MnmE is an evolutionarily conserved, three domain GTPase involved in tRNA modification. In contrast to Ras proteins, MnmE exhibits a high intrinsic GTPase activity and requires GTP hydrolysis to be functionally active. Its G domain conserves the GTPase activity of the full protein, and thus, it should contain the catalytic residues responsible for this activity. In this work, mutational analysis of all conserved arginine residues of the MnmE G-domain indicates that MnmE, unlike other GTPases, does not use an arginine finger to drive catalysis. In addition, we show that residues in the G2 motif (G29GTTRD253), which resides in the switch I region, are not important for GTP binding but play some role in stabilizing the transition state, specially Gly249 and Thr251. On the other hand, G2 mutations leading to a minor loss of the GTPase activity result in a non-functional MnmE protein. This indicates that GTP hydrolysis is a required but non-sufficient condition so that MnmE can mediate modification of tRNA. The conformational change of the switch I region associated with GTP hydrolysis seems to be crucial for the function of MnmE, and the invariant threonine (Thr251) of the G2 motif would be essential for such a change, because it cannot be substituted by serine. MnmE defects result in impaired growth, a condition that is exacerbated when defects in other genes involved in the decoding process are simultaneously present. This behavior is reminiscent to that found in yeast and stresses the importance of tRNA modification for gene expression.

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GTPases are involved in many different and essential cellular processes, including protein synthesis and translocation, membrane trafficking, signal transduction, and cell cycle control (1, 2). The common property shared by these proteins is the presence of a structural module, the G-domain, which is involved in the switching of the protein between a GTP-bound and a GDP-bound conformation. This conformational switch is crucial for the function of all GTPases (3).

Three-dimensional structures of members from the G protein superfamily indicate that the conserved residues of the G domain (grouped in motifs G1 to G4, Fig. 1) are invariably involved in binding of guanine nucleotides and Mg2+, hydrolysis of GTP, or controlling the conformational change. This change is primarily confined to two highly flexible regions called switches I and II, which include the G2 and G3 motifs, respectively.

The GTPase cycle is regulated by the intrinsic properties of each GTPase, as well as by the specific factors that the GTPase interacts with. Thus, GAPs stimulate GTPase reaction by several orders of magnitude and guanine nucleotide exchange factors and guanine nucleotide-dissociation inhibitors regulate the nucleotide exchange (3). Regulatory GTPases, like Ras-related proteins, are in an active state when GTP-bound; the binding of GTP causes a conformational change in the proteins that allows interaction with a target molecule or effector; upon GTP hydrolysis, they become inactive. The GTPase cycle of Ras proteins requires participation of GAPs and guanine nucleotide exchange factors, because these GTPases typically show a very low intrinsic hydrolytic activity and a very high affinity for guanine nucleotides. In most members of Ras family, GAPs stabilize the transition state of the reaction by supplying a so-called arginine finger (which stabilizes the negative charge development at the phosphate groups of GTP during the hydrolysis reaction) and enhancing stability of amino acids located in switches I and II (3–7). A glutamine adjacent to motif G3 is also crucial to stabilize the transition state and orient the attacking water molecule, being assisted in this respect by the arginine finger. Ga subunits of trimeric G proteins use a similar mechanism for GTP hydrolysis, but the catalytic invariant arginine is provided in cis from a helical domain of the GTPase polypeptide (3). Other cases of GTPases carrying putative cat-

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1 The abbreviations used are: GAP, GTPase-activating protein; P, inorganic phosphate; cmnm, carboximethylaminomethyl; cmnm5s2U34, 5-carboximethylaminomethyl-2-thiouridine at position 34; mant, methylanthraniloyl; GTP;§§ guanosine 5’-3-O-thiotriphosphate.
The evolutionarily conserved MnmE protein is a GTPase with a G-domain motif in Esherichia coli (12, 13). MnmE is involved in the modification of the wobble-position uridine (U34) of tRNAs that read codons ending with A or G, in the mixed codon family boxes, i.e., tRNAVal<sup>cmnm5s2UUG</sup>, tRNAAsp<sup>Glu</sup><sup>cmnm5s2UUU</sup>, tRNA<sup>Glu</sup><sup>Val</sup><sup>cmnm5s2UUU</sup>, tRNA<sup>Leu</sup><sup>Glu</sup><sup>cmnm5s2UUU</sup>, and tRNA<sup>Arg</sup><sup>cmnm5s2UUU</sup>. In the modification pathway of these tRNAs, MnmE controls, together with GidA, the addition of the cmnm group in position 5 of the U34, although the precise role of both proteins in the modification reaction is still unknown (Fig. 2). In tRNA<sup>Leu</sup><sup>cmnm5s2UUU</sup> and tRNA<sup>Glu</sup><sup>cmnm5s2UUU</sup>, MnmC transforms the cmnm5 group into the final cmnm5 modification, meanwhile MnmA, together with IscS, carries out thiolation in the 2-position of the wobble uridine (14–16). Modifications in the 2- and 5-positions occur independently of each other; thus, thiolation may precede or follow the synthesis of the side chain at position 5. Note that non-thiolated derivatives are present in tRNA<sup>Leu</sup><sup>cmnm5s2UAA</sup> and tRNA<sup>Arg</sup><sup>cmnm5s2UUC</sup>, whereas tRNA<sup>Glu</sup><sup>cmnm5s2UUU</sup> contains cmnm<sup>s2</sup>.<sup>2</sup> When selenium is available in the growth medium, sulfur at position 2 may be replaced by selenium in a reaction dependent on SelD and YbbB (17–19). It has been shown that modifications at position 2, but not at position 5, are important for aminocylation of tRNAs (20–22), whereas modifications in both 2- and 5-positions function in the codon recognition process (17, 18, 22–27).

Unlike the G-domain-only proteins of the Ras family, MnmE is a medium-size protein of 50 kDa that consists of three regions, an ~220-amino acid N-terminal region, required for self-assembly, a middle GTPase domain of about 160 residues, and an ~75-amino acid C-terminal region, which contains the only Cys residue present in the protein (12) (see Fig. 1). Moreover, MnmE can be distinguished from other GTPases like Ras, which were previously described (13). To determine values for $K_m$ and $V_{max}$, the data were fitted to the Michaelis-Menten equation using non-linear regression (GraphPad Prism version 3.00 for Windows, GraphPad Software, Inc.). MnmE was titrated against fluorescent mann nucleotides (Jena Bioscience) until saturation was reached. The mann nucleotides (2 μM) were excited at 380 nm, and the fluorescence was monitored at 440 nm (LS 50 B spectrophotometer, PerkinElmer Life Sciences). Unless otherwise indicated, all binding assays were performed at 25 °C in GTPase buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 5% glycerol). The binding constants ($K_i$ values) were calculated by fitting the curves with non-linear regression, using GraphPad Prism software.

