Proofreading \textit{in vivo}: Editing of homocysteine by methionyl-tRNA synthetase in \textit{Escherichia coli}

\textit{(in vivo $^3$S-labeling/homocysteine thiolactone/translational accuracy/aminoacyl adenylate/methionine biosynthesis)}

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\textbf{ABSTRACT} Previous \textit{in vitro} studies have established a pre-transfer proofreading mechanism for editing of homocysteine by bacterial methionyl-, isoleucyl-, and valyl-tRNA synthetases. The unusual feature of the editing is the formation of a distinct compound, homocysteine thiolactone. Now, two-dimensional TLC analysis of $^3$S-labeled amino acids extracted from cultures of the bacterium \textit{Escherichia coli} reveals that the thiolactone is also synthesized \textit{in vivo}. In \textit{E. coli}, the thiolactone is made from homocysteine in a reaction catalyzed by methionyl-tRNA synthetase. One molecule of homocysteine is edited as thiolactone per 100 molecules of methionine incorporated into protein \textit{in vivo}. These results not only directly demonstrate that the adenylate proofreading pathway for rejection of misactivated homocysteine operates \textit{in vivo} in \textit{E. coli} but, in general, establish the importance of error-editing mechanisms in living cells.

The synthesis of functional proteins depends on accurate transcription and translation of genetic information. To accomplish this with the least possible expenditure of energy, cells would have to evolve mechanisms capable of achieving accuracies better than 1 error in $10^{8}$ - $10^{10}$.

Two steps of protein synthesis are important for accurate translation of genetic information: the selection of amino acids for aminoacylation of tRNA by synthetases and the selection of aminoacyl-tRNA in the codon-programmed ribosomal A site. The accuracy of initial amino acid selection for tRNA aminoacylation is far greater than the accuracy of subsequent ribosomal processes (1). During selection of amino acids, synthetases very often have to distinguish the cognate substrate from a homologue having just one methyl group less in its structure. The binding energy of a methyl group is estimated to contribute only a factor of 10$^2$ to the specificity of binding (2), yet synthetases distinguish such closely related amino acids with a discrimination factor of $10^8$ - $10^{10}$ (3, 4). Examples of this include isoleucine vs. valine, alanine vs. glycine, threonine vs. serine, and methionine vs. homocysteine.

Aminoacylation of tRNA is a two-step reaction. In the first step, an amino acid (AA) is activated to form enzyme (E)-bound aminoacyl adenylate.

\[ E + AA + ATP \rightarrow E:AA-AMP + PP_i \]

In the second step, the amino acid is transferred from the adenylate to tRNA.

\[ \text{E:AA-AMP + tRNA}^{AA} \rightarrow E + \text{AA-tRNA}^{AA} + \text{AMP} \]

Extraordinary fidelity of the tRNA aminoacylation reaction is due to enzymatic proofreading or editing activities of aminoacyl-tRNA synthetases (5, 6). It has been proposed that aminoacyl-tRNA synthetases can exercise kinetic proofreading of incorrect substrates (7, 8). Editing can occur by the hydrolysis of the noncognate aminoacyl adenylate (9-11) or the aminoacyl-tRNA (12-15). Both editing mechanisms have been directly demonstrated \textit{in vitro}. Although it has been inferred from the \textit{in vitro} studies that editing is also important \textit{in vivo}, direct evidence for this was missing.

