Temporal Analysis of General Control of Amino Acid Biosynthesis in *Saccharomyces cerevisiae*: Role of Positive Regulatory Genes in Initiation and Maintenance of mRNA Derepression

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Received 6 September 1983/Accepted 6 December 1983

In *Saccharomyces cerevisiae*, starvation for a single amino acid results in the derepression of enzyme activities in multiple amino acid biosynthetic pathways. Derepression is a consequence of increased transcription of the genes encoding these enzymes. Analysis of the kinetics of mRNA elevation established that derepression occurs within 5 min of a shift of the culture from rich medium to starvation medium. Any starvation condition was sufficient to trigger an initial high mRNA elevation; however, it was the severity of starvation which determined the steady-state mRNA levels that were subsequently established. The products of the positive regulatory genes *AAS101*, *AAS103*, and *AAS2* were shown to be required in the initiation phase of this response, whereas the *AAS102* gene product was required to maintain the new elevated steady-state mRNA levels. The *AAS101* and *AAS102* genes were cloned. Consistent with their respective roles in initiation and maintenance of derepression, *AAS101* mRNA was found to be expressed at high levels in both rich and starvation media, whereas *AAS102* mRNA was derepressed only under starvation conditions. The derepression of *AAS102* mRNA is dependent on the *AAS101* gene product.

Analysis of the mechanism by which a change in the extracellular environment is sensed by a cell, processed into an intracellular signal, and ultimately used to effect an increase in the rate of transcription of a specific subset of genes is fundamental to an understanding of gene expression. One model system for the determination of the molecular events by which mRNA levels are modulated in response to external conditions is the general control of amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*. This regulatory system mediates the expression of at least 100 unlinked genes encoding amino acid biosynthetic enzymes (reviewed in reference 18), as well as enzymes such as tRNA synthetases, which catalyze reactions involved in protein biosynthesis (26). Coregulation of these genes results in derepression of enzyme activities in multiple amino acid biosynthetic pathways when cells are starved for a single amino acid (7, 32, 37, 50). Increased enzyme activities are correlated with increased levels of the corresponding mRNAs (14, 25, 33, 42, 51). Indirect evidence suggests that the elevated mRNA levels are a consequence of increased transcription rather than specific message stabilization (39, 51).

Coregulation of genes subject to general control is mediated by several molecules which have either repressing or enhancing effects on the expression of these genes. Genetic analyses have identified three negative regulatory genes, *GEN^c*, *CDR1*, and *TRA3*, which limit transcription of amino acid biosynthetic genes to basal levels when *S. cerevisiae* is grown in rich medium (22, 29, 50). Five unlinked positive regulatory genes, *AAS101*, *AAS102*, *AAS103*, *AAS104*, and *AAS2*, are required for the derepression of the mRNAs of these biosynthetic genes in response to starvation conditions (33, 50). The roles that these positive regulators play in eliciting the general control response are further analyzed in this report.

The molecular signal that activates mRNA derepression of the genes subject to general control has not been identified.

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Changes in internal amino acid pools and in tRNA charging are consequences of growth under starvation conditions (23, 24, 30, 46, 48). It has been suggested that such changes might provide common effectors by which concentrations of multiple amino acids could be monitored simultaneously (24). Efforts to identify the common physiological signal, the regulatory molecules involved in its generation, and the molecules which are directly affected by it must include an analysis of the time period during which the initial regulatory interactions occur.

Our investigations of the temporal aspects of general control mRNA derepression led to two novel observations. (i) Derepression of mRNA of amino acid biosynthetic genes was rapid, occurring within 5 min of a shift of cultures from rich medium to amino acid limitation medium. The *AAS101*, *AAS103*, and *AAS2* gene products were shown to be essential for this initial increase in mRNA levels. (ii) *aas102* mutants were capable of derepressing general control-regulated mRNAs to wild-type levels for only the first 2 h after the media shift. Thereafter, these mRNA levels were dramatically reduced. Thus, the *AAS102* gene product is essential for maintaining the derepressed steady-state levels of these mRNAs. We have cloned the *AAS101* and *AAS102* positive regulatory genes and studied their mRNA expression in response to starvation conditions. The temporal pattern of regulation of mRNA expression is consistent with their respective roles in the initiation and maintenance of the general control response.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* HB101 (4) was used for bacterial transformation and plasmid growth. The bacterial growth medium was as described by Miller (27).