**Readthrough Measurements**—Misreading of the UAG stop codon carried by the lacZ105 gene was monitored by using the β-galactosidase assay as described (13). Special precautions for very low β-galactosidase activities were taken (21, 24).

**Analysis of tRNA<sup>Glu</sup> Modification by Northern Blot**—Total tRNA from strains grown to an $A_{600}$ of 0.6–0.8 was extracted and deacylated (22). The tRNA was quantified by absorbance measurement at 260 nm with a Unicam UV-visible spectrophotometer (Helios-β). Northern blots were performed as described (13).

**Analysis of Modified Nucleosides in tRNA**—Bacterial strains were grown in Luria-Bertani broth at 37 °C to about 5 × 10<sup>8</sup> cells/ml ($A_{600}$ ~ 0.8). The cells were lysed, and total RNA was prepared (31), dissolved in 2 G. R. Björk, unpublished observation.

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2 G. R. Björk, unpublished observation.

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3 M. Martinez-Vicente, L. Yim, M. Villarroya, M. Mellado, E. Pérez-Puyà, G. R. Björk, and M.-E. Armengod, unpublished data.
mutational analysis of the MnmE Switch I region

In the switch I region of GTP-binding proteins, an invariant Thr is the only residue of the G2 motif conserved among GTPase families (Fig. 1). It is usually involved, via its side-chain hydroxyl in the coordination of the crucial Mg^{2+} and, via its main chain NH, in contacting the γ-phosphate. Other residues of the G2 motif are highly conserved within each GTPase family, but not between different families. Alignment of members of the MnmE family indicates that motif GTTRD, between G1 and G3, is absolutely conserved and, therefore, it appears as the putative G2 signature sequence of this family (Fig. 1). To analyze its role in the biochemical properties of MnmE, we firstly changed each residue in this motif to alanine. To determine whether the mutant proteins were capable of binding guanine nucleotides, we carried out binding studies in solution with fluorescent nucleotides. Mant-GTP probes of G-protein activation and conformational state (33–37) have been shown to be useful for G-proteins capable of binding guanine nucleotides, we carried out binding studies in solution with fluorescent nucleotides. Mant-GTP probes of G-protein activation and conformational state (33–37) have been shown to be useful for studying G-proteins. For example, mant-GTP was used as a probe for determining the affinity of G-Protein-coupled receptors for their ligands (33–37). In some cases, mant-GTP was found to be a more sensitive probe than NADH or FAD for monitoring the activation of G-proteins (33–37).

RESULTS

Mutational Analysis of the MnmE Switch I Region—In the switch I region of GTP-binding proteins, an invariant Thr is the only residue of the G2 motif conserved among GTPase families (Fig. 1). It is usually involved, via its side-chain hydroxyl in the coordination of the crucial Mg^{2+} and, via its main chain NH, in contacting the γ-phosphate. Other residues of the G2 motif are highly conserved within each GTPase family, but not between different families. Alignment of members of the MnmE family indicates that motif GTTRD, between G1 and G3, is absolutely conserved and, therefore, it appears as the putative G2 signature sequence of this family (Fig. 1). To analyze its role in the biochemical properties of MnmE, we firstly changed each residue in this motif to alanine. To determine whether the mutant proteins were capable of binding guanine nucleotides, we carried out binding studies in solution with fluorescent nucleotides. Mant-GTP probes of G-protein activation and conformational state (33–37) have been shown to be useful for studying G-proteins. For example, mant-GTP was used as a probe for determining the affinity of G-Protein-coupled receptors for their ligands (33–37). In some cases, mant-GTP was found to be a more sensitive probe than NADH or FAD for monitoring the activation of G-proteins (33–37).

Titration experiments were also performed to determine the dissociation constant of the wild-type MnmE protein for mant-GDP, which was calculated to be 4.1 μM (data not shown). This means that, under our experimental conditions, MnmE binds with a 3-fold higher affinity to mant-GTP-γ-S than to mant-GDP, which is in agreement with previous studies suggesting a higher affinity of MnmE for GTP-γ-S than for GDP (12). The moderate affinity of MnmE for nucleotides as well as its apparent preference for GTP over GDP might play a role in the control of the GTPase cycle of this protein. To investigate whether some G2 mutation has a significant effect on the GDP-binding ability of MnmE, we again made use of a fluorescent analogue. Upon binding of mant-GDP (2 μM) to each mutant MnmE protein (10 μM), an increase in fluorescence intensity similar to that obtained with the wild-type protein was observed (Fig. 3, lines b), which suggests that mutant proteins roughly conserve the mant-GDP binding properties of wild-type MnmE. In all these experiments, a variant in the G1 motif of MnmE (G228A), which had been previously shown to be substantially impaired for GTP binding (13), was used as a negative control. Protein G228A did not enhance the mant-GTP or mant-GDP emission intensity at 440 nm (data not shown), indicating that G228A-mant-nucleotide complexes did not form.

MnmE is a GTPase that requires very high substrate concentrations for half-maximal reaction velocity (12, 13, 38). Thus, to determine the enzymatic parameters of GTP hydrolysis by mutant MnmE proteins, GTP concentration was varied from 0 to 4 mM, and the inorganic phosphate produced in the reaction was quantified. As shown in Table III, the wild-type MnmE protein exhibited a GTPase activity of about 200 nmol of GTP/min/mg of protein, being the K_m for GTP hydrolysis very high, as usual. The intrinsic GTPase activity of MnmE was reduced by replacement of each G2 residue with alanine, being the magnitude of the effect more pronounced in the order T251A, G249A, D253A, R252A, and T250A. MnmE, like GTPases Era, Obg, and Cgt, has two adjacent threonine residues in the G2 motif; because the rate of hydrolysis (V_max) was decreased about 100-fold for T251A and only 4-fold for T250A (Table III), we conclude that Thr^{251} is much more important for catalysis. In this respect, behavior of MnmE is somewhat similar to that described for CgtA (34) in which substitution of the second threonine (Thr^{192}) by alanine has more drastic effects for GTP hydrolysis than substitution of the first one (Thr^{195}) that does not have any effect. The more important role of Thr^{251} for MnmE GTPase activity strongly suggests that it corresponds to the invariant threonine characteristic of all GTPases.

