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Association of RAP1 Binding Sites with Stringent Control of Ribosomal Protein Gene Transcription in *Saccharomyces cerevisiae*

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An amino acid limitation in bacteria elicits a global response, called stringent control, that leads to reduced synthesis of rRNA and ribosomal proteins and increased expression of amino acid biosynthetic operons. We have used the antimetabolite 3-amino-1,2,4-triazole to cause histidine limitation as a means to elicit the stringent response in the yeast *Saccharomyces cerevisiae*. Fusions of the yeast ribosomal protein genes *RPL16A*, *CRY1*, *RPS16A*, and *RPL25* with the *Escherichia coli lacZ* gene were used to show that the expression of these genes is reduced by a factor of 2 to 5 during histidine-limited exponential growth and that this regulation occurs at the level of transcription. Stringent regulation of the four yeast ribosomal protein genes was shown to be associated with a nucleotide sequence, known as the UAS_{rpg} (upstream activating sequence for ribosomal protein genes), that binds the transcriptional regulatory protein RAP1. The RAP1 binding sites also appeared to mediate the greater ribosomal protein gene expression observed in cells growing exponentially than in cells in stationary phase. Although expression of the ribosomal protein genes was reduced in response to histidine limitation, the level of RAP1 DNA-binding activity in cell extracts was unaffected. Yeast strains bearing a mutation in any one of the genes *GCN1* to *GCN4* are defective in derepression of amino acid biosynthetic genes in 10 different pathways under conditions of histidine limitation. These Gcn^- mutants showed wild-type regulation of ribosomal protein gene expression, which suggests that separate regulatory pathways exist in *S. cerevisiae* for the derepression of amino acid biosynthetic genes and the repression of ribosomal protein genes in response to amino acid starvation.

The stringent response of *Escherichia coli* has been extensively studied over the last 40 years (reviewed in reference 10). When *E. coli* are deprived of one or more of the 20 common amino acids, the expression of rRNA and ribosomal protein (rp) genes is reduced (negative stringent control) while that of amino acid biosynthesis and transport genes is increased (positive stringent control). Negative stringent control can be viewed as an attempt to conserve amino acids, in that continued synthesis of the translational apparatus is superfluous during amino acid starvation; positive stringent control can be viewed as an attempt to replenish amino acids. The signal for the stringent response is an accumulation of uncharged tRNA, not the lack of amino acids per se. This signal is transduced into the second messenger ppGpp (magic spot) by the *relA* gene product. Possibly all aspects of the stringent response are governed by the intracellular concentration of magic spot (10, 55). Aspects of the stringent response have been demonstrated in other bacteria (10) as well as in some eukaryotes (e.g., references 35, 47, and 67), although none of the studies with eukaryotes identified a second messenger such as ppGpp (8, 31, 46).

In the yeast *Saccharomyces cerevisiae*, a phenomenon analogous to positive stringent control is known as general amino acid control (reviewed in reference 21). In response to amino acid starvation, derepression of the transcriptional activator protein GCN4 leads to increased expression of more than 30 amino acid biosynthetic enzymes in 10 different pathways. The increase in *GCN4* expression under starvation conditions occurs at the translational level and requires the *GCN1*, *GCN2*, and *GCN3* gene products. The products of several *GCD* genes (21), as well as the α and β subunits of the translational initiation factor eIF-2 (70), are required for repression of *GCN4* translation under nonstarvation conditions. For general amino acid control, the signal may be uncharged tRNA since partial inactivation of an aminoacyl-tRNA synthetase leads to derepression of amino acid biosynthetic enzyme synthesis on rich medium (39). No second messenger has been identified, but the starvation signal may be recognized and transduced by the positive regulator GCN2 because it functions as a protein kinase in stimulating *GCN4* expression (52, 69) and contains a domain similar in sequence to histidyl-tRNA synthetases (68). Given the large body of literature on general amino acid control, we will use the term stringent control in *S. cerevisiae* only to mean negative regulation of ribosomal components.

The kinetics and magnitude of the stringent response in *S. cerevisiae* have been described in some detail (37, 44, 60, 66, 67). Again, the starvation signal appears to be uncharged tRNA. In response to amino acid deprivation or partial inactivation of an aminoacyl-tRNA synthetase, rRNA synthesis is inhibited by a factor of 3 to 6. For 16 of 20 ribosomal proteins studied, accumulation of translatable mRNA was reduced by a factor of 2 to 4 in response to amino acid starvation (67). So far, only one mutant defective for the stringent response (relaxed mutant) has been reported, but the molecular basis of its defect is not understood (62, 65).

We have initiated new studies on the stringent response of yeast cells by using experimental protocols that were applied successfully to the analysis of general amino acid control. Instead of imposing an absolute starvation by culturing an amino acid auxotroph on medium lacking the required amino acid, we used the antimetabolite 3-amino-1,2,4-triazole (3AT) to limit histidine availability. Histidine limitation caused by the addition of 3AT resulted in the cultures

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TABLE 1. *S. cerevisiae* strains

Strain	Relevant genotype	Alias or source
F113	*MATa can1 ino1-13 ura3-52*	TD28 (13)
H1314	*MATa can1 ino1-13 ura3-52 [RPL16A-lacZ URA3]*[a]	This study
H1519	*MATa can1 ino1-13 ura3-52 pep4::URA3*[a]	P. Miller
H1528	*MATa can1 ino1-13 ura3-52 [RPL25-CYC1-lacZ URA3]*[a]	This study
F35	*MATa can1 ino1-13 ura3-52 [HIS4-lacZ URA3]*[a]	L1511 (34)
H1049	*MATa gcn1-1 ura3-52*	This study
H1146	*MATa gcn1-1 leu2-3,-112 ura3-52 [HIS4-lacZ ura3-52]*	This study
H15	*MATα gcn2-1 leu2-3,-112 ura3-52*	L866 (34)
H1317	*MATα gcn2-1 leu2-3,-112 ura3-52 [CRY1-lacZ URA3]*[b]	This study
H1318	*MATα gcn2-1 leu2-3,-112 ura3-52 [RPL16A-lacZ URA3]*[b]	This study
F66	*MATa gcn2-1 ino1-13 ura3-52 [HIS4-lacZ URA3]*	L1636 (34)
H17	*MATα gcn3-102 leu2-3,-112 ura3-52*	L868 (34)
F69	*MATa gcn3-102 ino1-13 ura3-52 [HIS4-lacZ URA3]*	L1642 (34)
H24	*MATα gcn4-101 ura3-52*	L1356 (34)
F47	*MATa gcn4-101 ino1-13 ura3-52 [HIS4-lacZ URA3]*	L1583 (34)

[a] Isogenic to F113.
[b] Isogenic to H15.

growing exponentially with a doubling time that was threefold longer than in the absence of 3AT. We found that expression of several fusions of yeast rp genes with the *E. coli lacZ* gene was repressed under these partial starvation conditions and that this stringent control was similar to general amino acid control in both the kinetics and magnitude of the response.

Whereas general amino acid control is mediated by the transcriptional activator GCN4, stringent control appeared to be mediated by a different transcriptional factor known as RAP1. A RAP1 binding site (known as the RPG box, HOMOL1, and UAS_{rpg} [upstream activating sequence for rp genes]) is known to be required for rp gene expression during growth under nonstarvation conditions (51, 72) and to mediate a three- to fourfold increase in rp gene expression when glucose is added to a culture growing on a nonfermentable carbon source (20). Our results demonstrated that a pair of RAP1 binding sites from either of two different rp genes is sufficient to confer stringent control upon a heterologous yeast promoter. In contrast to GCN4, the level of which increases in response to amino acid starvation, we found that the level of RAP1 DNA-binding activity in vitro was unaffected by partial starvation for histidine.

In bacteria, mutants that fail to respond to amino acid starvation are called relaxed (Rel^-). All known Rel^- mutants in *E. coli* are defective for both negative and positive stringent control (reviewed in reference 10). In contrast to this, we found that yeast mutants that are defective for derepression of amino acid biosynthetic genes subject to general amino acid control (*gcn1*, *gcn2*, *gcn3*, and *gcn4*) have wild-type regulation of rp genes in response to amino acid levels. These results suggest that separate pathways exist in *S. cerevisiae* to mediate the positive and negative aspects of stringent control.