The aminoacyl-adenylate proofreading pathway originally discovered with valyl-tRNA synthetase (9, 10) has subsequently been established \textit{in vitro} to be of major importance with several other synthetases (11). In particular, methionyl-, isoleucyl-, and valyl-tRNA synthetases edited misactivated homocysteine by the adenylate pathway with the formation of homocysteine thiolactone. This feature of the homocysteine editing reaction provides a means to assay for editing \textit{in vivo} by looking for a special product of editing, i.e., homocysteine thiolactone. I. now report that homocysteine thiolactone is in fact synthesized \textit{in vivo} by the bacterium \textit{Escherichia coli}. The data indicate that the thiolactone synthesis in \textit{E. coli} is due to efficient \textit{in vivo} editing of homocysteine by methionyl-tRNA synthetase, thus establishing the existence and importance of proofreading \textit{in vivo}.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial Strains and Plasmids.} Wild-type \textit{E. coli} K-38 was obtained from N. Zinder (Rockefeller University). \textit{E. coli} K-12 \textit{metE} was from N. Brot (Rochelle Institute of Molecular Biology). The following strains were obtained from the \textit{E. coli} Genetic Stock Center (Yale University): RK4356 (\textit{metE metH}); CS50 (\textit{metG}); CA274 (\textit{trp49}); AB2575 (\textit{ilvD}); Hfr3000YTA73 (\textit{thrb}); CR147 was from M. Cashel (National Institutes of Health). Spontaneous Met$^+$ revertants of CS50 (\textit{metG146}) were selected on M9 (16) agar with leucine, proline, histidine, and threonine and without methionine. Strain HD1 harboring plasmid pRS734 was obtained from P. Schimmel and J. Burbarna (Massachusetts Institute of Technology). pRS734 contains the methionyl-tRNA synthetase gene cloned into pUC19 under the control of lac promoter. The plasmid contains also the lacI gene. The methionyl-tRNA synthetase gene produces a truncated protein that is fully active both \textit{in vitro} and \textit{in vivo} (17, 18).

\textbf{Growth and $^3$S-Labeling Conditions.} Cells were grown aerobically at 37°C in M9 medium plus auxotrophic requirements. After the culture reached a density of $4 \times 10^8$ cells per ml, cells were harvested by centrifugation in an Eppendorf microcentrifuge at room temperature for 30 sec, washed with M9 medium, resuspended in M9 with or without auxotrophic requirements, and maintained at 37°C with vigorous aeration. The cultures ($4 \times 10^8$ cells per ml) were labeled with 10 $\mu$M $[^3$S]$\text{Cysteine}$ at 0.05-0.1 mCi/ml (1 Ci = 37 GBq) (Amersham).

Abbreviation: IPTG, isopropyl $\beta$-D-thiogalactopyranoside.
For [35S]sulfate labeling experiments, low-sulfate M9 medium was used. The low-sulfate medium contained either 0.15 mM sulfate for growth or 0.15 mM [35S]sulfate at 0.1 mCi/ml (675 Ci/mol) (New England Nuclear) for labeling.

**Preparation of 35S-Labeled Extracts.** At specified time intervals, aliquots (20 μl) of the 35S-labeled E. coli cultures were removed and extracted with 5 μl of 5 M formic acid for 30 min at 0°C. The extracts were clarified by centrifugation in an Eppendorf microcentrifuge for 5 min at 4°C and analyzed immediately.

**Intracellular Concentrations of Sulfur-Containing Amino Acids.** E. coli cells were grown for four generations in low-sulfate M9 medium containing 0.15 mM [35S]sulfate (675 Ci/mol). Methionine auxotrophs, metE, and metG, pregrown to 4 × 10⁸ cells per ml in low-sulfate medium containing methionine, were centrifuged, washed, and suspended in low-sulfate M9 containing 0.15 mM [35S]sulfate. The cultures were maintained with aeration at 37°C for 5 hr. The labeled cells were collected on nitrocellulose filters (0.2 μm; Millipore) and extracted with 0.25 ml of 1 M formic acid on ice for 30 min. The filters were washed with two 0.12-ml aliquots of 1 M formic acid. The extracts and washes were combined, clarified by centrifugation, and lyophilized. The residues were taken up in 10 μl of water and analyzed by two-dimensional TLC.

**Two-Dimensional TLC Analysis of 35S-Labeled Compounds: Detection of [35S]Homocysteine Thiolactone in E. coli Extracts.** Extracts (5 μl) were applied as a spot on cellulose plates (10 × 10 cm; Sigma). Butanol/acetic acid/water (4:1:1, vol/vol) was used as the first-dimension solvent and 2-propanol/ethyl acetate/ammonia/water (25:25:1:4, vol/vol) was used as the second-dimension solvent. Standard sulfur-containing compounds were cochromatographed with the 35S-labeled samples. The standards were located under UV light and/or after staining with ninhydrin. 35S-labeled compounds were visualized by autoradiography using Kodak XAR-5 film. The 35S-labeled spots were quantitated by scintillation counting. Counting efficiency was 60%. Homocysteine was stable during extraction and TLC (half-life, 300 hr). The thiolactone was somewhat unstable in the ammonia-containing solvent used for the second dimension of the TLC (half-life, 1.5 hr) and its recovery was 70% during the 1-hr run.

