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Control of cell division in *Saccharomyces cerevisiae* by methionyl-tRNA

(nutritional control/growth control/protein synthesis)

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ABSTRACT **We suggest that two events are necessary for an asynchronous population of cells to undergo arrest in the G1 phase of the cell cycle upon nutrient starvation. First, passage through G1 must be prevented by a deficiency of some metabolic intermediate. Since this intermediate may act indirectly to arrest division, we designate it the "signal." We have found three conditions under which *Saccharomyces cerevisiae* cells arrest division in G1: sulfate starvation of a prototroph, methionine starvation of an auxotroph, or a shift of a conditional methionyl-tRNA synthetase mutant [L-methionine:tRNA^{Met} ligase (AMP-forming), EC 6.1.1.10] to a restrictive condition. We interpret these results to indicate that the signal for sulfate starvation in *S. cerevisiae* is generated near the end of the sulfate assimilation pathway (at or beyond the formation of methionyl-tRNA). As a unifying hypothesis, we propose that the signal for all nutrients is generated at the level of protein biosynthesis.**

A second event necessary for G1 arrest is the provision of sufficient protein synthetic capacity for cells to finish the cycles that are in progress when the signal is generated. This necessity is demonstrated by the failure of the methionyl-tRNA synthetase mutant to undergo G1 arrest when protein synthesis is abruptly terminated by a shift to 36° into methionine-deficient medium.

Many eukaryotic cells pass reversibly from a proliferative to a non-proliferative state and, in most cases that have been studied, division is arrested in the G1 interval of the cell cycle (1–3). Since growth is usually required for division, the cells of many organisms, including prokaryotic (4) and eukaryotic (5–9) microorganisms, metazoa (10–17), and metaphyta (18), control division in response to inorganic and organic nutrients. Some evidence exists to suggest that even the protein growth factors of mammalian cells such as serum factors and agglutinins may act by enhancing the availability of small molecular weight nutrients (19, 20).

Saccharomyces cerevisiae is no exception to this general rule, since starvation for any one of a number of nutrients, including ammonia, sulfate, phosphate, potassium, biotin, or a carbon and energy source, results in arrest of cell division at the first of three successive gene-controlled steps in G1 (ref. 8, and J. Pringle, personal communication). Completion of this step occurs when nutrients are sufficient and it initiates the cell cycle by permitting the duplication of the nuclear spindle plaque (21), budding, and acquisition of insensitivity to mating factor (22).

S. cerevisiae may be a useful organism in which to investigate the nutritional control of cell division because it is capable of growth in a defined medium, many gene–enzyme relationships have been determined (23), and a number of gene-controlled cell cycle steps have been defined (24). In this paper the role played by different intermediates of the sulfate assimilation pathway in the control of cell division in *S. cerevisiae* is investigated.

METHODS

Yeast Strains. All strains used in these experiments are listed in Table 2. Methionine-requiring strains were obtained from Drs. John Bassel and Robert K. Mortimer (Yeast Genetic Stock Center at the University of California, Berkeley, $met_{1,2,3,4,5,6,10}^-$); D. Hawthorne (University of Washington, met_{25}^-); H. de Robichon-Szulmajster (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France, $met_{14,16}^-$); A. Singh and F. Sherman (University of Rochester, met_{15}^-); and our collection (mes_1^-). Strains with the prefix *DU* were constructed by first mating *a* haploids to the α-haploid EMS-63 and then producing homozygosity of the mutation for methionine auxotrophy by x-ray-induced mitotic recombination (25). The haploid EMS-63 was supplied by Dr. Gerald Fink (Cornell University).