MATERIALS AND METHODS

Chemicals. Dextrose and 3AT were from Sigma Chemical Co. (St. Louis, Mo.); yeast nitrogen base was from Difco (Detroit, Mich.); Nuseive GTG agarose was from FMC Corp. (Rockland, Maine); protease inhibitors and ammonium sulfate were from Boehringer Mannheim (Indianapolis, Ind.); poly(dI/dC) was from Pharmacia (Piscataway, N.J.). Oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer.

Strains and genetic methods. Yeast strains are listed in Table 1; all are congenic with the common laboratory strain S288C. The *gcn1-1* allele (56) was introduced into H1049 and H1146 by genetic crosses and was confirmed by tetrad analysis, using sensitivity to 3AT as the phenotypic marker for the mutation. The *pep4::URA3* allele (1) in H1519 was introduced into F113 by transformation (45) and confirmed both by observing the loss of APE (*N*-acetyl-DL-phenylalanine β-naphthyl ester) hydrolytic activity (26) and by DNA blot hybridization analysis (61). Yeast cells were transformed by the lithium acetate method (25). Plasmids containing *lacZ* fusions were directed to integrate at *ura3-52* by linearizing the plasmids (45) in the *URA3* gene by digestion with *Sma*I, except for YIp55-669Z, which was digested with *Stu*I. The location and copy number of integrated plasmids were confirmed by DNA blot hybridization analysis. Except where noted, only transformants with a single integrated copy of the plasmid were used. *E. coli* DH5α was used for propagation of plasmids.

Plasmids. The *RPL16A-lacZ* fusion plasmid (pMR10) and the series of promoter deletion plasmids derived from it (see Fig. 6) (51), *RPL16A-CYC1-lacZ* (pMR672A; 51), *CRY1-lacZ* (pMR20; 49), *RPS16A-lacZ* (YIp55-669Z; 13), and *CYC1-lacZ* (pLG669Z; 16) have been described. Plasmids pMR10, pMR672A, pMR20, and pLG669Z are derivatives of YEp24 (9); these episomal plasmids were converted to the integrating plasmids pCM40, pCM60, pCM39, and pCM61, respectively, by deleting most of the 2μm DNA by digestion with *Spe*I followed by recircularization using T4 DNA ligase. The UAS_{rpg}-*CYC1-lacZ* fusions described in Fig. 7 were constructed by first replacing the 453-bp *CYC1 Xho*I fragment with the *Bgl*II-*Xho*I linker oligonucleotide (Fig. 1) to make pCM81; pCM83 was made in the same way except that the linker was inserted in the opposite orientation. The oligonucleotides L16, L25, and L25-mutant (Fig. 1) were cloned into the *Bgl*II-*Xho*I sites of pCM81 to make pCM92, pCM93, and pCM105, respectively. The oligonucleotide L16 was also cloned into the *Bgl*II-*Xho*I sites of pCM83 to make pCM95. The promoter deletions shown in Fig. 6 and the oligonucleotides shown in Fig. 7 were confirmed by double-strand DNA sequencing using a Sequenase kit (United States Biochemicals Corp., Cleveland, Ohio) according to the vendor's instructions.

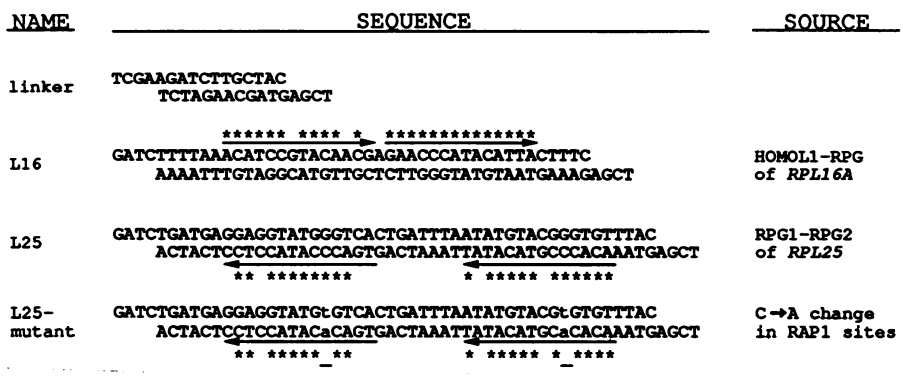


FIG. 1. Oligonucleotides. Asterisks denote identity with consensus sequence for RAP1 binding; arrows denote extent and direction of consensus sequence; underlining indicates positions of C-to-A changes.

Growth conditions. All yeast strains were cultured in SD medium (57) containing the required supplements only: 0.2 mM inositol, 2 mM leucine, 0.5 mM isoleucine and valine (the last two being added with leucine to obviate branched-chain amino acid imbalance; 42), and 1.5 mM arginine (needed for *gcn4* mutants; 22). For each experiment, cells were grown to stationary phase (about 40 h) with agitation at 30°C. The precultures were diluted 1:50 into fresh medium (time = 0), either 30 ml in a 125-ml Erlenmeyer flask or 60 ml in a 250-ml flask, and shaken vigorously at 30°C. After 2 h, filter-sterilized 3AT was added to certain flasks to 10 mM. At various times, cells from individual flasks were harvested by centrifugation and frozen at −20°C. Where indicated, filter-sterilized histidine was added to 5 mM to reverse 3AT-induced limitation.

Cell extraction and assays. Frozen cell pellets were resuspended on ice in breaking buffer (0.1 M Tris [pH 8.0], 20% glycerol, 1 mM β-mercaptoethanol; 40) and broken with glass beads (0.45-mm diameter) in a Braun homogenizer (B. Braun Instruments, Burlingame, Calif.) at 4°C, using 600-μl RIA vials (Sarstedt) and a cassette designed after that of Needleman and Tzagaloff (41). Cell extracts were clarified by centrifugation for 15 min in a Microfuge at 4°C. Assays for β-galactosidase activity were performed according to Miller (40) with the modifications of Lucchini et al. (33) or Menzel (38). Most assays were performed by the latter method, and the results of duplicate assays were usually within 5% of one another. Protein assays were performed by the method of Bradford (3), using reagent from Bio-Rad (Richmond, Calif.). Enzyme activities are expressed as units (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside [ONPG] cleaved per minute) per milligram of total protein.

In vivo labeling and IP. Two-milliliter cultures were grown in 18 × 150-mm culture tubes in the presence and absence of 3AT as described above. Proteins were labeled by adding 400 μCi of [^{35}S]methionine (1,000 Ci/mmol) for 10 min. The labeled methionine was chased for 5 min with a 1,000-fold excess of unlabeled methionine. Protein extractions and immunoprecipitations (IP) were performed as described previously (14), the latter being done with an equal number of trichloroacetic acid (TCA)-precipitable counts per minute from the plus-3AT and minus-3AT extracts. The volumes of all IP reaction mixtures were made equivalent by addition of the appropriate buffers. The antisera used were specific for GCN3 (19), β-galactosidase (Promega Biotec, Madison, Wis.), and RPL16A plus RPL16B, which differ by a single amino acid (gift of Y.-F. Tsay and J. Woolford, Carnegie Mellon University, Pittsburgh, Pa.). The extracts were precipitated with the GCN3, β-galactosidase, and RPL16 antibodies in a serial fashion. The immunoprecipitates were boiled in Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32) and fluorography (2). The amount of radiolabeled, immunoprecipitated protein present in each sample was quantified by scanning laser densitometry of the resulting fluorographs. The amount of labeled protein in each extract was estimated by liquid scintillation counting, and the estimate was confirmed by SDS-PAGE of a sample of each diluted extract and analysis both by fluorography and with an Ambis Systems (San Diego, Calif.) radioanalytic system.