**Measurements of Methionyl-tRNA Synthetase Activity.** E. coli strains were grown in LB medium to a cell density of 8 × 10⁶ per ml. The cells were harvested by centrifugation, washed with ice-cold 10 mM potassium phosphate buffer, pH 6.8/10 mM 2-mercaptoethanol/10% (vol/vol) glycerol, suspended in the buffer at a cell density of 2 × 10⁹ per ml, and disrupted by sonication (three strokes of 20 sec) on ice. The extracts were clarified by centrifugation in an Eppendorf microcentrifuge for 10 min at 4°C. Levels of methionyl-tRNA synthetase in the extracts were determined by tRNA aminoclaylation with [35S]methionine (15).

**RESULTS**

**Detection and Identification of [35S]Homocysteine Thiolactone in E. coli.** A metE E. coli strain, which is expected to accumulate homocysteine due to a mutation in the homocysteine transmethylase gene, was grown in M9 medium containing 0.2 mM methionine. Cells were harvested, washed, and resuspended in methionine-free M9 medium containing 10 μM (50 μCi/ml) [35S]cysteine. At time intervals up to 1 hr, 35S-labeled compounds were extracted from the metE cultures and resolved by two-dimensional TLC on cellulose plates. Autoradiograms of three of these chromatograms are presented in Fig. 1. Fig. 1A shows a schematic diagram of the resolution. A new 35S-labeled spot comigrating with a homocysteine thiolactone standard appeared after only 8 min of labeling (Fig. 1C); and its intensity increased by 48 min (Fig. **Fig. 1.** Two-dimensional TLC separation of sulfur-containing compounds from E. coli cultures. First dimension, butanol/acetic acid/water (4:1:1, vol/vol); second dimension, 2-propanol/ethyl acetate/ammonia/water (25:25:1:4, vol/vol). (A) Identities of the compounds based on comigration with standards. (B–F) Autoradiograms of the two-dimensional separation of formic acid extracts from the following [35S]cysteine-labeled E. coli cultures maintained in M9 medium: methionine-starved metE labeled for 1 min (B), 8 min (C), and 48 min (D); not starved (E) and arginine-starved (F) argA (strain CR147) labeled for 1 hr. Hcy, homocysteine; MTA, S-methylthioadenosine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine. 1D). In contrast to what was expected, the methionine-starved metE mutant did not accumulate homocysteine (Fig. 1B–D). Apparently, excess homocysteine was eliminated as the thiolactone. Similar results were obtained with a metE metH double mutant (data not shown).

**Methionyl-tRNA Synthetase Mutants Are Defective in Homocysteine Thiolactone Synthesis.** The metG mutant has a defect in methionyl-tRNA synthetase, which results in a higher Km for methionine and leads to methionine auxotrophy (20). As shown in Fig. 2, the metG mutant is also defective in the synthesis of homocysteine thiolactone. The rate of the thiolactone synthesis in the metG culture was 10-fold lower than that in the wild type, which in turn was 25-fold lower...
FIG. 2. Time course of homocysteine (Hcy) thiolactone synthesis in E. coli cultures. The levels of [35S]homocysteine thiolactone (pmol per 2 x 10^9 cells) in the following E. coli cultures were determined at indicated time intervals: metE (○), wild type (●), metG (●), and a spontaneous Met^+ revertant of the metG strain (metG^+·-●).

than that in the metE strain. A Met^+ revertant of the metG strain, metG^+·-●, did not synthesize any detectable levels of thiolactone (Fig. 2).

