Methionine analogs and cell division regulation in the yeast
Saccharomyces cerevisiae

(G1 phase/cell cycle/rRNA metabolism/ethionine/selenomethionine)

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ABSTRACT Methionine analogs such as ethionine, selenomethionine, and trifluoromethionine all arrest growth and division of the yeast Saccharomyces cerevisiae. One analog, ethionine, caused cells of the yeast to arrest specifically within G1; reciprocal shift experiments showed that ethionine and α-factor arrested cells at the same step ("start"). The major effect of ethionine on synthesis of macromolecules was to reduce both the rate of appearance of 35S ribosomal precursor RNA and the rate of production of mature rRNA. Synthesis of protein was relatively unaffected by ethionine. Selenomethionine and trifluoromethionine caused cells to arrest randomly in the cell division cycle. Although treatment of cells with either selenomethionine or trifluoromethionine also reduced the rate of total RNA synthesis, each of these analogs had other effects that presumably prohibited completion of the cell cycle. We propose that the rate of rRNA production is an important regulatory event in the cell cycle.

The yeast Saccharomyces cerevisiae, like most eukaryotic cells, regulates its division in the G1 portion of the cell cycle. A temperature-sensitive mutant (cdc 28) affected in the ability to progress through G1 and the mating pheromone α-factor have been used to define a period within G1 called "start" (1). The performance of start results in the initiation of both budding and deoxyribonucleic acid (DNA) synthesis. Before the start event can be completed, many physiological requirements must be met. Starvation for required nutrients causes cells of S. cerevisiae to arrest in G1 prior to completion of start.

Recently, Unger and Hartwell (2) demonstrated that starvation for sulfate caused yeast cells to arrest at a defined point in G1. Moreover, they demonstrated that depriving methionine auxotrophs of methionine, or incubating a strain conditionally defective in methionyl-tRNA synthetase at the nonpermissive temperature, also caused cells to accumulate within G1. We have further examined the molecular basis for G1 regulation by determining the effects of methionine analogs on growth and cell division. We have used three analogs—L-ethionine, DL-selenomethionine, and L-trifluoromethionine—all of which have been shown (3) to have similar effects on methionine metabolism. All three analogs repress endogenous methionine biosynthesis, may be esterified both in vitro and in vivo to tRNA, and may be incorporated into protein.

We have found that one of these analogs, ethionine, causes cells to accumulate rapidly in G1. Selenomethionine and trifluoromethionine, on the other hand, cause cells to arrest randomly in the cell division cycle. We report here our investigations into the molecular alterations correlated with the ethionine-mediated G1 arrest.

MATERIALS AND METHODS

Strains and Media. The diploid strain S. cerevisiae AG1-7 (ura1 his6) and its isogenic haploid segregant GR2 (a ura1 his6) have been described (4, 5). Cells were grown in liquid synthetic medium (YNB) described previously (6), supplemented with uracil (20 mg/ml) and histidine (40 mg/ml). Radioactive uracil, histidine, and methionine were all obtained from New England Nuclear Corporation. The mating pheromone α-factor was prepared by the method of Bucking-Throm et al. (7).

Cell number was determined as described elsewhere (8).

Analysis of Macromolecular Metabolism. The incorporation of labeled precursors into macromolecular material was measured as previously described (4). Degradation of RNA and protein was measured by quantitating release of acid-soluble radioactivity into the trichloroacetic acid-extractable pools.

For RNA preparation, cells were frozen in dry ice/ethanol and lyzed, and the RNA was extracted and fractionated, as previously described (5). Labeled material was resolved by electrophoresis through 2.5% or 10% polyacrylamide gels (5), and gels were processed for scintillation counting as described (4).

RESULTS

Effects of Methionine Analogs on Cell Division. When cells of strain AG1-7 were treated with either DL-ethionine (30 mg/ml) or L-ethionine (10–15 mg/ml), cell number increased approximately 1.8-fold and 85–90% of cells in the final arrested population were unbudded. This unbudded condition indicated that the cells were in the G1 portion of the cell cycle (1). Cell cycle arrest was not found for cells treated with D-ethionine (Fig. 1). When cells were treated with either selenomethionine (10 mg/ml) or trifluoromethionine (10 mg/ml), cell number showed little increase and cells in the final arrested population displayed random bud morphology, indicating that cells were arrested throughout the cell division cycle. At no concentration did either of these analogs cause G1 arrest.