Gel mobility shift assay for RAP1 binding to DNA. DNA-binding assays were performed on whole cell extracts essentially as described in Buchman et al. (6). Buffer A′ (25 mM K^+-HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 5 mM $MgCl_2$, 50 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 2 μM leupeptin) was made as a 1.1× stock without the last four ingredients, which were added from concentrated stocks immediately before use. Cells were grown exactly as for β-galactosidase assays. Twenty 60-ml cultures were grown in 250-ml flasks with or without 3AT and harvested 75 min after the addition of 3AT. Cell pellets were washed in cold deionized water, resuspended in cold buffer A′, broken with glass beads, and clarified as for enzyme assays. The extracts (approximately 2 ml) were frozen on dry ice and stored at −70°C. Later, the extracts were thawed on wet ice and made 0.3 M ammonium sulfate with a 3 M stock solution. After 30 min of gentle rocking, the samples were centrifuged at 2°C for 3 h at 110,000 × *g* in an SW60 rotor. The supernatants were dialyzed two times for 3 h at 4°C against 15 to 20 volumes of buffer A′. The dialysates were clarified and protein concentrations were determined as described above. Samples were divided into aliquots, frozen on dry ice, and stored at −70°C. Probes for DNA binding reactions were labeled to 8,000 cpm/fmol by end filling with [^{32}P]dCTP and Klenow polymerase (36). Reactions were performed in 20 μl of buffer A′ containing 1 to 14 fmol of probe, 0.5 or 1.0 μg of poly(dI/dC), and 2 to 16 μl of whole cell extract. Initial assays were performed with 1 μg of calf thymus DNA per 20 μl of reaction mixture, but this was found to be extremely inhibitory to complex formation. Use of 0.25 to 12 μg of poly(dI/dC) per assay had a negligible effect on specific complex formation but eliminated extrane-

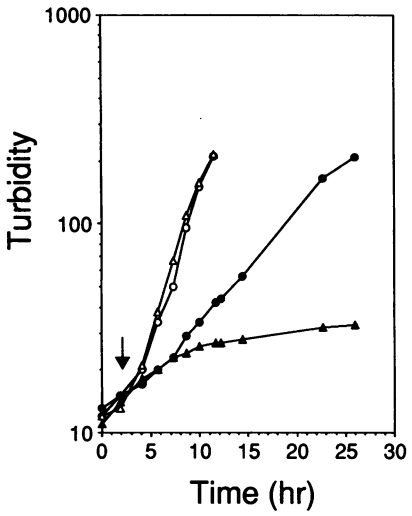


FIG. 2. Effect of 3AT on the growth of Gcn$^+$ and Gcn$^-$ strains. *GCN2* (H1314; circles) and *gcn2-1* (H1318; triangles) strains were grown to stationary phase in minimal medium and at time = 0 diluted into fresh medium. Growth of the cultures was monitored as turbidity in a Klett-Summerson colorimeter. Open symbols, no 3AT treatment; closed symbols, 3AT added to 10 mM; arrow, time of 3AT addition.

ous complexes observed with no carrier DNA (data not shown). Reaction mixtures were loaded directly (without tracking dye) on 4% Nuseive GTG agarose–0.25× TBE (22 mM Tris-borate [pH 8.3], 0.5 mM EDTA) gels and subjected to electrophoresis. The gels were fixed in 10% acetic acid–10% methanol and dried under vacuum.

RESULTS

Use of 3AT to elicit the stringent response. The method used here to elicit the stringent response to amino acid limitation is the same as that commonly used to derepress biosynthetic enzymes subject to general amino acid control. Cells are grown to stationary phase and then diluted into fresh medium (time = 0). At various times after the cultures have resumed exponential growth, 3AT is added at 10 mM to cause histidine limitation by inhibiting the *HIS3* gene product (30). Treatment with 3AT results in a histidine limitation sufficient for a strong metabolic response while still allowing exponential growth, albeit at a reduced rate. Partial histidine starvation caused the doubling time of a Gcn$^+$ strain to increase nearly threefold, namely, from 120 to 340 min (Fig. 2). Histidine-limited growth of a *gcn2* strain was similar to that of a *GCN2* strain for 5 to 6 h, or about one doubling, and then growth essentially ceased (Fig. 2). The results shown in Fig. 2 were supported by growth rate estimations based on measuring total protein concentration in cultures from several similar experiments (data not shown). Alternative methods of eliciting the stringent response, such as withholding an amino acid from an auxotroph or using a temperature-sensitive aminoacyl-tRNA synthetase, would be less useful because the former eventually blocks all protein synthesis and the latter introduces the confounding effect of heat shock on rp gene transcription (29).

Repression of rp gene-lacZ fusions in histidine-starved cells. Fusions of yeast rp genes with the *E. coli lacZ* gene served as convenient reporters to study the kinetics and magnitude

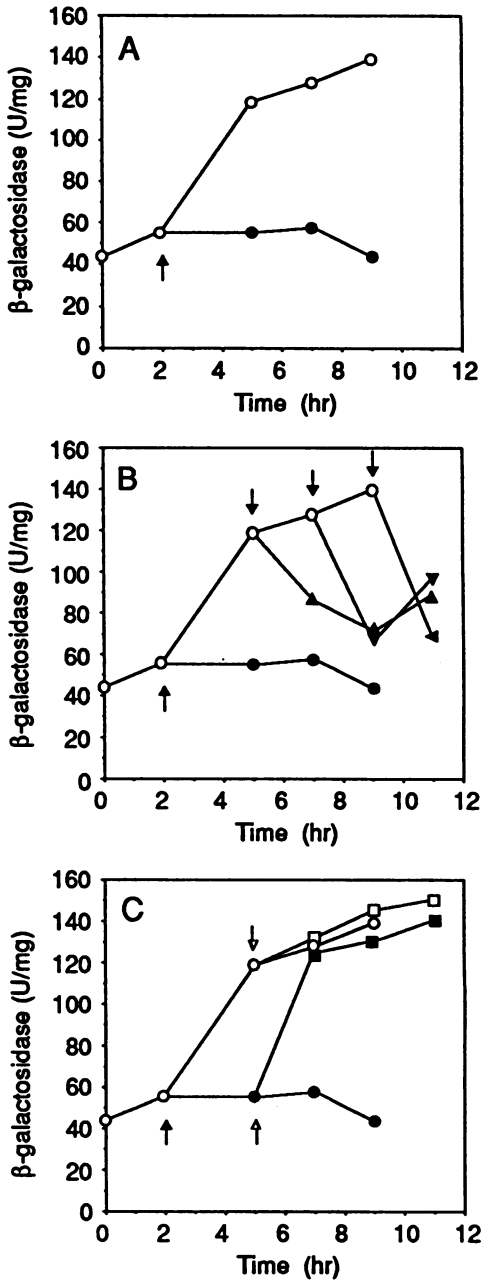


FIG. 3. Effect of 3AT and histidine on *RPL16A-lacZ* expression. Transformants of strain F113 containing the *RPL16A-lacZ* fusion were grown to stationary phase in minimal medium and at time = 0 diluted into fresh medium. At the indicated times, aliquots were taken and analyzed for β-galactosidase activity in whole cell extracts. Open symbols, no 3AT treatment; closed symbols, 3AT added to 10 mM; squares, histidine added to 5 mM; closed arrows, time of 3AT addition; open arrows, time of histidine addition. (A) 3AT added after 2 h of growth; (B) 3AT added after 2, 5, 7, or 9 h of growth; (C) 3AT added after 2 h and histidine added after 5 h of growth.

of the stringent response. Figure 3A shows a typical result for an *RPL16A-lacZ* fusion described previously (51) that we integrated in single copy at the *URA3* locus in wild-type strain F113. As the cells enter exponential growth, expression of fusion enzyme activity is stimulated threefold. The

low enzyme activity seen from 0 to 2 h reflects the reduced rp gene expression found in stationary-phase cultures (reviewed in reference 66; also see reference 53). The addition of 3AT prevents the stimulation of *RPL16A-lacZ* expression as cells enter exponential growth. Importantly, the cultures containing 3AT increased in protein content two- to threefold over 7 h, whereas the untreated cultures increased in protein content six- to sevenfold during the same time span (data not shown). These increases in protein content mirror the increases in cell density (turbidity) shown in Fig. 2. Thus, the failure to induce rp gene expression in the presence of 3AT clearly cannot be attributed to a cessation of protein synthesis.

As can be seen from Fig. 3B, the stringent response could be elicited at any time during the course of the experiment and was not limited to the very beginning of exponential growth. In addition, the repressing effect of 3AT on *RPL16A-lacZ* expression was completely reversed by addition of histidine to 5 mM (Fig. 3C), even after 20 h of growth in the presence of 3AT (data not shown). This control experiment rules out the possibility of a toxic effect of 3AT unrelated to inhibition of histidine biosynthesis.

Two other rp gene fusions were found to be repressed by 3AT under the same growth conditions: enzyme activities from the *CRY1-lacZ* and *RPS16A-lacZ* constructs were reduced by factors of 4 and 2, respectively, comparable to the magnitude of repression observed with *RPL16A-lacZ* (Fig. 4A and B). A similar result was obtained with these genes on 2μm-based plasmids (data not shown). In contrast to the rp-*lacZ* genes, *HIS4-lacZ* expression increased 3.3-fold in response to 3AT treatment (Fig. 4C; the last result exemplifies general amino acid control [21]), while a *CYC1-lacZ* fusion was virtually unresponsive to histidine limitation at any of several time points examined during exponential growth (Fig. 4D). Stringent regulation of the *RPS16A* construct is particularly informative because none of the mRNA from this fusion is derived from the rp gene. This eliminates the possibility of posttranscriptional control of gene expression and indicates that stringent control is exerted at the level of transcription initiation for *RPS16A* and possibly for the other rp genes as well.