The pathway of methionine biosynthesis has been discussed by Masselot and Robichon-Szulmajster (26). The methionine auxotrophs used in this paper are representative of the possible levels in the methionine biosynthetic pathway. Only three gene–enzyme assignments have been documented; the met_2^- mutant is defective in homoserine-*O*-transacetylase, met_{25}^- is defective in homocysteine synthetase, and mes_1^- is defective in methionyl-tRNA synthetase [L-methionine:tRNA^{Met} ligase (AMP-forming), EC 6.1.1.10] (26, 27). Strains carrying the *mes* mutation exhibit two phenotypes, a methionine auxotrophy and a temperature-sensitivity. We have undertaken a genetic and biochemical study of this mutation. The results show that both phenotypes are due to a single lesion and provide strong evidence that the lesion is in the structural gene for the synthetase [contrary to a report (28) in the literature]. The remaining mutants have been classified on the basis of feeding tests by both Masselot and Robichon-Szulmajster (26) and ourselves. Both of the following groups of mutants are defective in sulfate assimilation: strains with $met_{3,14,16}^-$ do not grow on sulfate but do grow on sulfite, and $met_{1,4,5,10}^-$ strains do not grow on sulfite but do grow on sulfide. The strain carrying met_6^- is defective in the methylation of homocysteine and it does not grow on homocysteine but does grow on methionine.

Chemicals. Bacto-yeast extract, Bacto-agar, Bacto-peptone, and Bacto-yeast nitrogen base without amino acids were purchased from Difco Laboratories, Detroit, Mich. All amino acids and vitamin supplements were obtained from the Sigma Chemical Co., St. Louis, Mo.

Growth Conditions. Minimal medium consisted of Wickerham's formula (29) plus succinic acid, 10 g/liter, and NaOH, 6 g/liter, as a buffer. To make a sulfate-deficient medium, NH_4Cl, 0.8 g/liter, and $MgCl_2$, 0.2 g/liter, were substituted for $(NH_4)_2SO_4$ and $MgSO_4$. Required amino acids were added at a concentration of 4 mg/liter and purines and pyrimidines, at a concentration of 1 mg/liter. Unless otherwise stated, all cultures were grown at 23° on rotary shakers. The cell number, the proportion of unbudded cells (at least 200 cells were counted for each point), and the incorporation of [*U*-^{14}C]leucine into protein were monitored as described previously (30).

Starvation Conditions. Cells were grown for at least 2 days

Table 1. Cell number increase and proportion unbudded cells under different conditions

	Conditions	Elapsed time (hr)	Cells/ml, $\times 10^{-6}$	Proportion unbudded
A.	Sulfate starvation of 2180a cells	0	0.27	0.48
		17	4.8	0.60
		20.5	6.8	0.73
		22.5	7.8	0.76
		36	8.7	0.97
	Cycloheximide added at 17 hr	17	4.6	0.59
		22.5	4.6	0.60
		36	4.1	0.64
	Cycloheximide added at 20.5 hr	20.5	6.8	0.84
		22.5	7.1	0.82
		36.0	6.8	0.82
B.	Readdition of sulfate to sulfate-starved 2180a*	0	2.5	0.98
		4	2.6	0.94
		8	3.6	0.34
	α-factor† added at 0 hr	0	2.3	0.97
		4	2.3	1.00
		8	2.2	0.95‡
C.	Readdition of methionine to methionine starved DU-mes cells*	0	3.0	0.85
		4	4.6	0.56
		8	14.0	0.46
	α-factor† added at 0 hr	0	3.3	0.82
		4	3.9	0.94
		8	3.9	—‡

* Cells were starved for 30 hr as described in *Methods*.
† α-factor was a gift of Dr. Russell Chan.
‡ Cells displayed the abnormal morphology characteristic of α-factor-treated cells.

in early log phase (less than 2×10^6 cell per ml) by frequent subculturing. At time zero, cells were collected on a Millipore filter (pore size of 0.45 μm) and washed with the appropriate starvation medium, and the filter was transfered to a flask containing starvation medium. This procedure took less than 1 min.