To locate the point of ethionine-mediated arrest in G1, we ordered this block with respect to the mating pheromone-sensitive step defined as start (1). The isogenic haploid strain GR2 (mating type a) was employed in these order-of-function mapping experiments (1). Cells of strain GR2 were first treated with α-pheromone. During the 4 hr of treatment, cell number increased slightly less than 2-fold; all cells in the final arrested population were unbudded, and many displayed the characteristic pear-shape morphology of α-factor-treated cells. Cells were then suspended in fresh medium without α-factor, and L-ethionine (to 10 mg/ml) was added to half of the culture. After a further incubation of 6 hr, cell number began to increase in the untreated population, and by 10 hr cell number had increased approximately 3-fold (Fig. 2C). In the ethionine-treated culture even after 10 hr there was no increase in cell number.

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treated cells placed in fresh medium with \( \alpha \)-factor showed a decrease in the proportion of unbudded cells after 6 hr of incubation. Ethionine-treated cells shifted into medium containing \( \alpha \)-factor, on the other hand, remained unbudded (Fig. 2B). Moreover, the unbudded cells began to show the characteristic pear-shape morphology of \( \alpha \)-factor treatment. The \( \alpha \)-factor-treated cells eventually overcame the transient cell cycle arrest (9) caused by the mating pheromone. Thus, ethionine caused cells to arrest either prior to or at the \( \alpha \)-factor-sensitive step. Considering the two reciprocal shift experiments together, we conclude that ethionine treatment causes cells to arrest at the same point as \( \alpha \)-factor (that is, at start).

**Synthesis of Macromolecules during Treatment with Ethionine.** Initially we determined those alterations in macromolecular synthesis that were correlated with ethionine-mediated G1 arrest. To one portion of a culture of strain AG1-7, which had been growing exponentially for several generations, was added L-ethionine to a final concentration of 10 \( \mu \)g/ml. A second portion served as a control. At intervals, the rate of incorporation of either \(^{3}H\)uracil or \(^{3}H\)histidine was determined as described (4, 5). The results of these pulse labeling experiments are shown in Fig. 3A. In ethionine-treated cells, the rate of protein synthesis remained unaffected for at least 4 hr, whereas the rate of incorporation of uracil into RNA showed an immediate and drastic decrease. During this period of ethionine treatment, we have been unable to detect release of previously incorporated \(^{3}H\)uracil or \(^{3}H\)histidine into trichloroacetic acid-soluble pools. The absence of demonstrable turnover of stable RNA makes it unlikely that the reduced level of uracil incorporation observed in this uracil-requiring strain was due to increased pool sizes from intracellular macromolecular degradation. Moreover, the specific activities of the pyrimidine triphosphate pools, resolved chromatographically (10), were not greatly altered by ethionine treatment (data not shown).

**RNA Synthesized during Treatment with Ethionine.** Because treatment with ethionine had a dramatic effect on the rate of RNA synthesis, we examined the types of RNA produced during treatment. Cells of strain AG1-7 were first grown for

![Graph](image1.png)

**Fig. 1.** Growth in the presence of ethionine. A growing population of cells was divided into four portions, which received no additions (●), D-ethionine to 15 \( \mu \)g/ml (□), L-ethionine to 15 \( \mu \)g/ml (●), or DL-ethionine to 30 \( \mu \)g/ml (●).

![Graph](image2.png)

**Fig. 2.** Order-of-function mapping of ethionine and \( \alpha \)-factor-sensitive steps. (A and B) Cells were arrested in G1 by treatment with L-ethionine. At the time indicated by the arrow, the ethionine-containing medium was replaced by media with and without \( \alpha \)-factor. (C and D) Cells were arrested in G1 by treatment with \( \alpha \)-factor. At the time indicated by the arrow, the \( \alpha \)-factor-containing medium was replaced by media with and without L-ethionine. O, Cells in the absence of G1-arresting conditions; ●, cells in the presence of ethionine; ▼, cells in the presence of \( \alpha \)-factor.
Thus, the effects on molecules, gels, treatment. Accumulation rRNA species ethionine. Untreated cells and incubated RNA species. Several generations (A) was samples of analog-treated and control cultures were removed to tubes containing either [3H]histidine (10 μCi/ml) or [3H]uracil (10 μCi/ml) (1 Ci = 3.7 × 10¹² becquerels). After a 5-min incubation, incorporation was stopped by addition of an equal volume of 10% trichloroacetic acid. (A) Effect of ethionine; (B) effect of trifluoromethionine; (C), effect of selenomethionine. °, [3H]Uracil incorporation in the absence of analog; •, [3H]uracil incorporation in the presence of analogs; ○, [3H]histidine incorporation in the absence of analogs; ●, [3H]histidine incorporation in the presence of analogs.