Synthesis of authentic RPL16 proteins is repressed by 3AT. Warner and Gorenstein (67) showed that the mRNA levels for a large number of yeast rp genes are decreased by a factor of 2 to 4 when an auxotroph is deprived of its required amino acid. We wished to show that a native rp gene is also repressed by the histidine limitation imposed in our studies using 3AT. Toward this end, we immunoprecipitated the RPL16A/RPL16B protein pair from extracts of wild-type cells pulse-labeled with [^{35}S]methionine after growth in the presence or absence of 3AT. For this experiment, 3AT was added either 2 or 4 h after dilution of the stationary-phase culture, and the pulse-labeling was done for 10 min after 1 or 2 h of growth in the presence of 3AT. As expected from the findings described above, synthesis of the native RPL16

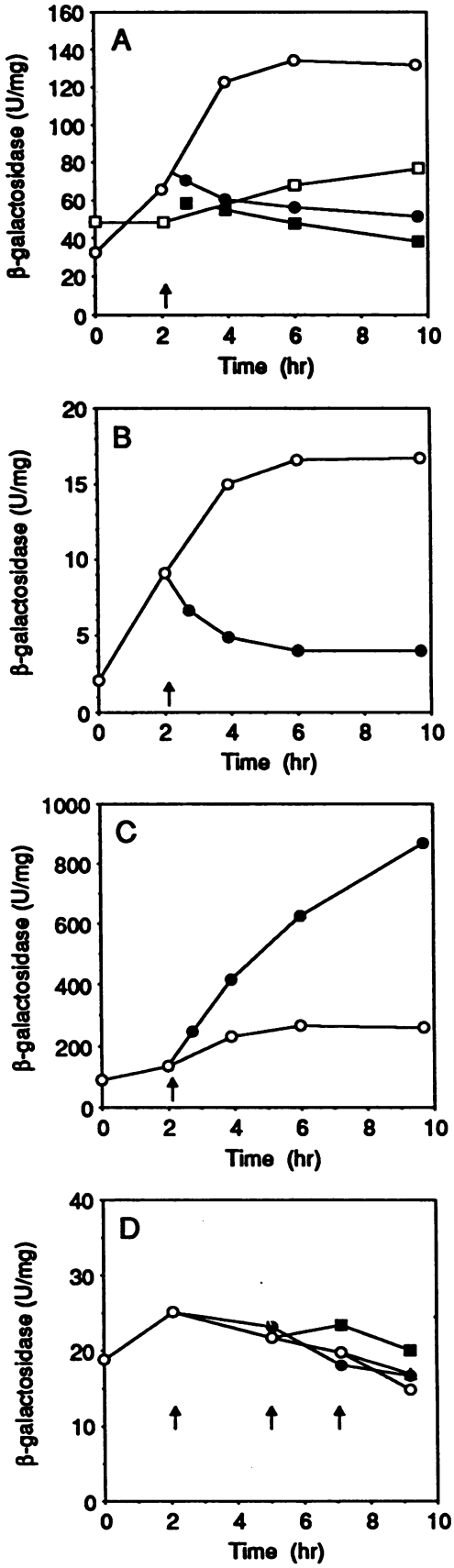


FIG. 4. Effect of histidine limitation on expression of three rp gene-*lacZ*, *HIS4-lacZ*, and *CYC1-lacZ* fusion genes. The experiment was conducted with transformants of strain F113 as for Fig. 3A. At the indicated times, aliquots were taken and analyzed for β-galactosidase activity in whole cell extracts. Open symbols, no 3AT treatment; closed symbols, 3AT added to 10 mM; arrows, time of 3AT addition. (A) *RPL16A-lacZ* on pCM40 (circles) and *RPS16A-lacZ* on YIp55-669Z (squares); (B) *CRY1-lacZ* on pCM39; (C) *HIS4-lacZ* (33); (D) *CYC1-lacZ* on pCM61.

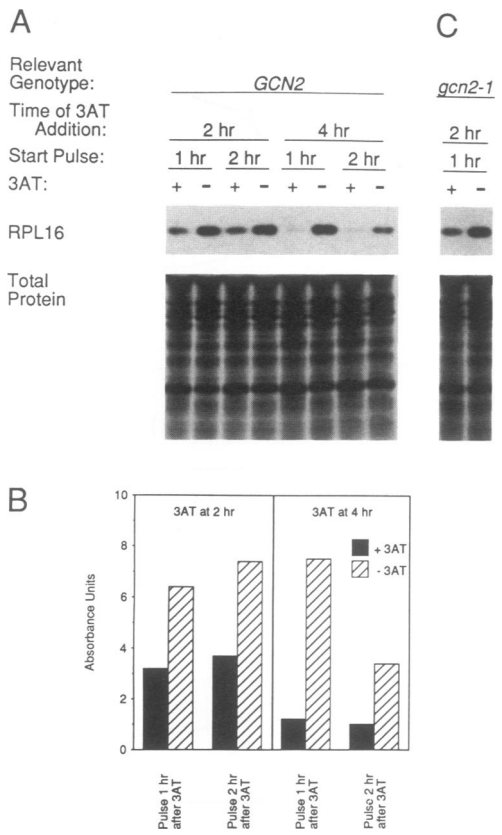


FIG. 5. Repression of authentic RPL16 protein synthesis by histidine starvation. Cells were grown as for Fig. 3A except that 3AT was added to some of the cultures at 2 or 4 h after dilution as indicated. The cultures were pulse-labeled for 10 min with [^{35}S]methionine at 1 or 2 h after the time of 3AT addition as shown. (A) RPL16A and RPL16B proteins (RPL16) were immunoprecipitated from 1 ml of whole cell extracts, each of which contained an equal number of TCA-precipitable counts per minute, and analyzed by SDS-PAGE. An autoradiogram of an SDS-PAGE separation of 30 μl of each whole cell extract of strain H1528 from the IP experiment is shown to verify proper normalization (Total Protein). (B) The relative amounts of labeled RPL16 in each sample, as determined by densitometry of the fluorograph shown in panel A, is plotted as a histogram. (C) RPL16A and RPL16B proteins (RPL16) were immunoprecipitated from 1 ml of whole cell extracts of strain H1318 (*gcn2*), both of which contained a number of TCA-precipitable counts per minute equal to that in panel A, and analyzed by SDS-PAGE. An autoradiogram of an SDS-PAGE separation of 30 μl of each whole cell extract from the IP experiment is shown to verify proper normalization (Total Protein).

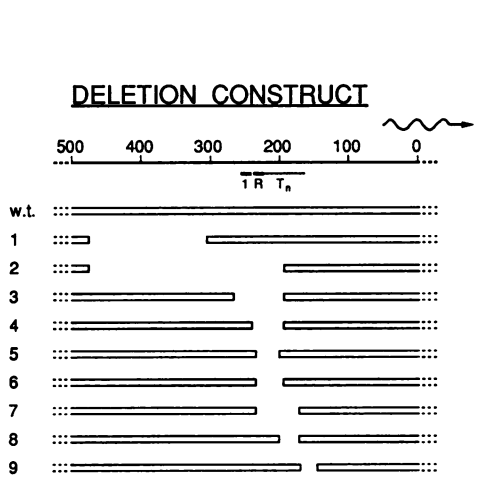
proteins, relative to that of total protein, was reduced significantly after 3AT addition (Fig. 5). The magnitude of the decrease in RPL16 expression is in accord with that reported previously using a different starvation regimen (cf. with rp39 in reference 67) and with that shown above using the *RPL16A-lacZ* fusion construct (Fig. 3B).