GTPases bind guanine nucleotides as their Mg^{2+} complexes. In this respect, it is surprising that the change T251A had a meaningless effect on the GTP-binding activity of MnmE (Table II) given the role of the invariant threonine in the coordination of Mg^{2+} and the γ-phosphate. However, similar findings have been reported from other GTPases, such as Era and EF-Tu (39, 40). Moreover, mutation T35A in Ras produces just only a 6-fold-reduction on GTP affinity (41). This moderate
TABLE I
E. coli strains and plasmids used in this study

| Strain or plasmid | Description | Origin and/or reference |
|-------------------|-------------|------------------------|
| #DEV16            | F- thi-1 rel-1 spoT1 lacZ105, mnmE192, U10 [MnmE Q192X, Val8] | 21 |
| #JC7623           | recB21 recC22 sbeB15 sbeC201, SalI | 29 |
| #V5701           | bgl (SalI') | 12, 29 |
| #MG1655           | F- | D. Touati |
| #IC3647           | JC7623 (AIC718) | See JC7623 (12) |
| #IC4126           | V5701 pIC755 | See V5701 (12) |
| #IC4130           | IC4126 mnmE::kan [MnmE] | This work |
| #IC4986           | IC4986 mnmE, tnaA::kan | This work |
| #IC4836           | IC4836 mnmE23, tnaA::kan [MnmE R252A, Kan8] | This work |
| #IC4837           | IC4837 mnmE102, tnaA::kan [MnmE R275A, Kan8] | This work |
| #IC4903           | IC4903 mnmE21, tnaA::kan [MnmE T250A, Kan8] | This work |
| #IC4904           | IC4904 mnmE22, tnaA::kan [MnmE T251A, Kan8] | This work |
| #IC4905           | IC4905 mnmE20, tnaA::kan [MnmE G249A, Kan8] | This work |
| #IC4955           | IC4955 mnmE24, tnaA::kan [MnmE D253A, Kan8] | This work |
| #IC5057           | IC5057 mnmE25, tnaA::kan [MnmE T2505, Kan8] | This work |
| #IC5082           | IC5082 mnmE26, tnaA::kan [MnmE T2515, Kan8] | This work |
| #IC5083           | IC5083 mnmE103, tnaA::kan [MnmE R288A, Kan8] | This work |
| #IC5109           | IC5109 mnmE37, tnaA::kan [MnmE T2509/T2515, Kan8] | This work |
| #IC5110           | IC5110 mnmE101, tnaA::kan [MnmE R256A, Kan8] | This work |
| #IC5118           | IC5118 mnmE100, tnaA::kan [MnmE R224A, Kan8] | This work |
| #IC5168           | IC5168 mnmE28, tnaA::kan [MnmE R252K, Kan8] | This work |
| #IC5357           | MG1655 tnaA::kan [MnmE, Kan8] | This work |
| #IC5358           | MG1655 mnmE::kan [MnmE, Kan8] | This work |
| #IC5360           | MG1655 mnmE103, tnaA::kan [MnmE R288A, Kan8] | This work |
| #IC5361           | MG1655 mnmE25, tnaA::kan [MnmE T2505, Kan8] | This work |
| #IC5362           | MG1655 mnmE26, tnaA::kan [MnmE T2515, Kan8] | This work |
| **Plasmids**      | **Chromosomal DNA fragment 980–9847 (containing mnmE and tnaA) inserted** between the HindIII and EcoRI sites on pT7–12 [MnmE+] | 12 |
| pIC684            | GST fusion of DNA fragment from 3033 to 5652 (GST-MnmE+ fusion) | 12 |
| pIC755            | HindIII fragment 980–3734 (containing mnmE) inserted into HindIII-cut pMAK700 (MnmE+, CmR, replicon Ts) | 12 |
| pIC914            | 1.3-kb SalI fragment (Kan8 determinant) from pUC4K inserted into tnaA (NruI site, nt 5652) on pFM1 | 13 |
| pIC935            | pIC884 derivative containing mnmE10 [MnmE G228A] | 13 |
| pIC938            | pIC884 derivative containing mnmE102 [MnmE R275A] | This work |
| pIC939            | pIC884 derivative containing mnmE23 [MnmE R252A] | This work |
| pIC1002           | pIC884 derivative containing mnmE21 [MnmE T250A] | This work |
| pIC1003           | pIC884 derivative containing mnmE22 [MnmE T251A] | This work |
| pIC1013           | pIC884 derivative containing mnmE24 [MnmE D253A] | This work |
| pIC1048           | pIC884 derivative containing mnmE26 [MnmE T2515] | This work |
| pIC1050           | pIC884 derivative containing mnmE103 [MnmE R288A] | This work |
| pIC1059           | pIC884 derivative containing mnmE25 [MnmE T250S] | This work |
| pIC1068           | pIC884 derivative containing mnmE100 [MnmE R224A] | This work |
| pIC1070           | pIC884 derivative containing mnmE27 [MnmE T250S/T2515] | This work |
| pIC1072           | pIC884 derivative containing mnmE101 [MnmE R256A] | This work |
| pIC1081           | pIC884 derivative containing mnmE28 [MnmE R252K] | This work |
| pIC979            | pIC914 derivative containing mnmE23 [MnmE R252A] | This work |
| pIC980            | pIC914 derivative containing mnmE102 [MnmE R275A] | This work |
| pIC1012           | pIC914 derivative containing mnmE20 [MnmE G249A] | This work |
| pIC1014           | pIC914 derivative containing mnmE24 [MnmE D253A] | This work |
| pIC1015           | pIC914 derivative containing mnmE21 [MnmE T250A] | This work |
| pIC1016           | pIC914 derivative containing mnmE25 [MnmE T250S] | This work |
| pIC1049           | pIC914 derivative containing mnmE26 [MnmE T251S] | This work |
| pIC1051           | pIC914 derivative containing mnmE103 [MnmE R288A] | This work |
| pIC1060           | pIC914 derivative containing mnmE25 [MnmE T250S] | This work |
| pIC1069           | pIC914 derivative containing mnmE100 [MnmE R224A] | This work |
| pIC1073           | pIC914 derivative containing mnmE101 [MnmE R256A] | This work |
| pIC1082           | pIC914 derivative containing mnmE28 [MnmE R252K] | This work |

*The numbering system used to define E. coli chromosomal fragments present in some plasmids is that employed by Cabedo et al. (12); in such a system, mnmE extends from position 3102 to 4466. Mutant MnmE protein and/or phenotype is indicated between brackets.