Figure 3. Rates of uracil and histidine incorporation during analog treatment. Analogs were added to 10 μg/ml at time zero. At intervals, samples of analog-treated and control cultures were removed to tubes containing either [3H]histidine (10 μCi/ml) or [3H]uracil (10 μCi/ml) (1 Ci = 3.7 × 10¹² becquerels). After a 5-min incubation, incorporation was stopped by addition of an equal volume of 10% trichloroacetic acid. (A) Effect of ethionine; (B) effect of trifluoromethionine; (C), effect of selenomethionine. °, [3H]Uracil incorporation in the absence of analog; •, [3H]uracil incorporation in the presence of analogs; ○, [3H]histidine incorporation in the absence of analogs; ●, [3H]histidine incorporation in the presence of analogs.

Figure 4. Polyacrylamide gel resolution of high molecular weight RNA. Cells were grown for several generations in the presence of [14C]uracil, transferred to unlabeled medium, and incubated for 2 hr. To half the culture was added ethionine to 10 μg/ml; 30 min later, [3H]uracil was added to each culture and incubation was continued for a further 20 min before cells were harvested and RNA was extracted. RNA was resolved on 5.5% polyacrylamide gels. (A) Labeled RNA from ethionine-treated cells; (B) labeled RNA from untreated cells. °, [3H]RNA; ○, [14C]RNA.

Table 1. [14C]Methyl to [3H]uracil ratio in RNA

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Control</th>
<th>Ethionine</th>
<th>Selenomethionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>25S</td>
<td>0.23 (1.00)</td>
<td>0.185 (0.80)</td>
<td>0.056 (0.24)</td>
</tr>
<tr>
<td>18S</td>
<td>0.215 (1.00)</td>
<td>0.175 (0.81)</td>
<td>0.06 (0.28)</td>
</tr>
<tr>
<td>4S</td>
<td>1.15 (1.00)</td>
<td>1.08 (0.94)</td>
<td>0.21 (0.18)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are ratio normalized to that of control cells.

In Fig. 4 is not the result of extensive deficiencies in the methylation process.

Rate of RNA Synthesis in Ethionine-Treated Cells. To determine the rate of transcription of RNA genes, we examined the rate of accumulation of [3H]uracil in 35S ribosomal precursor RNA. A culture of strain AG1-7, previously labeled with [14C]uracil, was divided, and L-ethionine (10 μg/ml) was added to one half. After 30-min incubation, cells were pulse labeled for periods of 2, 4, and 6 min with [3H]uracil. Extracted RNA from these cells was resolved by polyacrylamide gel electrophoresis (Fig. 5). In ethionine-treated cells, the appearance of labeled 27S and 20S ribosomal precursor RNA species, as well as mature rRNA species, was severely inhibited (Fig. 5B) when compared to untreated cells (Fig. 5A). Labeling of the 35S peak, containing the initial transcript of the RNA genes (11), was also reduced (note the difference in scales for A and B). For each gel the amount of [3H]label in the 35S region was determined after subtraction of the labeled heterogeneous background. The accumulation of label in the 35S peak for ethionine-treated cells and control cells is shown in Fig. 5C. The analog diminished the rate of label accumulation in this region to approximately half that found for untreated cells. The diminished rate of label accumulation is not due to altered rate of precursor uptake into cells, because[3H]uracil is taken up with similar kinetics by both ethionine-treated cells and untreated populations (data not shown).

The results shown in Fig. 5 suggest that in ethionine-treated cells the rate of transcription of ribosomal RNA genes is reduced. This type of analysis provides minimum estimates of the rate of transcription of ribosomal RNA genes, but does not account for removal of labeled 35S RNA molecules by cleavage during normal processing in untreated cells or by degradation of unprocessed ribosomal precursor RNA that may occur in ethionine-treated cells.