These results were extended by another experiment in which a transformant of strain F113 containing an integrated *HIS4-lacZ* fusion (strain F35) was treated with 3AT beginning 2 h after dilution of the culture. The RPL16, GCN3, and HIS4-LacZ proteins were immunoprecipitated serially from extracts of cells that were pulse-labeled 15 or 50 min after 3AT addition. All three protein species were analyzed by SDS-PAGE. GCN3 was examined as a control because its abundance was known to be relatively unaffected by histidine limitation (19). HIS4-LacZ was chosen as a second control because its expression was known to be rapidly derepressed by 3AT (14a). Treatment with 3AT reduced GCN3 expression by only a small amount (ca. 25%) and led to a marked increase in the level of HIS4-LacZ protein (greater than fivefold; data not shown). Relative to GCN3, RPL16 expression decreased by an amount similar to that shown in Fig. 5 (factors of 2.7 and 3.4 after 15 and 50 min, respectively, of growth in the presence of 3AT; data not shown). We conclude that the expression of authentic *RPL16* genes and the various rp gene-*lacZ* fusions analyzed in Fig. 3 and 4 are similarly repressed during histidine-limited exponential growth.

Stringent control of RPL16A 5′ deletion mutants. We used a set of *RPL16A-lacZ* constructs with deletions in the 5′ nontranscribed DNA constructed by Rotenberg and Woolford (51) in an effort to identify a discrete sequence element necessary for stringent control of this gene. Each of the constructs shown in Fig. 6 was stably integrated at the *URA3* locus in strain F113 and analyzed exactly as described for the experiment shown in Fig. 3A. For the sake of brevity, only the fusion enzyme levels determined after 7 h of growth in the presence and absence of 3AT are shown in Fig. 6. Two potential RAP1 binding sites, known as HOMOL1 and the RPG box (1 and R, respectively, in Fig. 6), have been identified at *RPL16A*. As shown previously (51), removal of the RPG box plus a portion of the thymidine-rich sequence (T_n in Fig. 6) reduced expression under nonstarvation conditions to about 1/10 of the normal level (constructs 4 to 6); however, these alleles are still repressed by histidine limitation, ruling out a requirement for multiple RAP1 binding sites and the 5′ half of the T_n element for stringent control. Constructs 1, 8, and 9 also ruled out an absolute requirement for sequences upstream of position −300 and the 3′ half of the T_n element for stringent control. When both copies of the RAP1 binding site were deleted (construct 3) or when the entire T_n element was deleted along with the RPG box (construct 7), *RPL16A-lacZ* expression was too low to ascertain whether these alleles are subject to stringent control.

The consensus sequence for RAP1 binding confers stringent control. To test directly whether the potential RAP1 binding sites at *RPL16A* are sufficient to confer stringent control, we took advantage of previously described high-copy-number plasmids containing *CYC1-lacZ* fusions with either the native *CYC1* UAS (16), no UAS, or the 126-bp fragment containing the *RPL16A* UAS (51). We constructed nonreplicating derivatives of these plasmids and integrated them in single copy at the *URA3* locus in strain F113. (For the sake of brevity, only the fusion enzyme levels determined after 7 h of growth in the presence and absence of 3AT are shown in Fig. 7A.) As expected, expression of β-galactosidase from the *CYC1-lacZ* fusion was not subject to stringent control (pCM61; Fig. 7A and B); expression from the UAS_{CYC1}-deletion constructs (pCM81 and pCM83) was too low to ascertain regulation. On the other hand, expression of β-galactosidase from the construct containing the 126-bp *RPL16A* fragment that encompasses both RAP1 binding sites and the T_n element (positions −298 to −172) was stringently regulated to roughly the same extent as the *RPL16A-lacZ* fusion analyzed above that contains 1 kb of 5′ noncoding DNA (pCM60 and pCM40, respectively; Fig. 7A).

We subsequently tested smaller fragments containing only a pair of RAP1 binding sites and 10 to 16 flanking base pairs

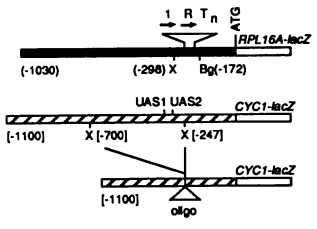


DELETION CONSTRUCT	PLASMID		β-galactosidase U/mg (S.E.) -3AT	β-galactosidase U/mg (S.E.) +3AT	RATIO -3AT/+3AT
w.t.	pCM40		170 (9.5)	42 (2.4)	4.0
1	pCM44	(475-305)	150 (5.5)	36 (3.5)	4.2
2	pCM43	(475-193)	1.1	0.6	1.8
3	pCM47	(266-193)	1.2	0.8	1.5
4	pCM48	(239-193)	22 (0.0)	5.7 (1.2)	3.9
5	pCM54	(233-199)	20 (3.0)	3.9 (2.4)	5.1
6	pCM49	(233-193)	20 (3.0)	4.1 (2.6)	4.9
7	pCM50	(233-171)	2.0 (0.1)	0.9 (0.4)	2.2
8	pCM52	(199-171)	100	20	5.0
9	pCM53	(168-144)	190 (22)	40 (5.5)	4.8

FIG. 6. Effect of histidine starvation on expression of 5′ deletion alleles of the *RPL16A-lacZ* fusion on pCM40. The scale indicates base pairs upstream of the ATG codon of *RPL16A*. The wavy arrow indicates the start site and direction of transcription. The previously described HOMOL1 (−253 to −240), RPG box (−238 to −225), and T-rich (−223 to −164) sequences are indicated below the scale as 1, R, and T_n, respectively. The deletions are indicated by gaps in the open boxes, and their exact boundaries are given by the hyphenated numbers in parentheses next to each plasmid name; some of these boundaries have been adjusted from those given in the original publication (51) on the basis of our sequence analysis. Transformants of strain F113 containing the indicated fusions were grown as described for Fig. 3A and analyzed for β-galactosidase activity in whole cell extracts; results from only the last time points are presented. Note that deletion 2 was present as a multicopy integrant and deletion 4 contained two repeats of the *Xho*I linker used to make the deletions. w.t., wild type.

for their ability to confer stringent control on *CYC1-lacZ* expression. A 38-bp fragment containing the HOMOL1 and RPG box tandem RAP1 binding sites from *RPL16A* displayed significant UAS activity under nonstarvation conditions when inserted in either orientation relative to the direction of transcription (pCM92 and pCM95). In addition, the transcriptional-promoting activity of this fragment was significantly reduced in histidine-starved cells, at least when

A



CONSTRUCT	PLASMID	β-galactosidase U/mg (S.E.) -3AT	β-galactosidase U/mg (S.E.) +3AT	RATIO -3AT/+3AT
	pCM40	170 (8.2)	41 (3.0)	4.1
	pCM61	16 (1.0)	16 (1.0)	1.0
	pCM81	2.0	1.5	---
	pCM83	1.0	0.8	---
RPL16A (1/R/T_n)	pCM60	250 (15)	100 (8.1)	2.5
L16 (reverse)	pCM95	22 (0.9)	8.5 (0.9)	2.6
L16	pCM92	62 (3.5)	45 (1.8)	1.4
L25 (>1 copy)	pCM93	36 (1.7)	13 (1.8)	2.8
L25	pCM93	11 (0.0)	3.9 (0.9)	2.8
L25-mutant	pCM105	<1.0	<1.0	---

B

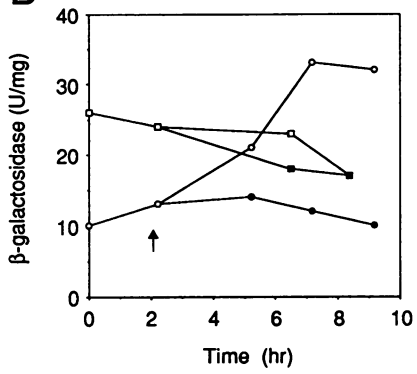


FIG. 7. Evidence that short oligonucleotides containing RAP1 binding sites are sufficient to confer stringent regulation upon *CYC1-lacZ* expression. (A) The first schematic depicts the *RPL16A-lacZ* fusion on pCM40 analyzed in previous figures. The second and third schematics depict, respectively, the wild-type 5′ end of *CYC1* fused to *lacZ* and deletion derivatives thereof lacking *CYC1* UAS elements 1 and 2 (16) and containing instead the oligonucleotide linker shown in Fig. 1. The remaining constructs contain various oligonucleotides derived from rp gene promoters inserted at the linker sequence of pCM81 or pCM83. The horizontal arrows show the direction of the RAP1 binding sites relative to the published consensus sequence (43). The RPL16A (1/R/T_n) fragment in pCM60 contains *RPL16A* sequences from −298 to −172 (5′ to the ATG codon) that include the two RAP1 binding sites (1 and R), the T_n element, and ca. 40 flanking nucleotides (51); L16 (reverse) in pCM95 is a subset of RPL16A that includes little more than the two RAP1 binding sites (Fig. 1) but is present in the opposite orientation as in RPL16A (1/R/T_n); L16 in pCM92 is the same DNA sequence but in the native orientation; L25 in pCM93 is from the *RPL25* sequence that includes little more than the two RAP1 sites formerly called RPG1 and RPG2 (72); L25 (>1 copy) indicates that more than one copy of the plasmid was integrated at the *URA3* locus; L25-mutant is the same as L25 except for the substitutions shown in Fig. 1. (B) Typical enzyme assay results obtained with the UAS_{L25}-*CYC1-lacZ* fusion on pCM93 (circles) and *CYC1-lacZ* fusion on pCM61 (squares). Open symbols, no 3AT treatment; closed symbols, 3AT added to 10 mM; vertical arrow, time of 3AT addition.