RESULTS

G1 arrest upon starvation for sulfate

When an exponentially growing culture of strain 2180a of *Saccharomyces cerevisiae* was shifted from a minimal medium to a minimal medium without sulfate, the cells continued to divide at the normal rate for several generations and then cell division stopped (Table 1A). At the time of the transfer, 0.48 of the cells were in the unbudded portion of the cell cycle, but by the time division had ceased, 0.97 of the cells were unbudded (Table 1); hence upon sulfate starvation the cells were arrested in a restricted portion of the cell cycle. Sulfate is the limiting component of the medium after starvation, since addition of an inorganic sulfur source, $(NH_4)_2SO_4$ or $MgSO_4$, or an organic sulfur source, methionine or cysteine, allowed division to resume and the cell number increased another 10-fold (data not shown).

Protein synthesis is required for the completion of cell cycles under conditions of sulfate starvation, as is shown by the following experiment. After 17 and 20.5 hr of growth in a minimal medium lacking sulfate, aliquots were removed to another flask containing 10 μg/ml of cycloheximide, a specific inhibitor of protein synthesis (31). Little or no change was observed in cell number or the proportion of unbudded cells (Table 1A).

Several observations indicate that the unbudded cells resulting from sulfate starvation are arrested at a specific point in the G1 interval of the cell cycle. Sulfate-starved cells are unbudded, contain a single nucleus, and have a single spindle plaque in the nuclear membrane (B. Byers, personal communication). This stage of the spindle plaque cycle is diagnostic of a cell cycle block early in G1 prior to the initiation of DNA synthesis (21, 22, 32). Furthermore, when sulfate-starved cells were shifted from a medium lacking sulfate to a medium containing sulfate and the yeast mating hormone, α-factor, they did not divide (Table 1B). The G1 interval of the *S. cerevisiae* cell cycle has been divided into a sequence of three consecutive steps, the first of which is mediated by the product of gene *cdc* 28 (22). The completion of this step results in the acquisition of insensitivity to the yeast mating hormone, α-factor. Sulfate-starved cells are arrested, therefore, at or prior to the first known event in G1 (22).

Rationale for a signal

The experiments presented above show that sulfate-starved cells of *S. cerevisiae* are arrested at a specific step in G1. In response to sulfate limitation a control mechanism must prevent completion of this step but not prevent completion of all other cell cycle events. The trivial explanation that protein synthesis is necessary specifically for this step but not for other steps in the cycle is ruled out by the observation that budded cells, as well as unbudded cells, require protein synthesis to complete the cell cycle (Table 1A).

How might such a control mechanism operate? We suggest that a decrease in the concentration of sulfate, or some intermediate in the sulfate assimilation pathway, results in the specific inhibition of an early G1 event. For the sake of discussion we shall term this intermediate the "signal". The signal might act directly upon the G1 event; e.g., a high concentration of the signal may be necessary as a cofactor for the completion of the early G1 event. On the other hand, it might act through one or more secondary compounds. The mechanism by which the signal inhibits the early G1 event is unspecified and is not the subject of this communication.

This hypothesis suggests a rationale for determining which intermediate in the sulfate assimilation pathway is the signal. When a methionine auxotroph is starved for methionine in the presence of sulfate, it should respond differently, depending on whether the auxotroph is blocked before or after the signal. Consider Fig. 1, in which met_a and met_b are different enzymatic steps in the biosynthesis of methionine and X is the signal. In case A when met_a^- is starved for methionine in the presence of sulfate, the cell will not be able to make compound X from sulfate and therefore the concentration of X should decrease.* Since the concentration of the signal decreases, the cell will be arrested in G1 under our hypothesis. In case B the cell is blocked after the signal. When met_b^- is starved for methionine in the presence of sulfate, the concentration of X will remain high because it can be synthesized from sulfate and the cell will not be arrested in G1. The starved cells will, of course, cease division in either case when they run out of methionine, but the arrest should be specific for G1 in A and nonspecific in B.

Cell cycle arrest of methionine auxotrophs

A variety of methionine auxotrophs were starved for methio-

* We assume that the pathway is reversible from methionine to X so that in the presence of methionine sufficient X is present to permit cell division. Otherwise, the auxotrophic block would result in a permanent G1 arrest and would therefore be lethal.