TABLE II
Fluorescence titration of mnmGTPγS with wild-type and mutant MnmE proteins

| MnmE         | $K_d$ (μM) |
|--------------|------------|
| WT           | $1.51 \pm 0.28$ |
| G249A        | $2.27 \pm 0.02$ |
| T250A        | $1.77 \pm 0.12$ |
| T251A        | $1.68 \pm 0.13$ |
| R252A        | $1.97 \pm 0.27$ |
| D253A        | $2.35 \pm 0.13$ |

effect suggested that the invariant threonine can be easily replaced by a water ligand without great disturbance of the nucleotide-binding site (41). In fact, several studies have indicated that in Ras the coordination of Thr35 to magnesium is rather weak and that the coordination of this residue to the γ-phosphate might be transient in solution (Refs. 40 and 42, and references therein). In MnmE, coordination of Mg$^{2+}$ and γ-phosphate might be even weaker than in Ras, because affinity of mutant T251A practically equals that of the wild-type protein (Table II).
It is intriguing that the \( K_m \) for GTP hydrolysis by MnmE is in the range of 500 \( \mu \)M (Refs. 12, 13, and 38 and Table III), whereas the \( K_d \) for mant-GTP-S is 1.5 \( \mu \)M. This difference between the \( K_m \) and \( K_d \) values suggests that GTP hydrolysis by MnmE may be a multistep reaction (for example, \( E+S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E+P \), with \( E \) being enzyme; \( S \) substrate; and \( P \) products), where the relation between \( K_m \) and the rate constants that define each individual reaction step can be considerably more complex than that resulting from reactions in which formation of only central complex can be assumed (i.e. \( E+S \rightleftharpoons ES \rightleftharpoons E+P \)). Interestingly, whereas inorganic phosphate is released instantaneously after GTP hydrolysis by Ras, a significant delay has been observed between GTP hydrolysis and release of inorganic phosphate in EF-G and IF2, supporting the notion that the functional cycle of these translational factors differs from that of conventional switch GTPases (43–45). Thus, it is possible that \( P_i \) release is also delayed in MnmE and this may modulate progression of its GTPase cycle and its kinetic parameters. If so, the calculated \( K_m \) does not indicate how tightly GTP binds to MnmE. Consequently, the \( K_m \) decrease produced by some G2 mutations (see Table III) could be more related to some step subsequent to GTP hydrolysis (i.e. release of the reaction products) than to ability of the mutant proteins to bind GTP. In fact, G2 mutations do not greatly alter the binding properties of MnmE to mant-GTP\( \gamma \)S (Table II).

To gain further insight into the role of Thr\(^{250} \) and Thr\(^{251} \) in GTPase activity of MnmE, we decided to change them to serine. As shown in Table III, the rate constant of the GTP-hydrolysis reaction (\( k_{\text{cat}} \)) is only decreased 1.8-fold for T250S and 4-fold for T251S, which indicates that each threonine residue in G2 may be substituted by serine without significant impairment of the MnmE GTPase activity. However, \( k_{\text{cat}} \) decreased more than 20-fold for the double mutant T250S/T251S. This reduction is somewhat greater than that corresponding to an additive effect, and suggests that both threonines may partially substitute one for other in some aspect of the GTP hydrolysis reaction. No substantial differences between nucleotide binding properties of the double mutant and wild-type proteins were detected (data not shown).

To further explore the role of G2 residues in GTPase activity, mutants were examined by a fluorescence assay originally designed to detect the formation of the transition-state analogue for Go and Ras proteins (for a short review, see Ref. 3). For Go subunits of heterotrimeric G proteins the formation of a com-

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**TABLE III**

**Kinetic parameters of wild-type and mutant MnmE proteins**

| MnmE       | \( V_{\text{max}} \) nmol/min/mg | \( k_{\text{cat}} \) min\(^{-1} \) | \( K_m \) \( \mu \)M |
|------------|-------------------------------|-----------------|------------------|
| WT         | 203.0 ± 8.3                   | 10.8            | 754.0 ± 79.1     |
| G249A      | 9.0 ± 1.4                     | 0.5             | 105.0 ± 19.5     |
| T250A      | 50.6 ± 4.3                    | 2.7             | 497.0 ± 15.1     |
| T251A      | 2.2 ± 0.3                     | 0.1             | 12.7 ± 5.7       |
| R252A      | 28.0 ± 1.7                    | 1.5             | 119.1 ± 4.0      |
| D253A      | 23.0 ± 3.1                    | 1.2             | 57.2 ± 10.8      |
| T250S      | 113.5 ± 2.6                   | 6.0             | 494.4 ± 17.1     |
| T251S      | 52.3 ± 3.5                    | 2.8             | 645.8 ± 12.7     |
| T250S/T251S| 9.1 ± 2.2                     | 0.5             | 464.0 ± 36.3     |
| R275A      | 34.7 ± 2.4                    | 1.8             | 403.2 ± 81.0     |
| R258A      | 121.6 ± 2.5                   | 6.5             | 511.4 ± 71.5     |
| R224A      | 137.1 ± 8.0                   | 7.3             | 693.7 ± 13.3     |
| R256A      | 100.4 ± 3.5                   | 5.3             | 680.1 ± 11.1     |
| R252K      | 84.8 ± 9.7                    | 4.5             | 759.6 ± 23.4     |

\( a \) Activity of this mutant protein was at limit for detection under our assay conditions.

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**FIG. 3.** Binding of MnmE proteins to mant-GDP and effect of the G2 mutations on formation of the transition state complex between MnmE-mant-GDP and aluminum fluoride. Fluorescence spectra (excited at 360 nm) of 2 \( \mu \)M mant-GDP only (lines a), and after subsequent addition of 10 \( \mu \)M of the corresponding MnmE protein (wild-type or mutant) (lines b), 10 mM NaF (lines c), and 70 \( \mu \)M AlCl\(_3\) (lines d). Fluorescence emission spectra were recorded 15 min after AlCl\(_3\) addition. \( \lambda \), wavelength; \( I_f \), fluorescence intensity; a.u.: arbitrary units.
plex was shown among the protein, GDP (including Mg$^{2+}$), and aluminum fluoride. This complex is believed to mimic the transition state of GTP hydrolysis, because it is stabilized by the catalytic arginine and glutamine, and the aluminum fluoride complex has a planar conformation, as supposed for the γ-phosphate group in the transition state. Small GTPases are only able to form such a complex in the presence of their respective GAPs, demonstrating that the active site of Ras-like proteins needs to be complemented by residues from GAP (46). Interestingly, proteins from the dynamin family, which exhibit a high intrinsic activity, also form a complex, in their GDP-bound form, with aluminum fluoride (36), but in this case, participation of an in-cis arginine-finger mechanism in GTP catalysis is not clear (36, 47–49).

We have found that MnmE alone is able to form a complex with GDP and aluminum fluoride, as expected, but the capability of the G2-motif mutants to form such a complex is impaired in different degree; thus, as shown in Fig. 3, addition of NaF and AlCl$_3$ increases the fluorescence produced by binding of MnmE to mant-GDP more than 50% (compare lines c and d), whereas the interaction with G249A and T251A mutants, in their mant-GDP form, causes a fluorescence increase lower than 5%. The increase detected for mutants R252A and D253A was ~10%, whereas for T250A the increase was ~25%. These results suggest that the G2 residues may play some role in stabilization of the transition state, especially Gly$_{249}$ and Thr$_{251}$. For such a role, Thr$_{251}$ could be partially substituted by a structurally conservative residue, given that mutation T251S only reduces 4-fold catalysis (Table III). In contrast, the fact that mutation G249A greatly perturbs formation of the transition-state mimic (Fig. 3) suggests that Gly$_{249}$ lies close to the catalytic center of MnmE and that even the smallest possible amino acid change (to alanine) would sterically interfere with the geometry of the transition state.

