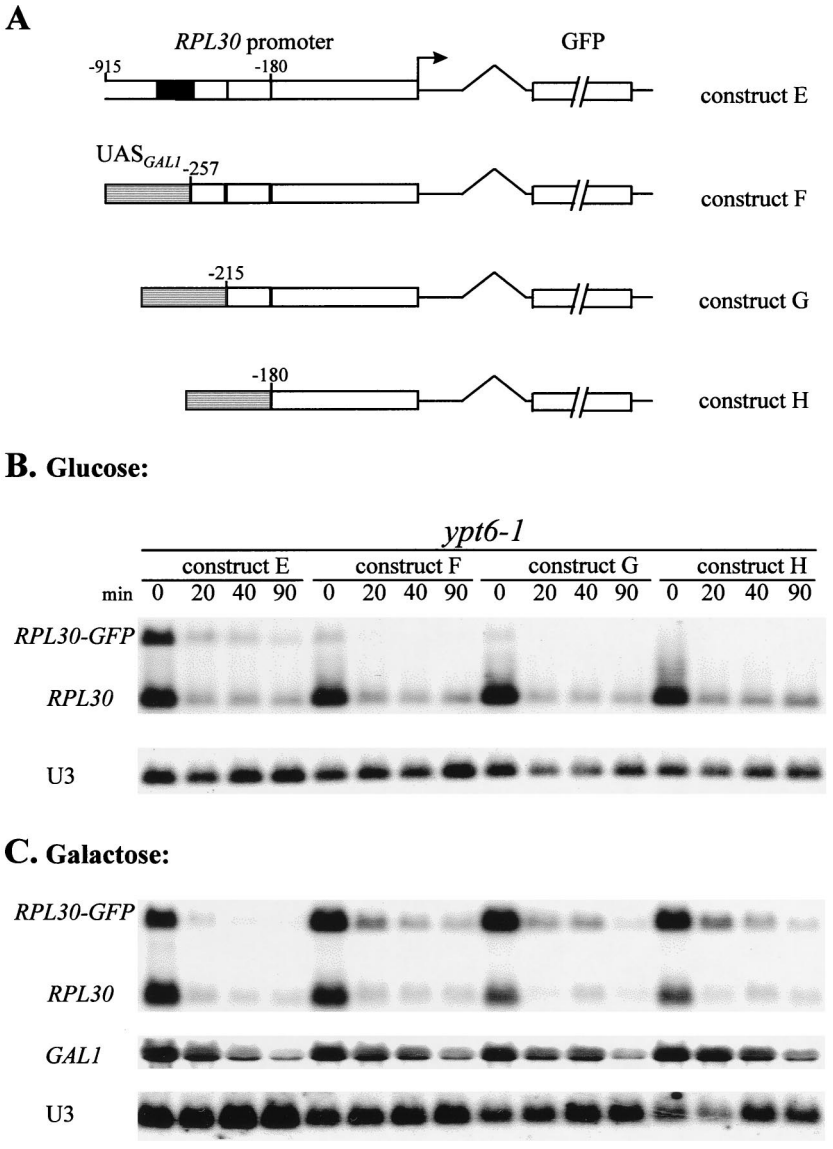

FIG. 9. (A) Construction of fused genes between *GAL1* and *RPL30-GFP* (constructs E to H). The stippled area represents sequences from the *GAL1* UAS. The nucleotide boundaries of the *RPL30* sequences are shown, and numbering follows the conventions described in the legend to Fig. 3A. The black boxes represent the *RPL30* Rap1p binding sites. (B) The 180-bp *cis* element mediates the repression of RP gene transcription in a *sec* mutant. *ypt6-1* cells carrying constructs E to H were grown to log phase in uracil-free medium containing 2% glucose at 23°C. An aliquot was harvested, the rest of the cultures were shifted to 37°C, and aliquots were harvested at intervals. Total RNA was isolated and analyzed as described previously. mRNAs for *RPL30* and *RPL30-GFP* were detected with an RNA probe that is complementary to the first exon of *RPL30*. The snoRNA U3 was detected with an oligonucleotide probe. (C) The same as panel B except that cells were grown in 2% galactose to induce the *GAL1* UAS. *GAL1* mRNA was detected with an antisense RNA probe.