Effect of Selenomethionine and Trifluoromethionine on RNA and Protein Metabolism. At concentrations of ethionine
with cells Arrows indicate the metabolism or analogs in RNA. We rate the cell ionone, the RNA [3H]uracil. of previously shown sion through the effect of selenomethionine during trifluoromethionine described for stable RNA. From this, we could not distinguish between a real decrease in methylation of RNA and a decrease in labeling due to competition by unlabeled methyl groups of selenomethionine.

To resolve this issue, the methylation of newly synthesized tRNA was quantitatively analyzed. Results of an analysis of 32P-labeled tRNA, synthesized from 30 to 60 min after the addition of selenomethionine, are given in Table 2. It is evident that methylated nucleosides in the tRNA synthesized in selenomethionine-treated cells are found at near-normal frequencies. Thus, the reduction in [14C]methyl labeling shown in Table 1 must stem from efficient competition by unlabeled methyl groups in selenomethionine-treated cells. Selenomethionine is known to be incorporated into the selenoadenosylmethionine analog of S-adenosylmethionine (3) and may donate unlabeled methyl groups in this way.

![Fig. 5](image-url) Appearance of labeled precursor ribosomal RNA during treatment with ethionine. A growing culture prelabeled with [14C]-uracil was divided; one half (A) received no additions and the other half (B) received L-ethionine to 10 μg/ml. After a 30-min incubation, cells were labeled for periods of 2 (G), 4 (N), and 6 (Q) min with [3H]uracil. RNA was extracted and resolved by polyacrylamide gel electrophoresis. Arrows indicate the positions of 14C-labeled 25S and 18S rRNA. (C) Accumulation of label in the 35S peak for ethionine-treated (●) and untreated (○) cells, normalized to 14C-labeled 25S RNA.

As shown in Fig. 3, treatment of cells with either selenomethionine or trifluoromethionine caused a rapid decrease in the rate of RNA synthesis. For cells treated with selenomethionine, the rate of protein synthesis remained high; in contrast, trifluoromethionine treatment caused a reduction in the rate of protein synthesis concomitant with the reduction in the rate of RNA synthesis. Because continued protein synthesis has been previously shown (2, 4) to be necessary for continued progression through the cell cycle, the absence of a uniform bud morphology during trifluoromethionine treatment may be due to the effect on protein synthesis.

The effects of selenomethionine on RNA and protein production were investigated in more detail. When RNA produced during selenomethionine treatment was analyzed as previously described for RNA produced during ethionine treatment, a similar pattern of accumulation of ribosomal precursor RNA was observed (data not shown). Other effects on RNA metabolism were also apparent. As shown in Table 1, selenomethionine treatment led to a reduction in the incorporation into stable RNA species of [14C]methyl groups from labeled methionine. From this experiment, we could not distinguish between a real decrease in methylation of RNA and a decrease in labeling due to competition by unlabeled methyl groups of selenomethionine.

To resolve this issue, the methylation of newly synthesized tRNA was quantitatively analyzed. Results of an analysis of 32P-labeled tRNA, synthesized from 30 to 60 min after the addition of selenomethionine, are given in Table 2. It is evident that methylated nucleosides in the tRNA synthesized in selenomethionine-treated cells are found at near-normal frequencies. Thus, the reduction in [14C]methyl labeling shown in Table 1 must stem from efficient competition by unlabeled methyl groups in selenomethionine-treated cells. Selenomethionine is known to be incorporated into the selenoadenosylmethionine analog of S-adenosylmethionine (3) and may donate unlabeled methyl groups in this way.

![Fig. 6](image-url) Effect of analogs on incorporation of labeled methionine and histidine into acid-precipitable material. A growing population was split into three portions. To one was added ethionine and to the second selenomethionine, and the third received no additions. At intervals, aliquots were moved to tubes containing either [14C]methionine or [14C]histidine. After 5-min incubation, incorporation was stopped by addition of an equal volume of 10% trichloroacetic acid. The ratio of [14C]methionine to [14C]histidine was taken as a measure of competition between the analog and methionine for incorporation into protein. ○, cultures receiving no additions; ●, cultures receiving selenomethionine; ●, cultures receiving ethionine.
Methionine analogs may be incorporated into protein in place of methionine (3, 14). To determine if analog-containing proteins were being synthesized under our conditions, aliquots of cells were labeled with either [14C]methionine or [14C]histidine. As shown in Fig. 6, selenomethionine treatment drastically diminished the methionine/histidine label ratio in newly synthesized proteins. It is unlikely that selenomethionine treatment induced the differential synthesis of a class of proteins with reduced methionine content; in fact, by one-dimensional gel analysis the complement of proteins synthesized in analog-treated cells differs little from that found in untreated cells (data not shown). Instead, selenomethionine probably competes effectively at the level of methionyl-trRNA synthetase to reduce the rate of [14C]methionine incorporation into protein. This conclusion is supported by the evidence that, for yeast methionyl-tRNA synthetase, selenomethionine is a competitive inhibitor with a K_I of 2 μM, a value equal to the K_m for methionine (3). In contrast, L-ethionine under G1-arresting conditions competes poorly with methionine at the level of tRNA synthetase and has no effect on the relative amount of [14C]methionine incorporated (Fig. 6). Thus, another effect of selenomethionine treatment not found during ethionine treatment is the extensive synthesis of methionine analog-containing proteins. Because synthesis of functional proteins must be required for progression through the cell cycle, the absence of a uniform cell cycle effect during selenomethionine treatment may be due to effects on functional protein production.