**GTP Hydrolysis Mediated by MnmE Does Not Involve an Arginine Finger Mechanism**—Our group has previously shown that the isolated G-domain of MnmE roughly conserves the guanine nucleotide binding and GTPase activities of the entire protein (12). Moreover, we have found that the isolated MnmE G-domain is able to form a complex with GDP and aluminum fluoride as efficiently as the entire protein (data not shown). Altogether these results indicate that the MnmE G-domain contains the catalytic residues that explain for the relatively high intrinsic hydrolysis activity of this GTPase. To identify putative catalytic arginines in this domain, we looked for conservation of arginine residues among members of the MnmE family. Only two arginines are highly conserved throughout the family: Arg$_{252}$ and Arg$_{270}$ (see Fig. 1). In addition, Arg$_{288}$ is fairly well conserved, being substituted in some family members by lysine. As shown in Table III, mutations R252A and R275A reduce about 7- and 6-fold, respectively, the GTPase activity of MnmE, whereas mutation R288A has a meaningless effect. It should be pointed out that mutation of the catalytic arginine completely abolishes activity in RabGAP and reduces catalysis 2000-fold in RasGAP, more than 50-fold in rhoGAP, and at least 40-fold in Go proteins (Ref. 11, and references therein). Thus, the effect produced by mutations R252A and R275A in MnmE is smaller than expected for a catalytic residue. In fact, change to alanine of some residues of the MnmE G2-motif (i.e. G249A and T251A) has a more drastic effect on catalysis (about 20- and 100-fold reduction, respectively) than that produced by R252A and R275A (Table III). The effect of both arginine mutations on GTP hydrolysis parallels their effect on formation of the transition-state mimic in the presence of GDP and aluminum fluoride; thus, proteins R252A and R275A are able to form the trimeric complex, although the increase in fluorescence is smaller than that produced by the wild-type protein (Fig. 3 and data not shown). In addition, a mutant R252K shows 40% GTPase activity in relation to the wild-type protein; this supports the idea that Arg$_{252}$ does not act as an arginine finger, because it has been shown that the crucial catalytic arginine cannot be replaced by any other amino acid, including lysine (46). The change by alanine of other less conserved arginine residues (i.e. Arg$_{224}$ and Arg$_{256}$) had little effect on MnmE GTPase activity. Altogether these results lead to the conclusion that MnmE does not use an arginine finger to drive catalysis.

**Cell Viability of mnmE Mutants—Loss-of-function mnmE mutations are lethal in the genetic backgrounds of strains JC7623 and V5701 but not in those of MC1000 and DEV16 strains (12, 13). Thus, it was postulated that JC7623 and V5701 should carry mutation(s) in some gene(s) involved in the translation process that are incompatible with loss-of-function mnmE mutations (a phenotype designated as synthetic lethal). At present, we have identified such genes and confirmed that proposal.** Here, it was of interest to analyze the effect of mnmE mutations affecting switch I region and conserved Arg residues of the MnmE G-domain, on cell viability of V5701 derivatives. This could inform on functionality of the new mutant proteins. As shown in Table IV, change to alanine of all G2 residues (G249A, T250A, T251A, R252A, and D253A) is lethal in V5701: in P1 transductions, mutations can be recovered on 1% or 10% for viable or non-viable mutations, respectively), and determination of Cm phenotype of colonies recovered at this temperature (next column). A percentage higher than 90% is indicative that survival at 42 °C depends on integration of plasmid (carrying a wild-type mnmE allele) into the bacterial chromosome and, therefore, that the mnmE allele recovered from P1 transduction is lethal in this background.

| mnmE allele$^a$ | GTP hydrolysis$^b$ | Recovery of Kan$^b$ transductants in recipient$^c$ | Survivors at 42 °C of the IC4126 derivative that were Cm$^b$$^d$ |
|-----------------|-----------------|--------------------------|----------------------------|
| WT              | 10.8            | +                        | +                         | ~5                        |
| mnmE-kan        |                |                          |                            |                          |
| G249A           | 0.5             | –                        | +                         | >90                       |
| T250A           | 2.7             | –                        | +                         | >90                       |
| T251A           | 0.1             | –                        | –                         | >90                       |
| R252A           | 1.5             | –                        | –                         | >90                       |
| D253A           | 1.2             | +                        | –                         | >90                       |
| T250S           | 6.0             | SG                       | –                         | ~5                        |
| T251S           | 2.8             | +                        | –                         | >90                       |
| R252K           | 4.5             | +                        | +                         | ~5                        |
| R224A           | 7.3             | +                        | +                         | ~5                        |
| R256A           | 5.3             | +                        | +                         | ~5                        |
| R275A           | 1.8             | +                        | +                         | ~5                        |
| R288A           | 6.5             | SG                       | +                         | ~5                        |

$^a$ mnmE allele carried by P1 lysates in the transduction experiments or by IC4126 derivatives in assays of survival at 42 °C.

$^b$ $k_{cat}$ values were taken from Table III.

$^c$ IC4126 is V5701 harboring plasmid pIC755, which contains a mnmE wild-type allele, and the Cm$^b$ determinant and is temperature-sensitive for replication. Experiments were carried out at 30 °C. “+” and “−” indicate that more than 50 Kan$^b$ transductants or no transductants, respectively, were recovered after 24 h of incubation. SG means “slow growth”; in this case, colonies of transductants were very small, in comparison with those carrying viable mutations, after 24 h of incubation.

$^d$ A Kan$^b$Sal$^b$ Cm$^b$ transductant of IC4126 was selected from each P1 infection and analyzed for the presence on the chromosome of the corresponding mnmE allele by appropriate PCR analysis. The strains thus obtained were used for analysis of survival at 42 °C (which was about 100% or 10% for viable or non-viable mutations, respectively), and determination of Cm phenotype of colonies recovered at this temperature (next column).

$^e$ A percentage higher than 90% is indicative that survival at 42 °C depends on integration of plasmid (carrying a wild-type mnmE allele) into the bacterial chromosome and, therefore, that the mnmE allele recovered from P1 transduction is lethal in this background.

$^f$ T250S is a mnmE-kan derivative of IC4126.

$^g$ mnmE-kan derivative that were Cm$^b$.

$^h$ IC4126 is V5701 harboring plasmid pIC755, which contains a mnmE wild-type allele, and the Cm$^b$ determinant and is temperature-sensitive for replication. Experiments were carried out at 30 °C. “+” and “−” indicate that more than 50 Kan$^b$ transductants or no transductants, respectively, were recovered after 24 h of incubation. SG means “slow growth”; in this case, colonies of transductants were very small, in comparison with those carrying viable mutations, after 24 h of incubation.