All yeast strains used were derivatives of *S. cerevisiae* wild-type S288C and are listed in Table 1. Minimal medium was as previously described (38). The rich medium (AA medium) consisted of minimal medium supplemented with all 20 amino acids at the following concentrations: 1.0 mM

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
S288C	<i>MATα</i> wild type
X57/2	<i>MATα</i> <i>his1-1</i>
X59/1	<i>MATα</i> <i>his6-1</i>
A941B	<i>MATα</i> <i>trp5</i>
BG68-2B	<i>MATα</i> <i>aas101-1 his6-1</i>
BG69-1C	<i>MATα</i> <i>aas102-15 his6-1</i>
BG70-1B	<i>MATα</i> <i>aas103-15 his6-1</i>
MP40A-2B	<i>MATα</i> <i>aas101-1</i>
MP41A-2D	<i>MATα</i> <i>aas102-1</i>
S288C <i>aas102-12</i>	<i>MATα</i> <i>aas102-12</i>
MP156-2B	<i>MATα</i> <i>aas102-15</i>
MP42-5C	<i>MATα</i> <i>aas103-1</i>
MP172-1A	<i>MATα</i> <i>aas103-11</i>
MP54-1C	<i>MATα</i> <i>aas2-1</i>
MP40A-8B	<i>MATα</i> <i>aas101-1 ura3-52</i>
MP41A-1D	<i>MATα</i> <i>aas102-1 ura3-52</i>
MP156-1A	<i>MATα</i> <i>aas102-15 ura3-52</i>
MP172-1B	<i>MATα</i> <i>aas103-11 ura3-52</i>
MP175-1A	<i>MATα</i> <i>aas103-14 ura3-52</i>
MP43-1D	<i>MATα</i> <i>aas104-1 ura3-52</i>
MP59-1A	<i>MATα</i> <i>aas2-1 ura3-52</i>

alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, methionine, and proline; 2.0 mM leucine, serine, and threonine; 0.75 mM isoleucine and phenylalanine; 0.5 mM arginine and tyrosine; 0.2 mM cystine; 0.3 mM histidine; 1.5 mM lysine; 0.4 mM tryptophan; and 2.5 mM valine. Also included were 0.15 mM adenine sulfate and 0.2 mM uracil. Where indicated, minimal medium was supplemented with 10 mM 3-amino-1,2,4-triazole (3AT), 10 mM leucine, or 1.25 mM 5-methyl-tryptophan.

Time course RNA preparation. Media shifts of exponentially growing yeast cultures at 30°C (absorbance at 550 nm, 0.2 to 0.3) were accomplished by the collection of cells on 0.45- μ m filter units, brief washing in water, and resuspension into equal volumes of prewarmed 30°C medium. This procedure required 5 to 10 min. Samples (40 ml) of cells were withdrawn at specified times after the media shift and were harvested by filtration or by centrifugation at 6,000 $\times$ *g* at 4°C for 3 min. Cells were resuspended in 2 ml of LETS buffer (0.1 M LiCl₂, 10 mM EDTA, 10 mM Tris-hydrochloride [pH 7.0], 1% sodium dodecyl sulfate) and immediately lysed by vortexing in the presence of 0.45 to 0.5-mm glass beads and an equal volume of phenol-chloroform-iso-amyl alcohol (25:24:1). RNA was further purified as previously described (40). In all experiments the strains were tested for viability throughout the time course. No decrease in viability was observed even when cells were cultured under severe amino acid starvation conditions.

Gel electrophoresis and hybridization analysis. RNA was resuspended in water, and the concentration of nucleic acids was determined by comparison of absorbances of sample dilutions at 260 and 280 nm. RNA (20 μ g) was loaded onto each lane, electrophoresed in 1.5% agarose gels containing 6% formaldehyde (21), and transferred to nitrocellulose paper (43). ³²P labeling of DNA sequences was accomplished by nick translation (35). Filter hybridization was carried out at 42°C in 50% formamide-5 $\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate), with 10⁶ cpm/ml. Filters were washed as previously described (44) and were exposed onto Kodak XAR-5 X-ray film with an intensifying screen.

Each time course series of RNA was probed with at least *HIS4* and *ARG4* sequences; most were also probed with *HIS3* and *TRP5* sequences. (These four genes are all subject

to general control.) To normalize for the amount of RNA loaded per lane, we probed each series with ³²P-labeled sequences of a gene not regulated by general control, *URA3* (39). Clones utilized as probes were: pMG1 (M. Greenberg and H. Greer, unpublished data), which contains the 1-kilobase (kb) *EcoRI*-*BglII* fragment internal to the *HIS4* structural gene (9); pYe(*arg4*)511 (5); pYe(*trp5*)1 (45); YIp5, which contains the *URA3* gene (3); and YIp1, which contains the *HIS3* gene (3). ³²P-labeled probes of the cloned AAS genes were obtained by eluting restriction fragments from low-melting-point agarose gels (47) and subsequent nick translation.

