Identification of Eukaryotic and Prokaryotic Methylthiotransferase for Biosynthesis of 2-Methylthio-N^6-threonylcarbamoyladenosine in tRNA

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Simon Arragain^1, Samuel K. Handelman^1,1, Farhad Forouhar^1,1, Fan-Yan Wei^2, Kazuhiro Tomizawa^3, John F. Hunt^4, Thierry Douki^1,‡, Marc Fontecave^1,‡, Etienne Mulliez,‡ and Mohamed Atta^3,‡

From the 1Institut de Recherches en Technologie et Sciences pour le Vivant IRTSV-LCBM, UMR, 5249 CEA/CNRS/UJF, Commissariat à l’Energie Atomique-Grenoble, 17 Avenue des Martyrs, 38054 Grenoble Cedex 09, France, the 2Northeast Structural Genomics Consortium and 3Department of Biological Sciences, Columbia University, New York, New York 10027, the 4Department of Molecular Physiology, Faculty of Life Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan, the 5De*SM/InAC/SCIB UMR-E3 CEA-UJF/Laboratoire “Lésions des Acides Nucleiques,” Commissariat à l’Energie Atomique-Grenoble, 17 Avenue des Martyrs, 38054 Grenoble Cedex 09, France, and the 6Collège de France, 11 Place Marcellin-Berthelot, 75005 Paris, France

Bacterial and eukaryotic transfer RNAs have been shown to contain hypermodified adenosine, 2-methylthio-N^6-threonylcarbamoyladenosine, at position 37 (A37) adjacent to the 3’-end of the anticodon, which is essential for efficient and highly accurate protein translation by the ribosome. Using a combination of bioinformatic sequence analysis and in vivo assay coupled to HPLC/MS technique, we have identified, from distinct sequence signatures, two methylthiotransferase (MTTase) subfamilies, designated as MtaB in bacterial cells and e-MtaB in eukaryotic and archaeal cells. Both subfamilies are responsible for the transformation of N^6-threonylcarbamoyladenosine into 2-methylthio-N^6-threonylcarbamoyladenosine. Recently, a variant within the human CDKAL1 gene belonging to the e-MtaB subfamily was shown to predispose for type 2 diabetes. CDKAL1 is thus the first eukaryotic MTTase identified so far. Using purified preparations of Bacillus subtilis MtaB (YqeV), a CDKAL1 bacterial homolog, we demonstrate that YqeV/CDKAL1 enzymes, as the previously studied MTTases MiaB and RimO, contain two [4Fe-4S] clusters. This work lays the foundation for elucidating the function of CDKAL1.

The methylthiotransferase (MTTase)^4 family, a subclass of the large radical AdoMet enzyme superfamily, has recently received special attention (1). Indeed, its members catalyze chemically challenging reactions, in all cases involving C–H to C–SCH3 bond conversion, through a radical mechanism that remains incompletely established. Furthermore, these reactions participate in important biological processes such as tRNA or ribosomal protein modification (2–5). Prototypes for the MTTase family are two bacterial enzymes as follows: MiaB, which modifies N^6-isopentenyladenosine (i^6A) to its 2-methylthio derivative (ms^2i^6A) in tRNA, and RimO, which acts on a specific aspartate residue of the ribosomal S12 protein. Both enzymes have been shown to contain two [4Fe-4S] clusters (4–6). The first one is chelated by the three cysteines of a conserved CXXXCCXC motif that is the hallmark signature of the radical AdoMet family (Scheme 1) (7). This cluster serves to bind and reduce AdoMet into methionine and the highly reactive 5’-deoxyadenosyl radical (Ado^•) (8). The latter is supposed to abstract an H atom of the substrate (tRNA or protein) selectively, thus generating an intermediate substrate radical that is amenable to C–S bond formation (9). It is believed that the thiolation step involves the second [4Fe-4S] cluster, chelated by three other conserved cysteines in the typical N-terminal UPF0004 domain (Scheme 1) (4–6). The final methylation step involves a second molecule of AdoMet (4–6). A specific signature of the methylthiotransferase subfamily not shared by the other radical AdoMet proteins is the presence of a C-terminal TRAM domain involved in substrate (tRNA or protein) recognition (Scheme 1) (5, 10).

The hypermodified 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A, where ms stands for methylthio, t for threonine, and A for adenosine) is an anticodon loop modification found at position 37 of tRNAs decoding ANN codons (Scheme 2). Despite the availability of extensive data on the physiological function of this hypermodified base (11–13), its biosynthesis pathway has only been partially characterized. The prerequisite carbamoylation of threonine is an ATP-dependent process requiring threonine and carbonate, but the genes involved in this pathway have remained uncharacterized (14). A recent publication (15) demonstrates that proteins from the YrdC/Sua5 family catalyze the first step in ms^2t^6A biosynthesis, i.e. the addition of a threonylcarbamoyl group at the N^6 nitrogen of adenosine converting adenosine 37 to t^6A-37. The second step in ms^2t^6A-37 biosynthesis, including both sulfur insertion and methylation at position 2 of t^6A-37 to yield ms^2t^6A-37, is likely to be catalyzed by one or more MTTases, potentially from the radical AdoMet family.