was carried out on cells growing in a synthetic medium at 23°C with a doubling time of 132 min, and thus the values are likely to be an underestimate compared to those based on cells growing in YPD at 37°C. Indeed, in our studies the $t_{1/2}$ of the *GAL1-RPL30* mRNA increases from 5 to 11 min as the temperature is lowered from 37 to 23°C (Table 2).

We suggest, therefore, that the intrinsic $t_{1/2}$ of the mRNA encoding L30 is between 5 and 7 min at 37°C. Thus, the decline of this mRNA after cells are shifted from 23 to 37°C can be ascribed solely to a repression of transcription, without the need to invoke an activation of turnover (15).

The data shown in Fig. 1 to 3 also suggest that the use of the *rpb1-1* mutant may lead to severely misleading estimates of the $t_{1/2}$ of mRNAs. This could be either a general effect due to the pleiotropic consequences of halting all Pol II transcription or a specific effect on the genes subject to severe repression.

One might ask why the $t_{1/2}$ of RP mRNAs is so short, since replacing them at frequent intervals seems an unnecessary use of resources. Indeed, the $t_{1/2}$s of the mRNAs encoding the abundant glycolytic enzymes are much longer (14). An explanation may be that the level of production of RPs must be closely monitored (27). Because the RPs participate in the assembly of a complex structure and because they are generally strong RNA-binding proteins, an excess of an RP may be far more deleterious to the cell than an excess of a glycolytic enzyme. Therefore, it seems likely that there is selective pressure to maintain a short $t_{1/2}$ for RP mRNAs in order to more closely control the relative production of the many RPs.

General considerations regarding transcription of RP genes. Before discussing our data regarding the control of transcription of RP genes, we argue on two grounds, magnitude and coordination, for the potential special nature of RP gene transcription and thus for the likelihood that it has unusual features.

(i) The transcription of ribosomal proteins is a major portion of the Pol II activity of the cell. As measured by SAGE analysis (57), RP genes account for 20 of the 30 most abundant mRNAs; each RP gene is represented, on average, by about 30 to 50 mRNAs per cell (our analysis of data provided by the authors of reference 57). The 137 RP genes, therefore, would account for >4,000 of the mRNAs in the cell. A more direct measurement has recently been reported by Holstege et al. (17). Based on an estimate of 15,000 mRNAs per cell, they determined that 132 of the 137 RP genes contributed 4,437 mRNAs, about 30% of the total. This is a reasonable number because the ~15,000,000 ribosomal proteins (~200,000 ribosomes/cell × 78 proteins/ribosome) make up about 15% of the protein mass of the cell, and even more of the protein number, since they average only ~150 amino acids in length. As shown in Table 2, the $t_{1/2}$ of an RP mRNA is 5 to 7 min. This value is consistent with our observations of the effect of heat shock on the mRNA levels of many RP genes (11). It is also consistent with the data recently reported by Eisen et al. (9), in which the mRNA level for most RP genes had declined to less than 20% of normal by 20 min after a heat shock. Yet, mRNAs encoding most other genes have a $t_{1/2}$ of >15 min (17) (based on the use of the *rpb1-1* allele, which admittedly may be misleading). Thus, if RP mRNAs account for 30% of the total mRNAs yet have a $t_{1/2}$ that is substantially shorter than those of most other mRNAs, we are led to conclude that the RP genes account for nearly 50% of all Pol II initiation events.