Cloning of AAS genes. An *S. cerevisiae* genomic library was constructed by using the vector YCp50. YCp50 is a derivative of YRp16-Sc4301 (41), constructed by C. Mann, which contains *URA3*, *ARS1*, and *CEN3*. *S. cerevisiae* DNA was prepared from S288C (6) and partially digested with *Sau3A*1, and 4- to 8-kb fragments purified from sucrose gradients (21) were ligated into the *Bam*HI site of YCp50. Bacterial transformation was as previously described (20). Plasmid DNA was prepared (1) and used to transform *aas ura3-52* strains to 3AT resistance and uracil prototrophy (16). DNA was isolated from such transformants (31), and the plasmids were propagated in *E. coli* to provide DNA for further analysis. Restriction endonuclease digests were performed according to the specifications of the manufacturers.

aas101-1 and *aas102-1* mutations were cloned by digesting DNA prepared from these strains with *EcoRI* and *Sall*, respectively, and by subsequent subcloning into YCp50. Colony hybridization in which the wild-type clones were used as probes identified plasmids containing *aas101-1* or *aas102-1* inserts (13). Subclones were compared with identical subclones of wild-type AAS sequences for complementation of the *aas* phenotype after transformation of the cognate mutant. Complementation was determined by scoring resistance on plates containing either 10 mM 3AT or 1.25 mM 5-methyl-tryptophan.

RESULTS

Rapid derepression of mRNA levels upon switch to severe starvation conditions. Amino acid limitation can be imposed on *S. cerevisiae* either by growth of an amino acid auxotroph in the absence of the required amino acid or by growth of a wild-type strain in the presence of an amino acid analog. Starvation by either method leads to the derepression of mRNA levels of genes subject to general control. Most analyses of this transcriptional effect have examined steady-state mRNA levels in cells which had been cultured in one type of medium for many hours. However, in one study, pulse-labeling experiments suggested that in cells starved for tryptophan, *TRP5* mRNA is elevated to peak levels after 1.5 h of amino acid-limited growth (51). To understand the physiological aspects of the general control derepression response in greater detail, we examined the kinetics of mRNA elevation when exponentially growing cells were shifted from growth in AA medium to an amino acid starvation medium.

The general control mRNA derepression response of a histidine auxotroph is presented in Fig. 1. In this experiment, a *his1* mutant grown in AA medium was shifted at time zero to minimal medium (which lacks the required histidine). Comparison of the relative mRNA levels of *HIS4* to those of *URA3* during the time course of the experiment revealed several novel features of the general control response. Most striking was the fact that derepression of *HIS4* mRNA occurs within 5 min of the shift to amino acid limitation

FIG. 1. Kinetics of *HIS4* mRNA derepression in auxotrophic cells shifted to starvation medium. Strain X57/2 (*his1-1*) was grown to mid-log phase in AA medium (AA lane). At time 0, the culture was filtered and resuspended into minimal medium. Samples of the culture were harvested by centrifugation at times indicated (0 to 60 min, 2 to 7 h), and RNA was prepared. RNA (20 μ g) from each time point was loaded onto formaldehyde-containing agarose gels, electrophoresed, and transferred to nitrocellulose paper. The filter was probed with 32 P-labeled *HIS4* DNA sequences. 32 P-labeled *URA3* was also used to normalize for the amount of RNA loaded per lane. The middle band corresponds to degradation products which comigrate with the 18S ribosomal RNA band.

medium. This rapid derepression was also observed for the other general control-regulated genes we examined: although cells were starved only for histidine, both *ARG4* and *TRP5* mRNA levels were elevated during early time points (data not shown). Derepression was not a consequence of experimental manipulations, since RNA samples from cultures returned to rich medium at time zero did not exhibit derepressed levels of these mRNAs. In an additional experiment, 1-min time points taken within the first 5 min of the media shift indicated that derepression was detectable between 3 and 4 min.

Further examination of the time course RNA experiment (Fig. 1) reveals that *HIS4* mRNA levels were maximally derepressed at 1 to 2 h after the shift to amino acid limitation medium. After this time, the levels were slightly lowered and were maintained at the new steady state for at least 7 h. Similar mRNA derepression patterns were observed irrespective of the starvation condition imposed. All auxotrophs that we tested, *his1*, *his3*, *his6*, and *trp5*, derepressed mRNA levels within 5 min of a shift from growth in AA medium to growth in minimal medium, with peak mRNA levels occurring after 1 to 2 h. The same pattern of mRNA elevation was observed when wild-type cells were shifted from growth in AA medium to growth in the presence of minimal medium plus any one of the following: 10 mM 3AT (3AT blocks histidine biosynthesis by competitively inhibiting imidazole glycerolphosphate dehydratase [19]), 1.25 mM 5-methyl-tryptophan (5-methyl-tryptophan falsely feedback inhibits anthranilate synthase which catalyzes the first step of tryptophan biosynthesis [28]), or 10 mM leucine (high concentrations of leucine interfere with the biosynthesis of valine and isoleucine [32]). Therefore, it appears that the particular amino acid that is limiting, the specific step that is blocked in a given pathway, and the particular metabolic intermediates which accumulate are irrelevant to the initiation response we observed. Each starvation condition utilized must generate a common intracellular derepression signal with approximately the same kinetics.