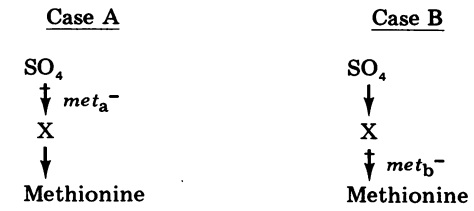

FIG. 1. Effect of auxotrophic blocks upon the signal.

nine and then examined for their position of arrest in the cell cycle (Table 2). For most of the *met*$^-$ complementation groups, the starvation was done in at least two different genetic backgrounds. All the auxotrophs, including the met_6^- strains, which are blocked in the last step in the biosynthesis of methionine, the conversion of homocysteine to methionine (26), are arrested as populations in which most (0.85–0.99) of the cells are unbudded. These results suggest that the signal is subsequent to the met_6^- step in the pathway and is therefore methionine or some product of methionine.

Cell cycle response of a strain carrying the *mes* mutation

One of the products of methionine is methionyl-tRNA. A mutation (mes_1^-) exists that reduces the activity of methionyl-tRNA synthetase in a temperature-sensitive manner (27). This mutation results in a requirement for methionine at the permissive temperature and is lethal at the restrictive temperature. McLaughlin and Hartwell have hypothesized that a single mutation has created an altered methionyl-tRNA synthetase that has a higher K_m for methionine at the permissive temperature and is thermolabile at the restrictive temperature (27). Although the mutant is able to synthesize methionine, additional methionine is required in the medium to overcome the unfavorable K_m for the substrate.

Mutants with the *mes*$^-$ mutation are unique among the methionine auxotrophs since it is possible to arrest growth in two different ways. In strains carrying the *mes*$^-$ mutation, either the removal of exogenously supplied methionine at 23° or a shift to the restrictive temperature of 36° arrests growth, presumably due to a deficiency of methionyl-tRNA. Fig. 2A and B shows that under either restrictive condition strains carrying the *mes*$^-$ mutation arrest as populations in which most of the cells are unbudded (from 0.31 at the time of the shift to 0.85 after methionine starvation or from 0.29 at the time of the shift to 0.78 after incubation at 36°). The unbudded cells produced by methionine starvation of the *mes*$^-$ mutants are arrested at or prior to the α-factor-sensitive step in the G1 interval of the cell cycle, since the unbudded cells in a starved culture did not divide upon the addition of methionine and α-factor (Table 1C). The proportion of unbudded cells does not reach 1.0 in these cultures probably because the cells continue to grow at a slow rate (Fig. 2B and C). Since a strain homozygous for the *mes*$^-$ mutation is arrested in the G1 interval of the cell cycle after either a methionine starvation or a shift to 36° in the presence of methionine, we conclude that if a unique signal exists for impending sulfate starvation, it is located after the step catalyzed by methionyl-tRNA synthetase.

Table 2. Strains, genotypes, and their cell cycle response to methionine starvation

Strain	Genotype	Proportion unbudded* +Met	Proportion unbudded* —Met
2180a	Wild-type	ND	ND
DU-1	met_1^-	0.43	0.86
DU-2	met_2^-	0.47	0.94
DU-3	met_3^-	0.50	0.96
DU-4	met_4^-	0.46	0.99
DU-5	met_5^-	0.43	0.98
DU-6	met_6^-	0.43	0.98
DU-25	met_{25}^-	0.49	0.98
DU-10	met_{10}^-	0.47	0.99
DU-14	met_{14}^-	0.41	0.99
DU-93	met_{15}^-	0.47	0.95
DU-90	met_{16}^-	0.42	0.98
H19-3-4	mes_1^-	ND	ND
DU-mes-1	mes_1^-	(Fig. 2)	(Fig. 2)

ND is not determined.
* The fraction of unbudded cells at start of starvation (+Met) and after 24 hr of methionine starvation (−Met) was determined.