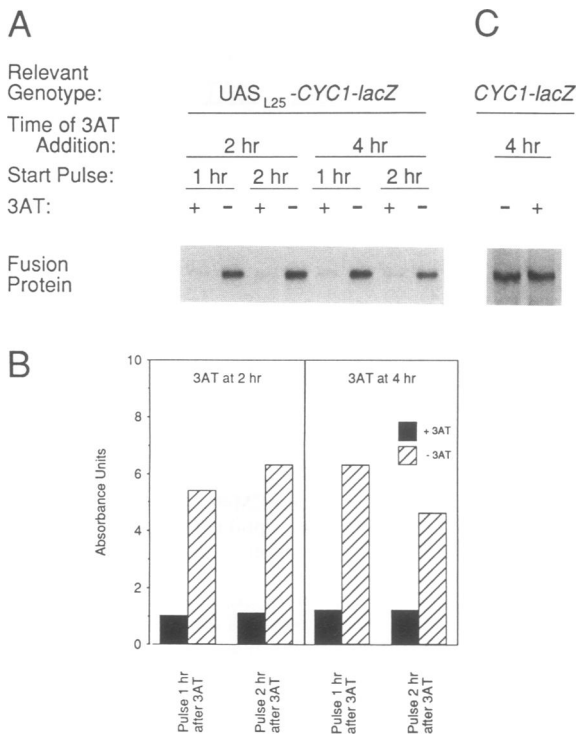


FIG. 8. UAS_{rpg}-dependent repression of CYC1-LacZ expression by histidine limitation. (A) Strain H1528 (UAS_{L25}-*CYC1-lacZ*) was grown as for Fig. 3A except that 3AT was added to some of the cultures at 2 or 4 h after dilution as indicated. The cultures were pulse-labeled for 10 min with [^{35}S]methionine at 1 or 2 h after the time of 3AT addition as shown. CYC1-LacZ protein (Fusion Protein) expressed from the UAS_{L25}-*CYC1-lacZ* construct was immunoprecipitated from 1 ml of whole cell extracts, each of which contained an equal number of TCA-precipitable counts per minute, and analyzed by SDS-PAGE. (These IP assays were performed with the same whole cell extracts used in Fig. 5A.) (B) Relative amounts of labeled fusion protein in each sample, as determined by densitometry of the fluorograph shown in panel A, plotted as a histogram. (C) Autoradiogram of the fusion protein that was immunoprecipitated from a strain bearing the *CYC1-lacZ* construct. After 4 h of growth, one aliquot of the culture was pulse-labeled immediately (−3AT sample) and another aliquot was pulse-labeled after treatment with 3AT for 1 h (+3AT sample). Unlike the UAS_{L25}-*CYC1-lacZ*-encoded protein, expression of the *CYC1-lacZ*-encoded protein was not affected by 3AT treatment. Expression of RPL16 and total protein was found to be the same as that shown in Fig. 5 (data not shown).

inserted in the orientation opposite that found in the native *RPL16A* gene (pCM95). Regulation was less evident when the fragment was inserted in the other orientation, suggesting that sequences flanking the RAP1 binding sequence affect regulation (7). Similarly, a 44-bp fragment containing the tandem RAP1 binding sites from *RPL25* had UAS activity that was subject to stringent control when inserted in its native orientation upstream from *CYC1-lacZ* (pCM93; Fig. 7 and 8). As can be seen from Fig. 7B, the L25 fragment confers the stimulation of transcription upon entry into exponential phase that is characteristic of rp genes (reviewed in reference 66), and this increase was abolished by histidine limitation in the same manner shown in Fig. 4 for three different rp-*lacZ* genes. Moreover, as shown above for the *RPL16A-lacZ* fusion (Fig. 3B), expression of the UAS_{L25}-*CYC1-lacZ* fusion was reduced when 3AT was added at times later than 2 h. This last result was established both by measuring enzyme activity (data not shown) and by IP of pulse-labeled UAS_{L25}-CYC1-LacZ protein (Fig. 8).

Figures 8A and B shows that by 3 h after dilution of the stationary-phase culture, expression of the UAS_{L25}-*CYC1-lacZ* fusion in the absence of 3AT is already at 85% of the maximum rate observed at 4 and 5 h after dilution. Therefore, expression of this fusion is essentially at equilibrium within 3 h of dilution of the culture, as was expression of authentic RPL16 in Fig. 5. Also, it is worth noting that the degree of repression of this fusion is the same whether 3AT is added at 2 or 4 h after dilution, as was observed for RPL16 in Fig. 5. Finally, Figure 8C shows that expression of the *CYC1-lacZ* control construct is not affected by histidine limitation.

As expected, two C→A changes introduced into the UAS_{L25}-*CYC1-lacZ* construct in pCM93 at the sole essential positions identified for UAS_{rpg} function (43) abolished the expression of this construct (pCM105; Fig. 1 and 7A). In fact, expression from the pCM105 construct was consistently lower than that from the pCM83 construct lacking any UAS. As shown below, these mutations also abolished RAP1 binding to its target sequence in vitro. Taken together, our results strongly suggest that stringent control of rp gene expression is mediated by the RAP1 protein.

The level of RAP1 binding activity is not under stringent control. We next addressed the possibility that stringent control of rp gene expression occurs by reducing the level of RAP1 protein, or its ability to bind to DNA, under partial starvation conditions. Whole cell protein extracts were prepared from strains H1314 (*RPL16A-lacZ PEP4*) and H1519 (*pep4::URA3*) grown in the presence or absence of 3AT and were assayed for RAP1 DNA binding activity by using the gel mobility shift assay (15). A double-stranded oligonucleotide containing the *RPL16A* tandem RAP1 binding sites (L16 in Fig. 1) was radiolabeled and used as the DNA probe. Protein extracts were prepared from a *pep4* mutant, which is deficient in the soluble vacuolar hydrolases (27), to minimize proteolytic degradation of RAP1. The *PEP4* strain H1314 was analyzed in parallel because it was unknown whether the *pep4* mutation would affect stringent control, e.g., by altering amino acid pools or susceptibility to 3AT. Cells were grown under the conditions described above and harvested 60 to 90 min after addition of 3AT because preceding experiments had shown that *RPL16A* expression was fully repressed by this time, yet the protein contents of the cells grown in the presence or absence of 3AT were still quite similar. As a control, aliquots of the H1314 culture were grown for an additional 5 h with and without 3AT. Assays of RPL16A-LacZ enzyme activity from extracts of these cultures showed the expected lower expression in the starved versus nonstarved cultures (data not shown).