Rapid derepression of mRNA levels upon switch to less severe starvation conditions. The aforementioned results suggested that less stringent starvation conditions, e.g., shifting wild-type cells from AA medium to minimal medium without

any amino acid analogs, might be sufficient to elicit the rapid derepression response. Results of such a time course experiment are presented in Fig. 2. Indeed, a rapid elevation of *HIS4* (as well as *ARG4*) mRNA was observed. For the first 30 min, the level of derepression approximately paralleled the response observed for wild-type cells shifted to minimal medium plus an amino acid analog or for an auxotroph shifted to minimal medium. For subsequent time points, mRNA levels remained only partially derepressed. (Steady-state mRNA levels in cells grown in minimal medium have been shown to be two- to fourfold lower than those in cells grown in an analog-containing medium which imposes severe amino acid limitation [33].) Thus, a change in the concentration of external amino acids is sufficient to trigger the initial high mRNA elevation. However, it is the severity of starvation which determines the steady-state mRNA levels that are subsequently established.

Withdrawal of all 20 amino acids is not necessary for general control-mediated derepression. Removal of a single amino acid, e.g., histidine, was sufficient to rapidly elicit derepression of both *HIS4* and *ARG4* (Fig. 3). It is interesting to note that for the first 30 min, the levels of mRNA were approximately the same as they were when all 20 amino acids were removed. The new steady-state mRNA level reached was lower under the less severe condition (in this case, omission of one amino acid) than under the more severe conditions (omission of all 20 amino acids), a finding consistent with the conclusion drawn from Fig. 2. This derepression was not caused by the absence of histidine transport per se. The same mRNA elevation resulted after a shift to medium supplemented with D-histidine and all 19 other L-amino acids. (D-Histidine is transported into the cell but cannot be charged onto tRNA [36].) These results suggested that exogenously supplied amino acids are rapidly utilized for some cellular process and their absence or inability to be employed in this process is rapidly sensed by the general control system.

Rapid repression of mRNA levels upon switch to rich medium. To estimate how long derepressed mRNA levels persist after the addition of amino acids, we shifted wild-type cultures back to rich medium after various periods of growth under 3AT-imposed histidine starvation. Time course analy-

FIG. 2. Kinetics of *HIS4* mRNA derepression in wild-type cells shifted from AA medium to minimal medium. Strain S228C (wild type) was grown to mid-log phase in AA medium (AA lane). At time 0, the culture was filtered and resuspended into minimal medium. Samples of the culture were withdrawn at times indicated (0 to 60 min, 2 to 6 h) and harvested by centrifugation. RNA (20 μ g) from each time point was loaded onto formaldehyde-containing agarose gels, electrophoresed, and transferred to nitrocellulose paper. The filter was probed with 32 P-labeled *HIS4* and *URA3* DNA sequences.

FIG. 3. Kinetics of *HIS4* and *ARG4* mRNA derepression in wild-type cells shifted from AA medium to medium supplemented with all amino acids except histidine. Strain S288C (wild type) was grown to mid-log phase in AA medium (AA lane). At time 0, the culture was filtered and resuspended in medium supplemented with all amino acids except histidine. Samples of the culture were harvested by centrifugation at times indicated (0 to 60 min). RNA (20 μ g) from each time point was loaded onto formaldehyde-containing agarose gels, electrophoresed, and transferred to nitrocellulose paper. The filters were probed with 32 P-labeled *URA3* and either *HIS4* or *ARG4* DNA sequences. The double band hybridizing to the *ARG4* probe may be an electrophoresis artifact due to comigration with the 18S rRNA. Alternatively, the *ARG4* mRNA may result from heterogeneous transcription initiation or termination or from post-transcriptional processing.

sis of RNA prepared from such a culture is presented in Fig. 4. Clearly, repressed mRNA levels of *HIS4* were attained within 30 min of the media shift. This result was independent of the amount of growth time under starvation conditions for at least 3 h. Thus, it appears that the internal starvation signal must be rapidly inactivated or reversed by the addition of amino acids to the medium.