Table 1 shows the intracellular concentrations of methionine, homocysteine, and homocysteine thiolactone in four E. coli strains. The concentrations of methionine and homocysteine in wild-type cells determined in this study are within a factor of 2 with respect to the concentrations determined in another wild-type E. coli (21). To my knowledge, the thiolactone has not been reported in bacterial cells before. However, as shown in Table 1, it is a significant component of the sulfur amino acids pool in E. coli.

Unexpectedly, a methionine-starved metE mutant did not accumulate homocysteine (Table 1). Instead, it accumulated the thiolactone. The intracellular concentration of homocysteine in metE was in fact 6.6-fold lower and the concentration of the thiolactone was 3.5-fold higher than in wild type. Some of the increase in rate of the thiolactone synthesis may also be due to a higher level of methionyl-tRNA synthetase in metE than in wild-type cells. Upon shift to medium lacking methionine, the level of methionyl-tRNA synthetase in metE cells decreased from about 5-fold over wild type before the shift, to 2-fold over wild type after 30 min without methionine (data not shown), as expected (22). Thus, the increase in the rate of the thiolactone synthesis in metE over wild type is mostly due to the absence of methionine in metE cells. As will be shown below, methionine prevents the thiolactone synthesis.

Intracellular levels of methionine were lower and the level of homocysteine was higher in a metG^− strain than in wild type (Table 1). This in itself would lead to higher rates of the thiolactone synthesis. Thus, the defect in the thiolactone synthesis is apparently due to the metG^− mutation, which results in 17-fold higher K_m values for the amino acid substrates (data not shown).

The intracellular levels of methionine and homocysteine in the metG^+·-● revertant were similar to those in wild type, yet there was no synthesis of the thiolactone in the revertant. Methionyl-tRNA synthetase in the metG^+·-● strain differs slightly from that in metG^−. The K_m for methionine decreased 1.5-fold and the V_max increased 1.9-fold relative to metG^−, resulting in 3-fold higher specificity (V_max/K_m) of the methionyl-tRNA synthetase present in metG^+·-● over that in metG^− (data not shown). Although the differences were small, they were reproducible. These results suggest that the metG^+·-● revertant acquired a second point mutation affecting either the amino acid binding site of the methionyl-tRNA synthetase or its interaction with tRNA, either of which could abolish the thiolactone-forming activity of the enzyme.

Previous attempts to genetically map mutations leading to prototrophy of the metG^− strain failed due to the lack of markers in the metG region of the E. coli chromosome (20) and therefore were not repeated here.

Rate of Homocysteine Thiolactone Synthesis Is Directly Proportional to Cellular Levels of Methionyl-tRNA Synthetase.

The cellular level of Met-tRNA synthetase was manipulated in E. coli strain HD1 harboring pRS734, a plasmid bearing the methionyl-tRNA synthetase gene under control of the lac promoter; the relationship between the synthetase concentration and homocysteine thiolactone synthesis was studied. The rate of the thiolactone synthesis in the HD1/pRS734 culture increased upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG, 3 hr) from 0.7 pmol/hr to 3.5 pmol/hr (Fig. 3A), which paralleled the increase in the levels of methionyl-tRNA synthetase from 0.5 unit to 2.75 units (data not shown). The rate of protein synthesis was the same in noninduced and induced cultures (Fig. 3B). Comparison of the rate of the thiolactone synthesis (Fig. 3A) with the rate of protein synthesis (Fig. 3B) in a noninduced culture allows one to quantify the frequency of homocysteine editing in E. coli. It can be estimated that 1 homocysteine molecule was edited as thiolactone per 10^9 molecules of methionine incorporated into protein.

Several different induction conditions were used: no IPTG added and IPTG added from 4 hr to 15 min before harvesting cultures at 2 x 10^9 cells per ml. Methionyl-tRNA synthetase