A slowly migrating complex was formed with the *RPL16A* probe (L16; Fig. 1), the amount of which increased in a linear fashion with increasing amounts of extract from the *pep4* mutant. The abundance of this complex formed per microgram of protein was indistinguishable between the minus-3AT and plus-3AT extracts (Fig. 9A). Identical results were obtained with extracts from the *PEP4* strain (data not shown). Evidence that this more slowly migrating species is a RAP1-DNA complex is provided by the competition experiment shown in Fig. 9B. Formation of the radiolabeled complex was inhibited equally well by unlabeled *RPL16A* probe and by an *RPL25* oligonucleotide that contains two

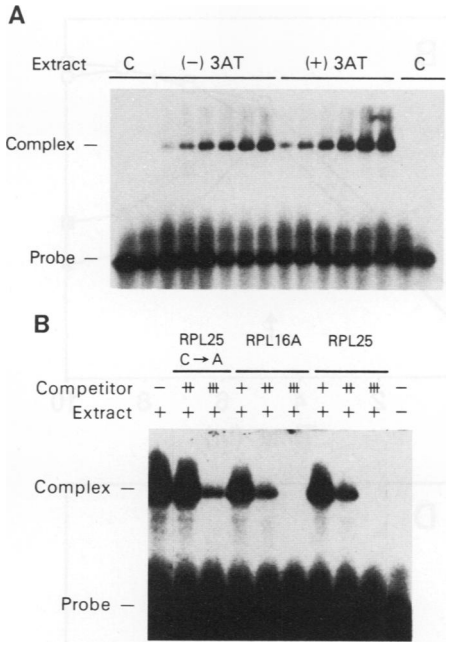


FIG. 9. Gel electrophoretic mobility shift assays of RAP1 binding in extracts from cells grown in the presence and absence of 3AT. Strain H1519 (*pep4*) was grown as for Fig. 3A. One hour after 3AT addition, whole cell extracts were prepared from nonstarved (−3AT) and starved (+3AT) cultures (see Materials and Methods). Aliquots of the extracts were incubated with 14 fmol of radiolabeled oligonucleotide L16 from the *RPL16A* UAS (shown in Fig. 1). Complex formation was analyzed by agarose gel electrophoresis followed by fluorography. (A) Histidine limitation does not affect the level of RAP1 DNA binding in vitro. From left to right in each set, 3.0, 4.5, 6.0, 7.5, 9.0, and 10.5 μg of total protein from the whole cell extracts of nonstarved [(−) 3AT] and starved [(+) 3AT] cultures; C, no extract controls. (B) Competition experiments show that complexes formed with the *RPL16A* UAS_{rpg} oligonucleotide contain RAP1 protein. Unlabeled competitors were mixed with 14 fmol of labeled L16 probe before addition of 7.5 μg of protein from whole cell extract from the nonstarved culture. Unlabeled competitors (shown in Fig. 1) were L25-mutant (*RPL25* C→A mutant), L16 (*RPL16A*), and L25 (*RPL25*); +, ++, and +++ refer to 20, 110, and 7,500 fmol, respectively.

RAP1 binding sites but no other obvious sequence similarities with the L16 probe (L25; Fig. 1). By contrast, approximately 100-fold-higher amounts of the *RPL25*-related oligonucleotide, containing the C→A substitutions that eliminate UAS function (L25-mutant; Fig. 1 and 7A), were required to inhibit formation of the radiolabeled complex. Our results are consistent with earlier experiments demonstrating that the C→A substitution is incompatible with RAP1 DNA-binding activity (7, 64).

Gcn^- mutants are not relaxed for stringent control. In bacteria, Rel^- (relaxed) mutants are defective for both negative and positive stringent control: during amino acid starvation, ribosome synthesis continues unabated and amino acid biosynthesis is not derepressed. The latter phenotype is seen in yeast strains bearing mutations in any of the genes *GCN1* to *GCN4* (21); therefore, these Gcn^- mutants are at least partly analogous to bacterial *rel* mutants. We tested whether *gcn* mutants are also defective for negative control of rp gene expression by integrating the rp gene-*lacZ* and *HIS4-lacZ* fusions shown in Fig. 4 at the *URA3* locus in strains containing a mutation in *GCN1*, *GCN2*, *GCN3*, or *GCN4*. The resulting transformants were tested for regulation of the integrated fusions in response to 3AT treatment. The results with the *gcn2* strain (H1318) shown in Fig. 10 are representative of all four mutants. Stringent regulation of the three rp-*lacZ* genes was very similar to that described above for a *GCN* strain (compare Fig. 4A and B with Fig. 10A and C). (The only difference we noticed was a higher basal level of expression for *RPS16A-lacZ* in the *gcn* strains; this could be a peculiarity of the *CYC1*-derived DNA in this construct, as previously reported [23].) As expected, no derepression of *HIS4-lacZ* expression occurred in any of the four *gcn* mutants (compare Fig. 4C with Fig. 10D). *RPL16A-lacZ* activity expressed from pCM40 was repressed equally in strains H1314 (*GCN2*) and H1318 (*gcn2*) whether 3AT was added 2 h after dilution of the culture, as in Fig. 4A and 10A, or 5 h after dilution of the culture, as in Fig. 10B. Finally, the amounts of native RPL16 protein immunoprecipitated from extracts of *GCN2* (H1528) and *gcn2-1* (H1318) strains grown under partial starvation or nonstarvation conditions were nearly identical (compare results in Fig. 5A and C, which originate from the same experiment and therefore are directly comparable). We conclude that none of the *gcn* mutants analyzed here are defective for stringent control of rp gene expression.

DISCUSSION

Evidence that the RAP1 binding site mediates stringent control of rp gene transcription. We have shown that making histidine limiting for growth of yeast cultures reduces the expression of rp gene-*lacZ* fusions constructed from four different rp genes (*RPL16A*, *RPL25*, *CRY1*, and *RPS16A*) by a factor of 2 to 5. Expression of the authentic RPL16 ribosomal proteins was repressed to a similar degree under the same starvation conditions. These results are consistent with the findings of Warner and Gorenstein (67), who demonstrated reductions by a factor of 2 to 4 in the levels of translatable mRNAs for 16 of 20 different yeast rp genes when a tyrosine auxotroph was cultured on medium lacking tyrosine. In particular, these workers showed that the amount of translatable *RPL16* (rp39) mRNA decreased by a factor of 2.3 in response to severe tyrosine starvation. Therefore, it appears that our condition of histidine-limited exponential growth is as potent as a complete starvation for tyrosine in eliciting the stringent response.

The previous study did not address whether reduced ribosomal protein synthesis under starvation conditions results from lower steady-state amounts of rp mRNAs or from lower translatability of these transcripts. We have shown that sequences from the 5′ nontranscribed regions of two rp genes are sufficient to confer stringent control upon a heterologous yeast gene, *CYC1*, whose expression does not normally respond to amino acid starvation. Expression of *CYC1-lacZ* constructs containing UAS_{rpg} elements (i.e., RAP1 binding sites) inserted in place of the UAS_{CYC1} was repressed by histidine limitation to roughly the same extent as that seen for authentic rp genes. These results strongly suggest that stringent control of rp gene expression operates primarily at the level of transcription initiation.

The UAS_{rpg} regulatory element was first identified by comparing the 5′ noncoding sequences of various rp genes (63). Subsequent mutational analyses demonstrated that UAS_{rpg} elements are essential components of many rp gene promoters (reviewed in reference 66). In accord with these findings, the UAS_{rpg}-containing fragments that we analyzed greatly increased expression of the *CYC1-lacZ* construct