Role of AAS genes in initiation and maintenance of the general control response. Previous studies have established that in an *aas* mutant background, mRNAs of genes subject to general control cannot be elevated to wild-type levels under starvation conditions (33). These analyses, however, examined mRNA levels after many hours of growth under starvation conditions. We performed time course RNA analysis on strains carrying representative mutations in each of the five AAS complementation groups. Starvation was accomplished by growth of *aas his6* strains in the absence of histidine or by growth of *aas* mutants in the presence of 3AT. Our findings divided the *aas* mutants into two classes: (i) *aas* strains that are unable to initiate mRNA derepression to wild-type levels; this class includes the *aas101*, *aas103*, and *aas2* mutants, and (ii) *aas* strains that are capable of initiating the elevation of amino acid biosynthetic mRNAs to wild-type levels for the first 2 h of the response but are unable to maintain high mRNA levels for longer periods of

starvation; *aas102* mutants fall into this class. Results for *aas104* mutants will be discussed elsewhere.

Representative time course experiments for both classes are presented in Fig. 5. An *aas101 his6* strain shifted to minimal medium exhibited derepression of the low basal level of *HIS4* mRNA expression. This derepression occurred within 5 min in experiments which included this time point (Fig. 5A). The mRNA elevation in this background, however, clearly did not reach wild-type levels at any point during the time course. *ARG4* and *HIS3* mRNA expression followed the same pattern. (At present, it is unclear whether the slight derepression observed in the *aas101* mutant background resulted from the independent action of other molecules involved in initiation or from partial function of the mutated *AAS101* gene product. We are constructing deletion mutations of the AAS genes we have cloned (see below) to address this question.) Even less derepression above basal levels is observed in *aas103* or *aas2* backgrounds. Thus, the *AAS101*, *AAS103*, and *AAS2* gene products are required for the initiation phase of the general control response. An implication of these results is that these regulatory gene products must preexist in the cell (possibly in inactive conformations) to effect such a rapid mRNA derepression.

Figure 5B depicts *ARG4* mRNA derepression exhibited in an *aas102 his6* mutant background. The mRNA levels of *ARG4* were elevated for the first 2 h after the media switch. At these time points, the levels of mRNAs paralleled those in the corresponding AAS⁺ starvation experiment. Subsequently, these mRNA levels dropped drastically. This derepression pattern was observed irrespective of the *aas102* alleles tested (*aas102-1*, *aas102-13*, or *aas102-15*) or the starvation condition imposed (*aas102 his6* strains grown in minimal medium or *aas102 HIS⁺* strains grown in 10 mM 3AT or 10 mM leucine). Thus, *aas102* mutants can initiate the general control response but fail to maintain elevated mRNA levels. It is noteworthy that the requirement for the

FIG. 4. Analysis of *HIS4* mRNA levels after a shift of wild-type cells from 3AT starvation medium to AA medium. Strain S288C (wild type) was grown to mid-log phase in AA medium (AA lane). At time 0, the culture was filtered and resuspended into minimal medium containing 10 mM 3AT. After 3 h of growth in this medium (3h), the culture was filtered and resuspended in AA medium (this time point is indicated by an arrow). Samples of the culture were harvested by centrifugation at times indicated (0 to 60 min, 2 to 3 h). Numbers begin at time 0 after each media switch. RNA (20 μ g) from each time point was loaded onto formaldehyde-containing agarose gels, electrophoresed, and transferred to nitrocellulose paper. Filters were probed with 32 P-labeled *HIS4* and *URA3* DNA sequences.

FIG. 5. Kinetics of general control-mediated mRNA derepression in *aas his6* mutant backgrounds. BG68-2B (*aas101-1 his6-1*) and BG69-1C (*aas102-15 his6-1*) strains were grown to mid-log phase in AA medium (AA lane). At time 0, the cultures were filtered and transferred to minimal medium to impose starvation conditions. Samples of the cultures were harvested by centrifugation at times indicated (0 to 60 min, 2 to 7 h). RNA (20 μ g) from each time point was loaded onto formaldehyde-containing agarose gels, electrophoresed, and transferred to nitrocellulose paper. RNA from an AAS⁺ *his6* strain grown for 60 min under starvation conditions is included for comparison of levels of derepression (wt 60). (A) RNA from BG68-2B probed with ³²P-labeled *HIS4* and *URA3* DNA sequences; (B) RNA from BG69-1C probed with ³²P-labeled *ARG4* and *URA3* sequences.

AAS102 gene product correlates with the time that the new derepressed mRNA steady state is established when AAS⁺ strains are exposed to severe starvation conditions (Fig. 1).