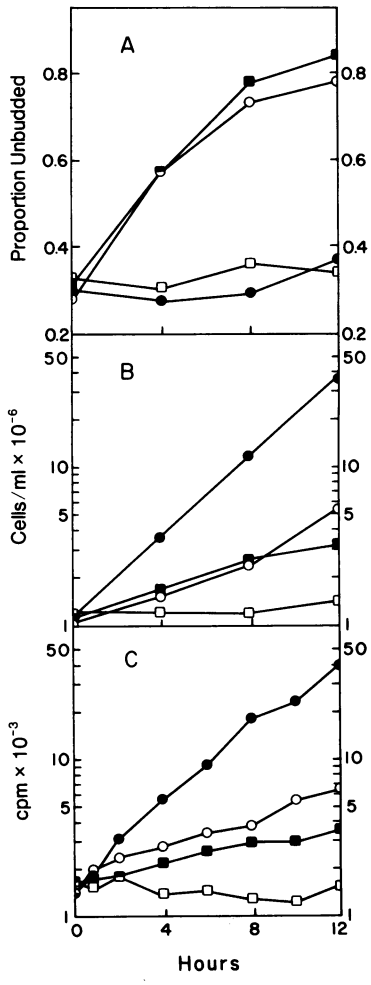

FIG. 2. Proportion of unbudded cells (A); cell number increase (B); and incorporation of [U-^{14}C]leucine into protein in strain DU-mes. For this experiment, minimal medium was supplemented with the following compounds at 4 mg/liter: histidine, isoleucine, phenylalanine, valine, tyrosine, serine, arginine, threonine, lysine, tryptophan, adenine, and uracil. The generation time and the proportion of unbudded cells are significantly decreased by this supplementation as compared to the unsupplemented medium. Final specific activity of leucine was 0.4 μCi/μmol. Symbols: (●) 23° with methionine; (■) 23° without methionine; (○) 36° with methionine; and (□) 36° without methionine.

Requirement for protein synthesis to complete cycles

Since protein synthesis is necessary to complete cell cycles under starvation conditions (Table 1A), it is necessary to consider how cells are able to complete cell cycles after starvation for sulfate, methionine, or methionyl-tRNA. One trivial possibility is that no methionine is needed to make the protein used to complete cell cycles. This possibility is excluded by the following experiment. When a strain carrying the *mes*$^-$ mutation was shifted to both restrictive conditions simultaneously (36° in the absence of methionine), little or no increase in cell number occurred (from 1.2×10^6 cell per ml at the time of the shift to 1.4×10^6 cell per ml after 12 hr), and little or no increase in the proportion of unbudded cells (from 0.33 at the time of the shift to 0.34 after 12 hr) occurred (Fig. 2A and B). We conclude not only that protein synthesis is necessary under starvation conditions, but also that methionine is required for this protein synthesis.

The difference in cell cycle response between a shift to either restrictive condition, as compared to a shift to both restrictive conditions, can be attributed to the degree of inhibition of the synthesis of methionyl-tRNA. As would be expected, protein synthesis in the *mes*$^-$ mutant is reduced from that in a control culture by either methionine starvation or a shift to 36° (Fig. 2C). Protein synthesis is even more drastically inhibited in a culture shifted to methionine-deficient medium at 36° (Fig. 2C). These results suggest that an accumulation of cells in G1 occurs when two conditions are met. First, the rate of formation of methionyl-tRNA must be rate limiting for growth to elicit the signal for cell cycle arrest. Second, there must be sufficient methionyl-tRNA to permit the synthesis of proteins at a reduced rate so that those proteins that are necessary for the completion of the cell cycle can be made. These conditions are apparently fulfilled upon sulfate starvation of prototrophs, methionine starvation of auxotrophs, and upon a shift of the methionyl-tRNA synthetase mutant to 36° or to a methionine-deficient medium. However, a shift of the methionyl-tRNA synthetase mutant to 36° in a methionine-deficient medium does not elicit G1 arrest, presumably because sufficient protein synthesis for the completion of cell cycles is not permitted under these conditions.