Table 1. Intracellular levels of sulfur amino acids in E. coli

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Intracellular concentration, pmol per 10^9 cells</th>
<th>Hcy/thiolactone</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Met</td>
<td>Hcy</td>
</tr>
<tr>
<td>Wild type</td>
<td>4.9</td>
<td>6.6</td>
</tr>
<tr>
<td>metE</td>
<td>&lt;0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>metG</td>
<td>2.6</td>
<td>11.2</td>
</tr>
<tr>
<td>metG^+·-●</td>
<td>4.3</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Wild-type and metG^+·-● cells were growing exponentially. metE and metG^− cells were nongrowing. Hcy, homocysteine.
levels and the rate of thiolactone formation were determined for each culture. Fig. 4 shows that there was a direct relationship between the level of methionyl-tRNA synthetase and the rate of homocysteine thiolactone synthesis in bacterial cells. The regression line describing the relationship extrapolated to 0.1 pmol of thiolactone at zero methionyl-tRNA synthetase, which indicates that at least 80% of the thiolactone synthesis in wild-type cells is due to the synthetase.

**Synthesis of Homocysteine Thiolactone Is Inhibited by Methionine and Norleucine.** Methionine, the natural substrate of methionyl-tRNA synthetase, totally abolished synthesis of the thiolactone by all strains tested (Table 2). Norleucine, an artificial substrate of the synthetase (15), also prevented synthesis of the thiolactone. However, inhibition of the thiolactone formation in this case resulted in accumulation of homocysteine (data not shown), which confirms that homocysteine is the direct precursor of the thiolactone in *in vivo*.

Ethionine, another artificial substrate for methionyl-tRNA synthetase, was much less inhibitory (data not shown), perhaps because of its limited transport into cells due to its larger size than norleucine and methionine. Several other amino acids (isoleucine, valine, leucine, and arginine) did not have any significant effect on the thiolactone synthesis by the *metE* strain (Table 2). The inhibitory effect of valine on thiolactone synthesis by the wild-type strain was probably due to valine-induced starvation for isoleucine (24), since addition of isoleucine reversed the inhibition. Isoleucine alone and leucine alone had only minor effects on the thiolactone synthesis by the wild-type strain, as did arginine (Table 2). That isoleucine, valine, and leucine did not inhibit synthesis of the thiolactone was further confirmed by observations that levels of homocysteine thiolactone in *ilv*, *leu*, and *thr* *E. coli* cultures were not increased by starvation. The thiolactone levels, in fact, decreased somewhat in starved *ilv*, *leu*, and *thr* cultures (data not shown), similar to effects observed in starved arg (Fig. 1 E and F) and wild-type cultures starved for isoleucine by valine addition (Table 2). These data exclude any significant participation of isoleucyl-, valyl-, or leucyl-tRNA synthetase in homocysteine thiolactone synthesis in *E. coli*. Instead, the results indicate that methionyl-tRNA synthetase is the primary enzyme involved in the thiolactone synthesis in bacterial cells.

**DISCUSSION**

This study confirms the *in vivo* existence of an efficient proofreading mechanism that prevents incorporation of homocysteine into tRNA and protein in the bacterium *E. coli*. It also directly shows that proofreading in general is important in living cells.

Both genetic and biochemical evidence presented above indicate that homocysteine is transformed into homocysteine thiolactone by methionyl-tRNA synthetase in *E. coli*, an editing reaction previously discovered *in vitro* (11). The thiolactone has not been reported in living cells before. As shown here (Table 1), it is a significant component of sulfur amino acid pools in *E. coli*. The thiolactone was formed at a rate of 1 pmol per 2 × 10^6 cells per hr (Figs. 2–4) and accumulated within cells at a relatively low concentration of 1.2 pmol per 10^6 cells (Table 1). Thus, most of the thiolactone was secreted from bacterial cells and was in fact found in cell-free medium (data not shown). *E. coli* has the capacity to metabolizing the thiolactone to methionine since some *met* mutants (e.g., *metA*) are able to grow on homocysteine thiolactone (unpublished data).