Molecular cloning of *AAS101* and *AAS102* genes. We have constructed a genomic library of *Sau3AI* partially digested S288C DNA ligated into the *Bam*HI site of the *S. cerevisiae*-*E. coli* shuttle vector YCp50. This plasmid contains the yeast centromere from chromosome III as well as a yeast *ARS* sequence. These regions limit replication of the plasmid to once per cell cycle such that it exists in a single copy per cell. The *S. cerevisiae URA3* gene is also inserted into this plasmid and can be used as a selectable marker in yeast transformations. This DNA library was used to transform *aas ura3-52* strains to amino acid analog resistance and uracil prototrophy. (*aas* mutants are unable to grow in the presence of amino acid analogs because they are unable to derepress amino acid biosynthetic enzymes to overcome deleterious analog effects.) By using such an approach, clones which complemented *aas101* and *aas102* mutations (C101-1 and C102-2, respectively) were isolated. Restriction analysis facilitated subsequent subcloning into YCp50, and subclones were assayed for retention of the capacity to complement the cognate *aas* mutations. Sequences required

for complementation by such analysis are depicted in Fig. 6 by solid bars. Transformation of representatives of all five *aas* mutant classes with these cloned sequences established that each gene only complements mutations in its respective gene. Homologous sequences cloned from the *aas* mutant backgrounds failed to complement the corresponding *aas* mutations, verifying the identity of the cloned sequences. Identical restriction maps of cloned sequences independently isolated have been shown to map to the corresponding AAS loci by integrative transformation (14).

mRNA expression of the regulatory genes *AAS101* and *AAS102*. The roles that the *AAS101* and *AAS102* genes play in effecting derepression of general control-regulated mRNAs can be further clarified by the determination of the physiological conditions under which they are expressed. The mRNA levels of these AAS genes were analyzed by using the cloned AAS genes as probes in time course experiments.

The mRNA of the *AAS101* gene was expressed at relatively high levels when cells were grown in rich medium (Fig. 7A). Assuming translation of this message, this high level of expression is consistent with the requirement for a preexisting *AAS101* gene product for the rapid initiation of the derepression response. Under amino acid limitation conditions this mRNA level was slightly derepressed. The high basal level expression of this mRNA in rich medium was unaffected in *aas102* mutant backgrounds. However, after 2 h in starvation medium, a marked derepression compared to the levels of an AAS⁺ strain is observed (Fig. 7B).

The *AAS102* gene, required for maintaining elevated mRNA levels after 2 h of starvation, was expressed at relatively low mRNA levels in rich medium (Fig. 7C). Under amino acid starvation conditions, this message was derepressed. The expression of this message depends on the *AAS101* gene product since *AAS102* mRNA failed to accumulate in an *aas101* mutant background (Fig. 7D). Thus, at least one regulatory gene required for maintaining mRNA levels under continuing starvation conditions is induced by the general control system.

DISCUSSION

Temporal analysis of general control-mediated mRNA derepression has revealed several novel aspects of the molecular events involved in this regulatory system. The response to amino acid limitation has been divided into two distinct, but coordinated, phases: (i) the initiation phase, in which elevation of mRNA levels occurs rapidly and mRNA derepression reaches maximum levels, and (ii) the steady-state phase, in which new mRNA levels that reflect the severity of the amino acid starvation condition imposed are established. The regulatory products of the *AAS101*, *AAS103*, and *AAS2* genes are required in the initiation phase, whereas the *AAS102* gene product is required for the maintenance of steady-state mRNA levels.

The sequence of regulatory events that are involved in general control mRNA derepression can be summarized as follows: a change in the amino acid composition of the external environment is rapidly sensed by the *S. cerevisiae* cell; this information is relayed to the nucleus via a signaling pathway that involves the activities of the *AAS101*, *AAS103*, and *AAS2* gene products, resulting in an increase in the transcription of amino acid biosynthetic genes, as well as the *AAS102* gene; and finally, the *AAS102* gene product accumulates and maintains the derepressed steady-state mRNA expression of genes under general control for as long as starvation conditions persist.

FIG. 6. Restriction maps of *AAS101* and *AAS102* clones. Cloned inserts are represented by thin lines. Wide-open bars represent flanking YCp50 DNA; *Sau3AI* sites are at each junction. Restriction sites shown are *Bam*HI (B), *Bgl*II (Bg), *Pst*I (Ps), *Pvu*II (P), *Eco*RI (R), *Sal*I (S), and *Xba*I (X). Wavy lines indicate sequences that were utilized as 32 P-labeled probes in Fig. 7. Thick bars indicate approximate gene positions. For C101-1, the 3.3-kb *SalI*-*EcoRI* fragment is capable of complementing *AAS101* mutations when subcloned into YCp50, whereas the 2.1-kb *Bam*HI-*EcoRI* fragment is not. For C102-2, deletion of the 1.2-kb *EcoRI* fragment eliminates complementation function. A subclone of the 4.6-kb *Bam*HI fragment into YCp50 also fails to complement *aas102* mutations. Transcription mapping of these sequences has shown that the 2.2-kb *Bgl*II fragment hybridizes to a different message from the 1.2-kb *EcoRI* fragment. Since the 0.4-kb *Bgl*II fragment hybridizes with the *AAS102* mRNA and the maximum size of the *AAS102* mRNA is 4.0 kb, the left end of the gene must fall within the *PstI*-*Bam*HI fragment.