$^i$ A Kan$^b$Sal$^b$ Cm$^b$ transductant of IC4126 was selected from each P1 infection and analyzed for the presence on the chromosome of the corresponding mnmE allele by appropriate PCR analysis. The strains thus obtained were used for analysis of survival at 42 °C (which was about 100% or 10% for viable or non-viable mutations, respectively), and determination of Cm phenotype of colonies recovered at this temperature (next column).

$^j$ A percentage higher than 90% is indicative that survival at 42 °C depends on integration of plasmid (carrying a wild-type mnmE allele) into the bacterial chromosome and, therefore, that the mnmE allele recovered from P1 transduction is lethal in this background.

$^k$ T250S is a mnmE-kan derivative of IC4126.

$^l$ mnmE-kan derivative that were Cm$^b$.
only when it harbors a plasmid carrying a wild type mnmE allele (strain IC4126). Such a lethality could be due to the reduced GTPase activity of mutant proteins, because the MnmE tRNA-modifying function requires efficient hydrolysis of GTP (13). However, given that mutant V5701(T257A) is viable despite the $k_{cat}$ of protein R275A is 1.8 min$^{-1}$, we think that lethality produced by mutations T250A and R252A is not related to the hydrolysis rate of the corresponding proteins ($k_{cat}$ 2.9 and 1.5 min$^{-1}$, respectively). Strikingly, the structurally conservative change T251S also produces a functionally inactive protein (as deduced from incapability to recover transducing in V5701 strain, Table IV) notwithstanding protein T251S exhibits a relatively high level of GTPase activity ($k_{cat}$ 2.8 min$^{-1}$). We think this is due to the incapability of protein T251S to switch between different conformations. In this respect, it should be pointed out that Spoerner et al. (42) have investigated the dynamic properties of the switch I region of Ras by using mutants of T35 (equivalent to Thr251 in MnmE) and demonstrated that this residue is necessary for the switch function of Ras, because even a change to serine drastically affect the dynamic behavior of the switch. Thus, it was proposed that the invariant threonine of the G2 motif is conserved among GTPases not for structural but rather dynamic considerations: the methyl group of this residue is important for the capability of GTPases to dynamically switch between different conformational states (42). The dynamic of the switch I region is crucial for the function of Ras in its interaction with effectors and regulators (41, 42). It is noticeable that Ras is active while bound to GTP, whereas MnmE requires GTP hydrolysis to be functional (13). Thus, we infer that mutation T251S in MnmE alters the conformational change of switch I, as occurs in Ras, but given the critical role of GTP hydrolysis for the MnmE function, we suspect that the active conformation of the MnmE switch I is associated with hydrolysis rather than binding of GTP. Interestingly, mutation T250S, which affects only slightly the GTPase activity ($k_{cat}$ 6.0 min$^{-1}$), seems to result in a partial loss-of-function, as deduced from the slow growth of mutants, whereas mutation R252K has a smaller effect on growth (Tables IV and V). This means that Thr250 may be hardly substituted by a structurally conservative residue, which suggests that the methyl group of Thr250 is also dynamically important for the MnmE function. In conclusion, because impaired GTPase activity does not appear as the main cause for the functional defect of mutant proteins T250A, T250S, and T251S, we suggest that both threonine residues of the G2 motif are crucial for mediating a conformational change associated with GTP hydrolysis that is required for MnmE function. Further experiments should be performed to directly probe that changes in Thr250 and Thr251 alter the dynamic behavior of the MnmE switch I region.

In relation to the role of the remaining residues of the G2 motif, the important effect of mutation G249A on GTPase activity of MnmE might be responsible for the functional impairment of the mutant protein, as inferred from incapability of recovering a V5701(G249A) transductant (Table IV). Obviously, it cannot be excluded that Gly250, in addition to be crucial for GTP hydrolysis, plays a role in the conformational change concomitant or subsequent to this reaction. Residues Arg252 and Asp253 may play some role in stabilization of transition state, as deduced from the effect of changes R252A and D253A on GTP hydrolysis and formation of aluminum fluoride complex (Table III and Fig. 3); however, such an effect is not enough to explain for lethality conferred by these mutations, because, as argued above, R275A results in a similar GTPase activity but is viable in the V5701 background (Table IV). Therefore, we think that residues Arg252 and Asp253 may be also involved in the conformational change of switch I associated with GTP hydrolysis, which, as above suggested, would be crucial for the MnmE function.

On the other hand, mutant R288A appears as a partial loss-of-function mutant, as also deduced from its extremely slow growth, although it exhibits an almost normal GTPase activity (Tables III–V). Because some MnmE family members carry a lysine in place of Arg288, it is reasonable to think that substitution of lysine for R288 in MnmE should not have consequences on its function and that either Arg or Lys at position 288 must play a role structurally important for MnmE function.

**Effect of Mutations on the tRNA-modifying Function of MnmE—**To analyze the co-relation between cell viability and tRNA-modification capability associated with the new mutations, we firstly made use of a well established read-through assay. MnmE-mediated modification is known to be required for efficient read-through of the UAG codon present in the mutant lacZ gene lacZ105, which is easily quantified by determination of $\beta$-galactosidase activity (13, 21, 50). DEV16, a strain in which the loss-of-function mnmE mutations are not lethal, carries both the null mnmE allele Q192X and the lacZ105 gene. Thus, we substituted the Q192X allele on the DEV16 chromosome by the new mutant mnmE alleles and analyzed their effect on the misreading of the lacZ UAG codon.

As shown in Fig. 4, the change to alanine of all G2 residues generates MnmE proteins apparently unable to modify tRNA, as inferred from the extremely low level of $\beta$-galactosidase activity detected in the corresponding DEV16 derivatives, which is similar to that produced by the null Q192X mutant. Mutation T251S also produces a non-functional MnmE protein, as expected, whereas mutations that are viable in V5701 (R224A, R252K, R256A, and R275A; see Table IV) resulted in $\beta$-galactosidase activity levels similar to that of the wild-type allele. Surprisingly, $\beta$-galactosidase activity of derivatives carrying the presumptive partial loss-of-function mutations T250S and R288A was similar to that produced by loss-of-function mutations. Western blot analysis indicated that the cellular levels of the mutant proteins were indistinguishable from that of the wild-type MnmE (data not shown). Therefore, the effects produced by some mutant proteins cannot be attributed to a lower accumulation of these proteins into the cell. The similar behavior of full and partial loss-of-function mutations in the readthrough assay could be then explained assuming that proteins T250S and R288A generate a small population of appropriately modified tRNAs whose activity cannot be detected by
this assay, but allows slow growth of V5701 derivatives (see Tables IV and V).