DISCUSSION

A population of *S. cerevisiae* cells growing asynchronously arrests division upon sulfate starvation at or before the α-factor-sensitive step in the G1 interval of the cell cycle (Table 1B). Protein synthesis is required during starvation for the cells to complete the last cell cycle before arrest (Table 1A). Thus, the yeast cell must have a mechanism, or mechansims, by which it is signalled of impending sulfate starvation. Furthermore this signal, which produces a specific inhibition of cell cycle progress at the α-factor-sensitive step in G1, must occur at a time when sufficient protein synthetic capacity exists for the other steps of the cycle to be completed.

Methionine auxotrophs have been used to identify the level in metabolism at which this signal is generated. The results reported in Table 2 and Fig. 2 suggest that if only one signal exists in the sulfate assimilation pathway for impending sulfate starvation, it is located after the step controlled by the methionyl-tRNA synthetase. If more than one signal for sulfate starvation exists, then one of the signals must be after this step. Our results are compatible with the signal's being generated at any level of protein biosynthesis. The signal might be methionyl-tRNA, a product of the ribosome (like ppGpp, ref. 33), or the accumulation of a specific protein.

Since yeast cells arrest growth at the same point in the G1 portion of the cell cycle after starvation for sulfate, ammonia, phosphate, potassium, biotin, or a carbon and energy source, they must have a mechanism that signals the availability of each of the six nutrients. The simplest hypothesis to explain how the cell controls division in response to a limitation in any one of these nutrients is that the signal for all of these is the same and the signal lies at the level of protein biosynthesis. Furthermore, since bacterial cells undergo G1 arrest upon starvation for ammonia (4) and mammalian cells undergo G1 arrest upon starvation for some amino acids (10–13), we suggest that the signal for the nutritional control of cell division may lie at the level of protein biosynthesis in all cells.

This unifying hypothesis predicts that an early step prior to the initiation of DNA synthesis will be inhibited in cells when protein synthesis is inhibited by starvation for a variety of nutrients including amino acids or by an inhibition of amino acid activation onto tRNA. Preliminary experiments with an isoleucyl-tRNA synthetase and various protein synthesis mutants suggest that a limitation of protein synthesis by any one of several means can under certain conditions lead to a G1 arrest. A defect in amino acid biosynthesis or activation might be responsible for the phenotype of the yeast *tra*$_3$ mutant, which is constitutively derepressed for the enzymes of the histidine, arginine, lysine, and tryptophan biosynthetic pathways and arrests division after a shift to 36° in the G1 interval of the cell cycle (34).

The hypothesis that nutritional control is mediated by a signal generated at the level of protein synthesis does not, however, predict that a population of cells will necessarily become arrested in G1 when protein synthesis is inhibited by any means for the following reason. To achieve G1 arrest a population of cells must fulfill a second requirement in addition to receiving the hypothetical signal, namely, they must be able to mobilize sufficient protein synthetic capacity after the signal has been received to permit completion of cycles in progress. The distinction between these two requirements was first recognized by Everhart and Prescott in their experiments with leucine deprivation of Chinese hamster cells (12). Abrupt withdrawl of leucine arrested cells in G1, S, and G2, but trace levels of leucine permitted cells in S and G2 to complete the cycle so that most of the cells accumulated in G1. Their explanation of this observation as a differential sensitivity of the G1 interval to a reduced rate of protein synthesis is entirely consistent with both our results and our interpretation. It seems reasonable to imagine that control mechanisms will have evolved to assure the fulfillment of this second requirement for those types of starvations which an organism is accustomed to encountering. In this context it is noteworthy that G1 arrest is a general response of micro-organisms to starvation for essential nutrients (refs. 5–9, and J. Pringle, personal communication). Auxotrophic mutants of normally prototrophic micro-organisms and mammalian cells, which are accustomed to a relatively constant nutritional environment, however, may not be able to fulfill this second requirement in all cases.

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