FIG. 7. mRNA expression of the *AAS101* and *AAS102* genes. (A) Strain S288C (wild type) was grown to mid-log phase in AA medium (AA lane). At time 0, the culture was filtered and transferred to minimal medium containing 10 mM 3AT. Samples of cells were harvested by centrifugation at times indicated (0 to 120 min), and RNA was prepared. RNA (20 μ g) was loaded onto formaldehyde-containing agarose gels, electrophoresed, and transferred to nitrocellulose paper. The 1.2-kb *SalI*-*Bam*HI fragment from C101-1 was 32 P labeled and utilized as a probe for *AAS101* mRNA expression. 32 P-labeled *URA3* sequences were included for normalizations. (B) RNA samples (20 μ g) from the *aas102-15 his6-1* starvation experiment detailed in the legend to Fig. 5 and from the *AAS⁺ his1-1* starvation experiment detailed in the legend to Fig. 1 were electrophoresed in formaldehyde-containing agarose gels and transferred to nitrocellulose. Filters were probed with the 32 P-labeled 1.2-kb *SalI*-*Bam*HI fragment from C101-1. Samples had been previously normalized for *URA3* mRNA levels (Fig. 1 and 5). (C) RNA samples (20 μ g) from the experiment described in (A) were electrophoresed and transferred to nitrocellulose paper. Time points are in minutes (0 to 60) or hours (2 h to 7 h). The 1.2-kb *EcoRI* fragment from C102-2 was 32 P labeled and utilized as a probe for *AAS102* mRNA expression. 32 P-labeled *URA3* sequences were included for normalization. (D) RNA samples (20 μ g) from the *aas101-1 his6-1* starvation experiment detailed in the legend to Fig. 5 were electrophoresed in formaldehyde-containing agarose gels and transferred to nitrocellulose. Filters were probed with the 32 P-labeled 1.2-kb *EcoRI* fragment from C102-2 and with 32 P-labeled *URA3* DNA sequences.

Initiation phase of the general control response. The rapid mRNA derepression phenomenon and the growth conditions which elicit this response have several implications for the nature of the signal sensed by the regulatory molecules that mediate general control. Although pools of amino acids are large in *S. cerevisiae*, the bulk of amino acids are sequestered in vacuoles and are metabolically inactive (49). Even the cytoplasmic amino acid fraction would not be substantially diminished within 5 min, given the reported rates of protein synthesis (2). Consistent with this conclusion is the observation that protein synthesis continues at the same rate for 45 to 60 min after a shift of *S. cerevisiae* into medium lacking 1 of the 20 amino acids (12). Thus, cellular depletion of a particular amino acid is unlikely to be the cause of rapid mRNA derepression. Nevertheless, external omission of even one amino acid is rapidly sensed and vigorously responded to by the yeast general control system.

Changes of amino acid concentrations in specific subcellular compartments are likely to trigger the signaling events in this response. Protein-labeling experiments in several eucaryotic systems have established that exogenously added amino acids are preferentially utilized in protein biosynthesis before equilibrating with cellular stores (10, 11, 17). This observation implies that amino acids are rapidly charged onto the cognate tRNAs as they enter the cell. If such preferential dependence on exogenously added amino acids was operative in *S. cerevisiae*, withdrawal of amino acids from the medium could result in rapid depletion (falling below critical levels) in this metabolically important subpool of the cytoplasmic fraction.

Such subcellular changes in amino acid concentrations could directly or indirectly signal the activation of the regulatory molecules of the general control system. Preliminary results from our laboratory and published data from other groups suggest that tRNA molecules serve as couplers in the activation pathway. Messenguy and Delforge have documented derepression of enzyme activities subject to general control when a temperature-sensitive isoleucine tRNA synthetase mutant is grown at the restrictive temperature (24). Under this condition, less than 10% of tRNA^{Ile} remains charged, although cellular amino acid pools do not change significantly. Our preliminary analysis of *aas103* mutants suggests that this positive regulatory gene monitors or generates tRNA discharging. We suggest that the withdrawal of amino acids from the external environment transiently results in the discharging of some or all tRNA species in a biosynthetically active cellular compartment. Uncharged tRNAs may then activate one (or all) of the AAS gene products involved in the initiation response or inactivate a negative regulator.