(ii) As would be expected for genes encoding components of a molecular machine, the 137 RP genes appear to be regulated in lockstep (reviewed in reference 44). This is true of responses to heat shock (11, 24), to a defect in the secretory pathway (34 and this paper), to growth conditions such as C source (16, 22), to levels of cAMP (25, 39), and to the deprivation of amino acids (37, 62). During the growth cycle, RP genes are repressed as the cells enter late log phase (6, 21) and are induced dramatically within 10 min after stationary cells are diluted into a fresh medium (unpublished data). By using classical methods no exceptions have been found among the 20 or so proteins whose mRNAs have been studied or among the 50 or so proteins whose synthesis has been studied in a few situations (11). Very recent data from a genome-wide analysis of a few conditions, e.g., heat shock (9) and diauxie (6), suggest that none of the RP genes escapes from this coordinate regulation, although a few genes with apparently intermediate results will require more direct analysis.

It is intriguing to consider why *S. cerevisiae* has evolved to utilize transcription as its primary method to regulate the production of RPs, while both eubacteria (reviewed in reference 64) and vertebrates (reviewed in reference 33) have chosen to regulate RP synthesis largely at translation, albeit in very different ways. Indeed, a recent transcriptome analysis of mouse fibroblasts during the transition from stationary to growth phase found almost no change in the levels of RP mRNAs (19), although there is a substantial increase in the rate of RP synthesis (55).

Role of Rap1p in the repression of RP gene transcription. Rap1p is the major factor activating the transcription of nearly all of the RP genes, as well as many other genes with abundant transcripts, such as those encoding elongation factor 1α (EF1α) and the enzymes of the glycolytic pathway. Rap1p is also the primary element of the complex that silences both the silent mating type loci and genes adjacent to telomeres (49). Therefore, Rap1p is a likely candidate for effecting the silencing of the RP genes in response to stress. Indeed, we find that just 40 bp containing the two Rap1p binding sites of the *RPL30* gene are sufficient to make the *ACT1* promoter respond like an RP gene to a defect in the secretory pathway (Fig. 5, construct D). Furthermore, in cells carrying the *rap1-17* allele, which encodes a truncated form of Rap1p that retains both its DNA binding domains and its activation domain but not its silencing domain, the RP genes are no longer silenced in response to a defect in the secretory pathway (35), although temporary silencing in response to a temperature shift still occurs. This effect of the *rap1-17* allele is also true for genes that have no Rap1p binding sites, such as *RPL3* (35) and construct G shown in Fig. 9A (data not shown). This observation not only implicates Rap1p in the pathway between the secretory system and the RP genes but also suggests that a different pathway is used for the repression of RP gene transcription in response to a temperature shift.

Yet several facts suggest that conventional silencing by Rap1p is not responsible for the repression of RP genes. Abf1p, rather than Rap1p, is the major transcription factor for several RP genes that are also repressed in response to a defect in the secretory pathway, e.g., *RPL3* (Fig. 1). In addition, Rap1p-mediated silencing at telomeres and at silent mating type loci requires Sir3p and Sir4p (31), neither of which is necessary for the silencing of RP genes (Fig. 6). Finally, sequences downstream of the Rap1p binding sites, adjacent to the promoter of *RPL30*, will silence the transcription driven by Gal4p, in response to a temperature shift or to a defect in the secretory pathway (Fig. 9). These sequences contain the putative TATA box and presumably bind TBP and its associated TAFs, as well as the sequences adjacent to the transcription initiation site.

Thus, our attempts to identify the *cis*-acting sequences of RP genes that mediate the repression of transcription have led to an apparent contradiction. Either the Rap1p binding sites or the promoter-proximal sequences can play such a role. Yet the effect of the *rap1-17* allele suggests that Rap1p is necessary for silencing in response to a defect in the secretory pathway. Our conclusion, then, is that for Rap1p to activate the transcription of an RP gene it must bind to upstream sequences, yet for Rap1p to repress the transcription of an RP gene it need not bind to the gene directly. It remains to be seen how this fascinating protein can pull off such a trick.

Whatever the mechanism, the sudden silencing of the RP genes, which account for 50% of Pol II activity, must have a dramatic effect on the overall transcriptional economy of the cell. What influence does this sudden release of transcriptional potential have on the transcription of other genes?

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