The modification state of tRNAs that are substrates in the MnmE-dependent reaction can also be analyzed by using electrophoresis on acidic polyacrylamide gel and Northern blot hybridization. The mnm5 group adds a positive charge to the tRNA at pH 5.0, and thus tRNA containing this group migrates slower than tRNA lacking it (13, 22). We also made use of this approach to analyze tRNA\textsubscript{mnm5}s\textsubscript{UUC} activities from DEV16 derivatives were measured as described under "Experimental Procedures." Values are the average of six independent experiments. The dotted lines indicate the \(\beta\)-galactosidase activity from the null mutant (DEV16) or wild-type strain (IC4770). Strains used were: DEV16 (Q192X), IC4963 (G249A), IC4906 (T250A), IC5055 (T250S), IC4907 (T251A), IC5018 (T251S), IC5112 (T250S/T251S), IC4864 (R252A), IC4956 (D253A), and IC4770 (WT), in panel A, and DEV16 (Q192X), IC4864 (R252A), IC5111 (R224A), IC5169 (R252K), IC5113 (R256A), IC4865 (R275A), IC5019 (R288A) and IC4770 (WT), in panel B.

![Fig. 4. Readthrough of the lacZ UAG codon. \(\beta\)-galactosidase activities from DEV16 derivatives were measured as described under “Experimental Procedures.” Values are the average of six independent experiments. The dotted lines indicate the \(\beta\)-galactosidase activity from the null mutant (DEV16) or wild-type strain (IC4770). Strains used were: DEV16 (Q192X), IC4963 (G249A), IC4906 (T250A), IC5055 (T250S), IC4907 (T251A), IC5018 (T251S), IC5112 (T250S/T251S), IC4864 (R252A), IC4956 (D253A), and IC4770 (WT), in panel A, and DEV16 (Q192X), IC4864 (R252A), IC5111 (R224A), IC5169 (R252K), IC5113 (R256A), IC4865 (R275A), IC5019 (R288A) and IC4770 (WT), in panel B.

MnmE is a 50-kDa GTPase, conserved between bacteria and humans. In \textit{E. coli}, MnmE, together with GidA, is involved in the modification of uridine bases at the wobble position (U34) of tRNAs decoding two-family box triplets (Fig. 2). \textit{MSS1} and \textit{MT01} have been found to be the respective homologues of the \(mnmE\) and \textit{gidA} genes in both human and yeast (51–54). The protein products of \textit{MSS1} and \textit{MT01} from yeast form heterodimers and localize in the mitochondria, and their mutants are associated with respiratory defects (52). Also in yeast, it has been recently reported that mitochondrial tRNAs\textsuperscript{\textsubscript{mnm5}s\textsubscript{UUC}} molecules isolated from \textit{MT01} and \textit{MSS1} deletion strains contain s\textsubscript{U} instead of the cmnm\textsubscript{5}s\textsubscript{U} found in the wild type tRNA\textsubscript{\textsuperscript{\textsubscript{mnm5}s\textsubscript{UUC}}} molecules; this indicates that \textit{MT01} and \textit{MSS1} genes are both involved in the biosynthesis of the 5-carboxy-methylaminomethyl group of cmnm\textsubscript{5}s\textsubscript{U} of mitochondrial tRNA\textsubscript{\textsuperscript{\textsubscript{mnm5}s\textsubscript{UUC}}} (55). Therefore, the function of MnmE and GidA seems to be evolutionarily conserved although the precise role of both proteins in the modification reaction is hitherto unknown. To get insight into this matter, the x-ray structure of MnmE from \textit{Thermotoga maritima} (450 residues), in the nucleotide-free form, has been recently solved (28). The structure reveals a three-domain protein composed of the N-terminal α/β domain (residues 1–118), a central exclusively helical domain formed by residues 118–210 from the middle region of the C-terminal residues 381–450, and the G-domain residues 211–380. The N-terminal domain induces dimerization and is homologous to the tetrahydrofolate-binding domain of \(N,N\)-dimethylglycine oxidase. Biochemical and structural data indicate that MnmE indeed binds 5-formyltetrahydrofolate (28). This strongly supports a direct participation of MnmE in the tRNA modification reaction, because formyltetrahydrofolate may be used as the methyl donor in the formation of the cmnm group, as previously suggested (14, 21). We have shown that the Cys residue (Cys\textsubscript{453} in \textit{E. coli} MnmE) from the C-terminal tetrapeptide motif Cys(ULV)GK conserved in all MnmE family members (see Fig. 1), is required for tRNA modification (13). Thus, we suggested that MnmE is a tRNA-modifying enzyme and that Cys\textsubscript{453} functions as a catalytic residue in the modification reaction. The addition of the cmnm group to C5 of U34 could take place through a mechanism similar to that proposed for known pyrimidine C5-modifying enzymes such as thymidylate synthase and RUMT, which use an enzymatic cysteine to activate pyrimidine carbon 5 for nucleophilic attack (13, 28). Interestingly, the structure of \textit{T. maritima} MnmE reveals that the C-terminal region of the protein contributes to form the central helical domain and locates close to the tetrahydrofolate-binding site (28); however, the C-terminal Cys and the formyl group are 11 Å apart and thus not close enough to act in common on the uridine base. It is possible that GTP hydrolysis by MnmE, which we have shown to be essential for tRNA modification (13; this paper), leads to changes of the overall protein conformation that bring the C-terminal Cys and formyl group in juxtaposition or, whichever the role of Cys\textsubscript{453} is, promote the participation of the MnmE remaining domains in the modification reaction. Such a behavior would be reminiscent of that observed in the biosynthesis factors where the structural changes involving the G domain are transferred to other domains. Thus, in the case of IF2/eIF5B, a molecular lever transmits the effects of GTP binding over a distance of 90 Å from the N-terminal G-domain to the C terminus of the molecule (56), and a large
FIG. 5. High performance liquid chromatography chromatograms of tRNA hydrolysates from wild-type and mnmE mutant strains. Strains used were: IC5357 (WT), IC5358 (MnmE::Kan), IC5361 (T250S), IC5360 (R288A), and IC5362 (T251S). The nucleosides were monitored at 314 nm to maximize the detection of thiolated nucleosides. mnm\(^5\)s\(^2\)U and s\(^2\)U were identified by comparing UV spectra with published spectra (32). AU, absorbance units.
structural change has been documented for EF-Tu and EF-G, being GTP hydrolysis needed for EF-G to be functionally active (43, 57–61). In the case of MnmE, the link between the N-terminal and helical domains seems to be flexible and accessible for proteases, which is a hint that this region could act as a hinge for the conformational change that brings the C- and N-terminal regions of the protein closer to each other (28).