As indicated above, the initiation phase of the general control response is characterized by a transient high derepression of mRNA levels, the duration of which depends on the severity of the starvation imposed. If the signal for this derepression is generated through changes in a specific subcellular compartment, its duration would depend on the time required for the establishment of a new equilibrium between this compartment and the rest of the intracellular pool. This equilibrium should occur faster under less severe starvation conditions than under more severe starvation conditions.

Regulation of the initiation phase. Our data indicate that the *AAS101*, *AAS103*, and *AAS2* gene products are required at least during the initiation phase of general control. We have established that the *AAS101* mRNA levels are relatively high under repressing conditions. This result suggests that

in rich medium, the *AAS101* gene product is present in the cell in an inactive conformation. Activation would then occur rapidly upon a switch to starvation medium, either by interaction with a specific positive effector (e.g., tRNA or metabolites generated in the signaling pathway) or by inactivation of a negative regulator (e.g., *TRA3*).

Genetic analysis has established that a functional *AAS101* gene product is necessary for constitutive derepression in the *tra3-1* mutant, whereas the other AAS gene products are not necessary (14, 50; M. Penn, unpublished data). This result suggests that the *AAS101* gene may be directly involved in the transcriptional activation of genes subject to general control, perhaps by interacting at the 5' regulatory regions of these genes (8, 15). The roles of the *AAS103* and *AAS2* gene products in the initiation response remain to be elucidated.

Steady-state phase of the general control response. After the initial period of high derepression resulting from a switch to starvation medium, the mRNA levels of genes subject to general control are established at a new steady state. The magnitude of mRNA derepression in this phase correlates with the severity of the imposed starvation conditions. In a wild-type strain the steady-state mRNA levels are higher when all amino acids are withdrawn from the culture than when only one amino acid is removed. It is possible that this depends on the levels of the equilibrium reached in the subcellular compartment that generates the signal for general control derepression.

Our data suggest that continuous starvation is required for the maintenance of elevated steady-state levels. A shift of cultures from starvation medium to medium containing all 20 amino acids results in a rapid (ca. 30-min) establishment of the repressed levels for these mRNAs (Fig. 4). Assuming that the 16-min half-life calculated for the average yeast message (34) is applicable to these general control-regulated mRNAs and is not affected by growth conditions, these results are consistent with a rapid shutdown of transcription followed by message turnover. This observation suggests that removal of the postulated activating signal (e.g., uncharged tRNAs) directly inactivates positive regulators of transcription or activates negative regulators.

Regulation of the steady-state phase. Maintenance of the steady-state derepressed mRNA levels of the amino acid biosynthetic genes requires the function of the *AAS102* gene product. The synthesis of the *AAS102* mRNA is induced under starvation conditions. However, as discussed above, the presence of the corresponding regulatory molecule must be coupled with continuous starvation conditions to effect mRNA derepression. At present, the mode of action of the *AAS102* gene product is unclear. It could function in any one of the steps in the general control regulatory pathway, affecting the maintenance of a metabolite starvation signal or directly enhancing transcription.

Maintenance of the elevated mRNA levels may also require some or all of the regulatory gene products that function in the initiation phase. A slight derepression of the *AAS101* mRNA is observed under starvation conditions. One interpretation of this result is that the *AAS101* gene product is also required during the steady-state phase. The larger derepression observed for *AAS101* mRNA in an *aas102* mutant background might occur in an effort to compensate for the lack of the *AAS102* product. Alternatively, the derepression of *AAS101* mRNA in this background might result if the *AAS102* gene product is a negative regulator of *AAS101* gene expression. Whatever the mechanism involved, such overproduction of the *AAS101* mRNA is

not sufficient to overcome the requirement for the *AAS102* product.

In conclusion, we postulate that general control of amino acid biosynthesis exists to maintain a balance of amino acids in metabolically active compartments. The ability of *S. cerevisiae* cells to monitor slight imbalances in their amino acid pools and to respond rapidly by enhancing transcription of genes encoding amino acid biosynthetic enzymes ensures that biosynthesis of these enzymes will be accomplished before potential depletion of cellular amino acids. The subsequent establishment of the steady-state phase limits the extent of mRNA derepression according to cellular needs. A detailed description of this system will require the identification of the regulatory gene products, the determination of their cellular localization, and the elucidation of their interactions with metabolites or other regulatory elements.

ACKNOWLEDGMENTS

We thank Gail Mazzara, Robert Karp, Brian Hauge, and Jai Lingappa for many helpful comments on the manuscript and Phyllis Maffa for essential services contributed.

This work was supported by Public Health Service grant GM25207 to H.G. from the National Institutes of Health.

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