In addition to methionyl-tRNA synthetase, several other aminocetyl-tRNA synthetases edit misactivated homocysteine by transforming it into thiolactone *in vitro* (11, 25). However, the *in vivo* results presented in this work indicate that most if not all of the editing of homocysteine in *E. coli* cells is via methionyl-tRNA synthetase. That the editing of misactivated homocysteine does not occur with the valyl-, isoleucyl-, and leucyl-tRNA synthetases in *E. coli* cells can be partially explained by the *in vitro* measurements of the efficiency of the reactions with homocysteine. Methionyl-tRNA synthetase is the most efficient (kcat/Km = 16.7 sec^-1·mM^-1) of the four synthetases (11). Isoleucyl-tRNA synthetase (kcat/Km = 9.3 sec^-1·mM^-1) is about 2-fold less efficient than the methionyl-tRNA synthetase. Valyl- and leucyl-tRNA synthetases are the least efficient with kcat/Km values 100-fold and 70-fold, respectively, lower than that for methionyl-tRNA synthetase (11, 25). This may explain why misactivation and editing of homocysteine essentially does not occur with valyl- and leucyl-tRNA synthetases *in vivo*. However, as the kinetic indices indicate, editing of homocysteine by isoleucyl-tRNA synthetase should occur *in vivo*. There is a potential mechanism that could prevent interaction of homocysteine with isoleucyl-tRNA synthetase. Possibly,

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**Table 2. Effect of amino acids on synthesis of homocysteine (Hcy) thiolactone by *E. coli***

<table>
<thead>
<tr>
<th>Amino acid(s)</th>
<th>E. coli culture</th>
<th>[35S]Hcy thiolactone synthesis rate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.2 mM) metE</td>
<td>Wild type</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Met</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Ile</td>
<td>106</td>
<td>65</td>
</tr>
<tr>
<td>Val</td>
<td>114</td>
<td>28</td>
</tr>
<tr>
<td>Ile, Val</td>
<td>118</td>
<td>73</td>
</tr>
<tr>
<td>Leu</td>
<td>101</td>
<td>83</td>
</tr>
<tr>
<td>Arg</td>
<td>104</td>
<td>86</td>
</tr>
<tr>
<td>Norleucine</td>
<td>8.3</td>
<td>18.6</td>
</tr>
</tbody>
</table>

The 100% values were 15 pmol/hr (*metE*) and 0.8 pmol/hr (wild type); 1 pmol = 4300 cpm.
interenzyme metabolite transfer may be involved in the metabolism of homocysteine in vivo. The interenzyme metabolite transfer would lead to compartmentalization of homocysteine reactions to within the methionine biosynthetic pathway. This would also account for the lack of homocysteine accumulation in metE cells observed in this study. Alternatively, tRNA may affect the amino acid specificity of isoleucyl-tRNA synthetase in vivo. Whatever the explanation, one cannot avoid the conclusion that some specific proofreading reactions that have been studied in vitro may not be relevant in vivo.

The relationship between the last step of the methionine biosynthetic pathway and the proofreading reaction is depicted in Scheme I. In the last step of the biosynthetic pathway, homocysteine (Hcy) is methylated to methionine by the product of the metE or metH gene. Any amount of homocysteine that cannot be processed to methionine is edited as homocysteine thiolactone by the metG gene product, whose other major function is to provide methionyl-tRNA\textsuperscript{Met} for protein synthesis. Quantitation of the metabolite flow through methionyl-tRNA synthesis indicates that 1 mol of homocysteine is edited as the thiolactone per 109 mol of methionine incorporated into protein. This is in a good agreement with predicted theoretical numbers of 1 homocysteine edited per 140 methionines incorporated based on in vitro measurements of $K_m$ and $V_{max}$ values for homocysteine and methionine with methionyl-tRNA synthetase (11) and on in vivo concentrations of the amino acids (Table 1).

The demonstration of in vivo editing in amino acid selection during protein synthesis relied on the distinct chemical nature of the edited amino acid: misactivated homocysteine was transformed into the thiolactone during editing. The thiolactone can be easily separated from homocysteine and other sulfur compounds by two-dimensional TLC. Another case where in vivo editing may be demonstrated is the misactivation of homoserine, a nonprotein amino acid, by valyl- and isoleucyl-tRNA synthetases. Misactivated homoserine is edited in vitro by the cleavage of homoserine adenylate with the formation of homoserine lactone (not shown). Cyclization will also occur with the adenylates of aspartate, glutamate, and lysine. Whether these amino acids are misactivated and edited is not known, but assaying for cyclic forms of these three amino acids in vivo may provide a means of establishing this point.

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