Role of the G2 Motif—The results here presented indicate that residues of the MnmE G2 motif are not needed for GTP binding but play some role in GTP hydrolysis (Fig. 3 and Table III). Specifically, change to alanine of Thr\textsuperscript{251} drastically reduces the GTPase activity of MnmE and abolishes its tRNA-modifying function (Table III and Fig. 4). This supports our previous conclusion from results obtained with mutation D270A affecting the G3 motif, that GTP hydrolysis (and not simply GTP binding) is a requirement for MnmE to reach its functionally active state (13). Interestingly, the structurally conservative mutation T251S leads to a minor loss of the MnmE GTPase activity but results in a non-functional protein (Table IV and Fig. 5). Therefore, GTP hydrolysis is a required but non-sufficient condition in order that MnmE can mediate modification of tRNA. Probably, the conformational change of switch I region associated with GTP hydrolysis is crucial for the function of MnmE, and Thr\textsuperscript{251} is essential for such a change. That Thr\textsuperscript{251} cannot be substituted by serine is in agreement with the assessment that the methyl group of the invariant Thr of G2 motif is important for the capability of GTPases to dynamically switch between different conformations (42). In the case of Ras proteins, the active conformation is reached while the protein remains bound to GTP, whereas in the MnmE case, the active conformation rises from GTP hydrolysis. In Ras proteins, the conformational change of switch I region is required for interactions with GAPs and effectors (41–42), whereas in MnmE, we suggest that the conformational change of switch I may be transferred to other protein domains in such a way that their role in the modification reaction results facilitated; however, at present, other possibilities cannot be ruled out; thus the conformational change of MnmE switch I could be required for interactions with other partners such as tRNA, Gid\textalpha or a still unknown component of the modification reaction.

In addition to Thr\textsuperscript{251}, residues Thr\textsuperscript{250}, Arg\textsuperscript{252}, and, probably, Asp\textsuperscript{253} are also involved in the conformational change associated with GTP hydrolysis, because their substitution by Ala produces non-functional proteins while reduces their GTPase activity to a level that is functional for other mutants, i.e., R275A (see Table IV). We cannot discard that Gly\textsuperscript{249} is also involved in this conformational change; however, its change to alanine has an important effect on catalysis, and this appears as the main cause of the functional impairment of protein G249A (Table IV).

MnmE Performs GTP Hydrolysis without an Arginine Finger—The MnmE G-domain conserves the relatively high GTPase activity of the entire protein (12). Therefore, this domain should contain the catalytic residues that stabilize the negative charge development at the phosphate groups of GTP during the hydrolysis reaction and correctly position the nucleophilic water molecule at the transition state, according to the current mechanisms of action proposed for GTPases (7). Go proteins have two invariant residues playing these roles: a Gln adjacent to G3 and an Arg just before G2; this arginine belongs to the inserted helical domain characteristic of these proteins. No built-in GTPase-activating protein domain, such as that carried by Go proteins, is found in the G-domain of MnmE. In fact, the structure of MnmE from T. maritima reveals that the G domain has the canonical Ras-like fold, with no insertion or deletion of secondary structural elements (28). In this work, to explore whether an arginine finger is involved in the catalytic mechanism employed by MnmE, we have changed to alanine the only two highly conserved arginines of the G domain, including that present in the G2 motif. Mutants R252A and R275A are, at the best, 7-fold less active than the wild type protein (see Table III). The residual activity exhibited by these mutants is large enough to conclude that neither of them plays a crucial role in catalysis. Moreover, given that the change to alanine of other less conserved arginines did not reduce significantly the GTPase activity of MnmE, it is reasonable to think that this protein does not employ an arginine finger to mediate GTP hydrolysis. Finally, because MnmE contains a Leu residue (Ile or Val in other MnmE family members) in place of the catalytic Gln carried by Ras and Go proteins, we conclude that MnmE follows a completely different mechanism to drive catalysis. Other GTPases also use mechanisms alternative to that employed by Ras and Go proteins. Thus, RanGAP and Rap1GAP mediate GTP hydrolysis without an arginine finger (10, 11). In addition, GTPases of poorly defined function, like those from Era and Obg families, as well as dynamin-related proteins contain, similarly to MnmE family members, a hydrophobic residue in place of the catalytic glutamine (2, 3). Further mutational analysis and structural data of these proteins will be necessary to elucidate their peculiar hydrolysis mechanisms.

MnmE Defects Result in Impaired Growth—Our results show that survival of V5701 derivatives carrying mnmE mutations depends on the capability of the MnmE mutant proteins to modify tRNA in some extent. Thus, among the mutations that impair the tRNA-modifying function of MnmE, only those conferring a partial loss-of-function (T250S and R288A) could be recovered in the V5701 background (Table IV and Fig. 5). Such derivatives exhibit slow growth, a defect that can also be detected, but in a minor degree, in the genetic background of strain DEV16 (Table V). In this background, mutations that produce full loss-of-function could be recovered, but they have the greatest effect on doubling time (Table V). In conclusion, scarcity of properly modified tRNAs causes a decoding disorder that results in impaired growth, a feature that is exacerbated in strain V5701, because it carries a mutation in a gene involved in the ribosomal dynamics that acts synergistically with mnmE mutations.\textsuperscript{4} Such a behavior is reminiscent of that observed in yeast, where disruption of MTO1 or MSS1 results in a respiration defect (consequence of inefficient mitochondrial protein synthesis) that is exacerbated when one of them is disrupted in the presence of mutation P\textsuperscript{H145} (51, 52, 55). This mutation resides in the mitochondrial 15 S rRNA and may alter the conformational changes involved in the decoding process (51, 57, 62). Interestingly, the effect produced by disruption of MTO1 or MSS1 is also exacerbated when one of them is inactivated along with MTU1, an mnmA homologous gene involved in the 2-thio modification of wobble uridines (55; see Fig. 2). Altogether these results stress the importance of the tRNA modification in the mRNA-decoding process. In this respect, several recent works strongly suggest that lack of wobble modification is a major causative factor of human mitochondrial diseases associated with specific mitochondrial tRNA mutations (63–67). This leads to the hypothesis that defects in tRNA-modifying proteins may play some role in human mitochondrial diseases.

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REFERENCES

1. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature \textbf{349}, 117–127
2. Leipe, D. D., Wolf, Y. I., Koonin, E. V., and Aravind, L. (2002) J. Mol. Biol. \textbf{317}, 41–72
3. Vetter, I. R., and Wittinghofer, A. (2001) \textit{Science} \textbf{294}, 1299–1304
4. Bourne, H. R. (1997) \textit{Nature} \textbf{389}, 673–674
5. Scheffzek, K., Ahmadian, M. R., Kabsch, W., Wiesmüller, L., Lautwein, A.,
Effects of Mutagenesis in the Switch I Region and Conserved Arginines of *Escherichia coli* MnmE Protein, A GTPase Involved in tRNA Modification
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