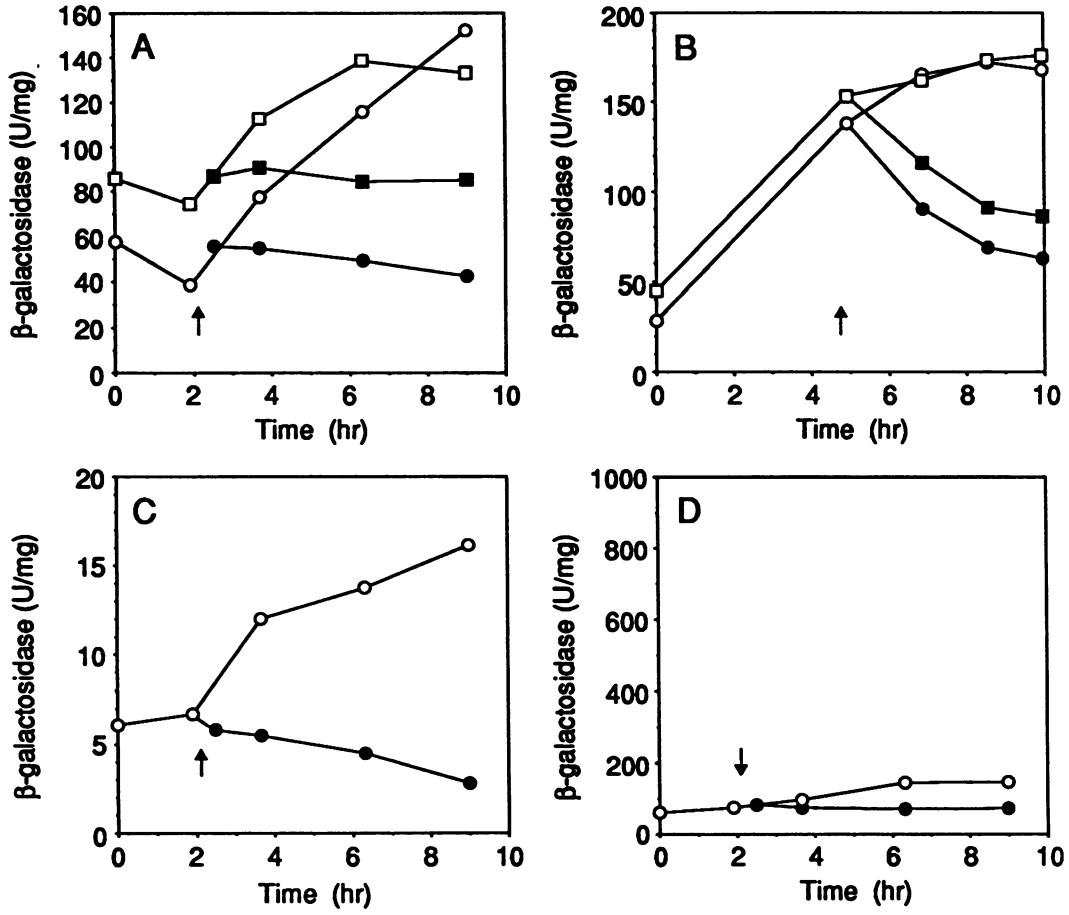


FIG. 10. Effect of histidine limitation on expression of three rp gene-*lacZ* and *HIS4-lacZ* fusions in a *gcn2-1* mutant. The experiment was conducted with the same fusion plasmids as in Fig. 4. Open symbols, no 3AT treatment; closed symbols, 3AT added to 10 mM; arrows, time of 3AT addition. (A) *RPL16A-lacZ* (circles) and *RPS16A-lacZ* (squares) in *gcn2-1* strain H1318 with 3AT added 2 h after dilution of the culture; (B) *RPL16A-lacZ* in *GCN2* strain H1314 (circles) and *gcn2-1* strain H1318 (squares) with 3AT added 5 h after dilution of the culture; (C) *CRY1-lacZ* in *gcn2-1* strain H1317; (D) *HIS4-lacZ* in *gcn2-1* strain H1146.

lacking its own UAS. In addition, transcriptional activation associated with these fragments was completely abolished by substitution of the invariant C nucleotides at the fifth position of each UAS_{rpg} (43). Substitutions at this position were shown previously to abolish UAS_{rpg} function in vivo (6). The small *RPL25* and *RPL16A* fragments that we found to be sufficient to confer stringent control upon *CYC1-lacZ* expression contain only UAS_{rpg} elements and a small number of flanking nucleotides that are not conserved between the two rp genes. This finding suggests that UAS_{rpg} elements mediate stringent control of rp gene expression. In this view, transcriptional activation by UAS_{rpg} elements would be less efficient during growth under amino acid-limiting conditions than under nonstarvation conditions.

UAS_{rpg} elements bind a protein factor present in yeast cell extracts that was first called TUF (24). Other DNA-binding factors identified by their interactions with certain RNA polymerase II promoters (4), telomeres (5), and the silent mating-type loci (59) were found to have the same binding specificity as TUF. It is likely that all of these DNA-binding factors are the same protein, which we refer to as RAP1 because the structural gene for this factor has been cloned and sequenced (58). A rough correlation has been established between the ability of various RAP1 binding sites to function as UAS elements in vivo and their binding affinity for RAP1 protein in vitro (6, 7, 43, 64). These results suggest that RAP1 binding is responsible for transcriptional activation of rp gene expression by UAS_{rpg} elements.

Accordingly, we examined the possibility that stringent control of rp gene expression might occur by decreasing the level of RAP1 binding activity in response to amino acid starvation. We observed no difference in the level of binding to an *RPL16A* DNA probe containing two UAS_{rpg} elements in extracts prepared from cells grown in the presence or absence of 3AT. As expected for RAP1, a fragment containing the UAS_{rpg} elements of *RPL25* efficiently competed with the *RPL16A* probe for protein binding. In addition, binding (measured indirectly in competition experiments) to the *RPL25* probe was greatly diminished by substitution of the invariant C nucleotides in the fifth position of the tandem UAS_{rpg} elements. We conclude from these experiments that the abundance of RAP1 and its affinity for the UAS_{rpg} in vitro is not significantly reduced by histidine limitation. Given that the small *RPL16A* and *RPL25* fragments that confer stringent control have no other obvious sequence similarity beyond UAS_{rpg} elements, it seems unlikely that binding of an additional factor at a site adjacent to UAS_{rpg} is involved in stringent control by RAP1. Therefore, the most

likely remaining possibilities are either that the in vitro assay does not reflect in vivo regulation of RAP1 binding activity or that interaction with some other effector molecule, that does not itself bind DNA, modulates the ability of RAP1 to activate transcription in response to amino acid availability.

The same comments apply to the regulation of RAP1 activation of rp gene transcription according to the carbon source. Expression of rp genes is roughly three- to fourfold higher when yeast cells are grown on glucose than when cells are grown on nonfermentable carbon sources (20). Experiments similar to those described here showed that this regulation is mediated by UAS_{rpg} elements; however, it is not clear whether the level of RAP1 binding activity does (54) or does not (11) differ significantly when yeast cells are grown on different carbon sources. The fact that RAP1 mediates regulation of rp gene expression in response to the availability of both amino acids and glucose represents another parallel with *E. coli*, in which the synthesis of ribosomal components is repressed by carbon and energy starvation as well as by amino acid limitation (10).

The results presented in Fig. 7B show that RAP1 binding sites are sufficient to mediate the differences in rp gene expression observed between exponential and stationary growth phases (53). It will be interesting to determine whether transcriptional repression of yeast rp genes under conditions of nitrogen starvation (66) are also mediated by RAP1. Another interesting question is whether the roles ascribed to RAP1 in transcriptional silencing of mating-type cassettes (59) and telomere function (6) are affected by amino acid or glucose deprivation.

Some rp genes such as *TCM1* do not have RAP1 binding sites but contain instead a sequence that binds a different abundant transcription factor called SUF or TAF (reviewed in reference 66). This factor is probably encoded by the *ABF1* (*BAF1*) gene (12, 17, 48). Despite the absence of RAP1 binding sites, *TCM1* is subject to stringent control (*TCM1* encodes rp1 in reference 67), raising the possibility that a common factor exerts stringent control over all rp genes by interacting with both RAP1 and ABF1. Interestingly, an 82-kDa protein was identified that could be UV cross-linked to DNA in both a RAP1 and TAF (ABF1) DNA-protein complex (18).

The starvation signal for stringent control. Repression of yeast rp gene expression in response to 3AT treatment occurred as the result of histidine starvation rather than an unrelated toxic effect of 3AT, since this repression was abolished by addition of histidine. Nevertheless, it could be argued that any insult to the cell that slows growth would reduce rp gene expression as a secondary consequence. For example, heat shock (29), growth on a poor carbon source (13, 28), and amino acid starvation all repress yeast rp gene expression. However, deletion of one member of a duplicated pair of rp genes (50) and partial inactivation of the large subunit of RNA polymerase I (71) both inhibit cell growth without affecting expression of rp genes. Consequently, it appears that only certain forms of stress generate a signal which is recognized by the regulatory molecules that control yeast rp gene expression.

In the case of amino acid starvation, this signal is probably uncharged tRNA, because a mutation that lowers aminoacyl-tRNA synthetase function is sufficient to repress yeast rp gene expression in the absence of amino acid starvation (34, 67). For the same reason, uncharged tRNA is thought to be the proximal signal for derepression of amino acid biosynthesis in yeast cells (39). The products of *GCN1*, *GCN2*, *GCN3*, and *GCN4* are required for increased expression of many amino acid biosynthetic genes under starvation conditions, and the possibility existed that one or more of these factors also would be required for repression of rp genes under the same circumstances. In particular, the similarity of adjacent domains of GCN2 with protein kinases (52) and histidyl-tRNA synthetases (68) suggested that GCN2 might play the same role as RelA in *E. coli* and initiate both the positive and negative pathways of stringent control in yeast cells. At odds with this possibility, we found that repression of rp gene expression occurs normally in *gcn1*, *gcn2*, *gcn3*, and *gcn4* mutants that are completely defective for derepression of *GCN4* and amino acid biosynthesis genes under its control. This finding suggests that *S. cerevisiae* differs from *E. coli* in possessing two separate signal transduction pathways to elicit the positive and negative aspects of stringent control.

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