**DISCUSSION**

We have examined the effect on cell division of three methionine analogs: L-ethionine, DL-selenomethionine, and L-trifluoromethionine. One of these, L-ethionine, at a concentration of 10 μg/ml caused cells, within one cell division cycle, to arrest in G1 at a step coincident with the start period. Cells treated with ethionine displayed a dramatic decrease in the extent of ribosomal precursor RNA labeling. Overall rates of protein synthesis were unaffected. Moreover, ethionine treatment retarded the rate of precursor ribosomal RNA processing. Methylation of stable RNA species was affected to a limited extent.

Two other methionine analogs, selenomethionine and trifluoromethionine, failed to cause a G1 arrest but instead arrested cells throughout the cell division cycle. With trifluoromethionine treatment there were other effects as evidenced by the decrease not only in the rate of RNA synthesis but also in the rate of protein synthesis. Selenomethionine treatment also caused alterations in addition to those affecting rRNA metabolism. In contrast to ethionine, selenomethionine effectively replaced methionine as a methyl donor and was incorporated into protein in place of methionine. Presumably, these other effects preclude continuation through the cell cycle of cells treated with selenomethionine or trifluoromethionine.

Previously, several workers using rat liver showed that ethionine treatment causes a preferential reduction in the rate of RNA synthesis (15). This effect was attributed to an alteration in energy metabolism resulting from decreased intracellular concentrations of adenine nucleotides. The ethionine-mediated G1 arrest that we observe was not abrogated by the addition of adenine or adenosine. This suggests that adenine nucleotide pool levels do not mediate the G1 arrest that we observe. Moreover, abrupt depletion of an energy source such as glucose (J. R. Pringle, personal communication) causes cells to arrest with random bud morphology. Because ethionine-treated cells are able to complete the cell division cycle and all the arrested cells are viable, we feel it is unlikely that ethionine treatment significantly affects energy metabolism.

One of the major effects of ethionine treatment is the reduction of free methionine pools (3). Two observations suggest that alterations in free methionine pools may be involved in bringing about G1 arrest during ethionine treatment. First, added methionine will overcome the growth inhibitory effect of ethionine (unpublished data); second, mutants resistant to ethionine (16) have increased intracellular methionine pools. We suggest that one regulatory effect of ethionine treatment may be to limit the intracellular supply of methionine.

Warner and his colleagues (17) have established that inhibition of synthesis of ribosomal precursor RNA leads to inhibition of synthesis of mRNA for ribosomal proteins; however, the converse is not true. That is, inhibiting ribosomal protein synthesis by inhibiting synthesis of mRNA does not seem to affect the levels of ribosomal precursor RNA production (18). From these findings it is unlikely that the decrease in the rate of rRNA synthesis we observe is due to altered rates of ribosomal protein synthesis.

The effects of ethionine on cells of the yeast are similar to those observed when we used the zinc-chelating agents 8-hydroxyquinoline or o-phenanthroline (5). With both of these agents we observed, under conditions giving rise to specific G1 arrest, an initial period in which ribosomal RNA synthesis was reduced and protein synthesis rates remained high. The similarity of the metabolic changes caused by zinc-chelating agents and by ethionine suggests that the alterations are part of a generalized response by cells to conditions that will lead to G1 arrest. Although alterations in the level of protein synthesis may well affect the cell division cycle, we feel that production of ribosomal precursor RNA may itself be an important regulatory event for progression through G1.

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