Bioinformatics analyses presented here demonstrate that five major families of homologous MTTases are encoded in...
vertebrate and bacterial cells. In addition to the previously characterized MiaB and RimO families, we identify three new families as follows: (i) MtaB found in eubacteria with the B. subtilis yqeV gene as its prototype; (ii) e-MtaB found in higher eukaryotes and archaebacteria with the murine CDKAL1 gene as its prototype; and (iii) MTL1 found so far exclusively in proteobacteria. CDKAL1 is of special interest because genetic polymorphisms in this gene increase the reduction of insulin secretion and increase the risk of developing type 2 diabetes (16). Using in vivo gene complementation, we show that proteins from both the MtaB and e-MtaB families are responsible for the conversion of N^6-threonylcarbamoyladenosine (t6A) into 2-methylthio-N^6-threonylcarbamoyladenosine (ms2t6A) in tRNA. In addition, using purified preparations of Bacillus subtilis YqeV as a representative of the MtaB family, we demonstrate that these enzymes contain two [4Fe-4S] clusters, like the MiaB and RimO families, the only other known radical sulfur-inserting enzymes.

### MATERIALS AND METHODS

#### Plasmids, Strains, and Growth Conditions—The Escherichia coli, B. subtilis strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in LB medium at 37 °C.

#### Phylogenomic Analyses—PSI-BLAST profiles (17) created via ClustalW alignment (18) of a set of MiaB and RimO homologs were used to search the human genome and the CRSH data base of proteins of likely equivalent function from 474 bacterial genomes. Sequences identified in this search exhibiting significant (e-value <0.05) sequence similarity to MiaB/RimO that aligned over all three domains were combined with the initial sequences to perform another PSI-BLAST search using the same significance threshold. This second search identified many proteins from other radical AdoMet enzyme families not containing the UPF0004 or TRAM domain, suggesting that all MTTases were likely to have been identified. Cladograms were reconstructed by the program MEGA 3.0 (19) based on 100 bootstrap replicates using the Unweighted Pair Group Method with Arithmetic mean with default parameters. Proteins were assigned to putative functional families via examination of the phylogenetic tree.

#### Cloning of the yqeV and CDKAL1 Genes and Construction of Overexpressing Plasmids—The open reading frame encoding the YqeV (MtaB1) protein (BSU25430) was PCR-amplified using B. subtilis genomic DNA, Pwo polymerase (Roche Applied Science), and the following primers: the N-terminal primer (YqeVN-ter, 5'-GAGGTGATCACATATGGCAACTGTTGCTTTC-3') was designed to contain a unique NdeI restriction site at the predicted initiation codon and the C-terminal primer (YqeVC-ter, 5'-GTGACAGCTCTTTTATTCTGCAGCTTACAAAC-3') to hybridize in the 3'-untranslated region and to contain a unique PstI restriction site. The PCR fragment was purified with the High Pure PCR kit (Roche Applied Science), double-digested with NdeI and PstI (Fermentas), and gel-purified before direct cloning into the pT7-7 expression vector, leading to the pT7-mtab plasmid. DH5α cells were transformed with the pT7-mtab plasmid, and one clone containing an insert with the expected size was selected for sequencing. The cloned yqeV was confirmed to be wild type when compared with the GenBank accession number BSU25430. Full-length mouse CDKAL1 cDNA (BC016073) was purchased from Invitrogen. The open reading frame of the

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CDKAL1 gene was PCR-amplified to contain SmaI and XhoI restriction sites at the N and C termini, respectively. The PCR fragment was cloned into pCR2.1-TOPO by TOPO-TA cloning (Invitrogen). CDKAL1 fragment was excised from pCR2.1-TOPO vector by double digestion with SmaI and XhoI (New England Biolabs) and cloned into pGEX6P-1 (GE Healthcare) leading to the pGEX6P-emtab plasmid.

yqeV Cloning in pDG148-Stu Plasmid—The following primers were used to amplify yqeV gene using pT7-mtab as template: pDG148yqeVN, 5'-H11032-AAGGAGGAAGCAGGTATGGCAACTTGTTGCTTTCCATACGCTTGGCTG-3' (forward), and pDG148yqeVC, 5'-H11032-GACACGCACGAGGTTTAAGAAGACAAACGCATGTGTTCAGTTATTTC-3' (reverse). The cloning procedure used is exactly as described previously (20). The obtained plasmid was named pDG148-mtab and sequenced to verify that no error has been introduced during the PCR experiment. The electrocompetent MGNA-C496 cells were prepared as described and transformed with pDG148-mtab by electroporation (21).

Site-directed Mutagenesis; Construction of the AXXXAXX Triple Variant of the Radical AdoMet Motif—Triple variants of cysteine residues of the CXXX/CXX motif were constructed by PCR using the following primers: yqeVCtoA1, 5'-H11032-ATACAAGGAGGAAGCAGGTATGGCAACTTGTTGCTTTCCATACGCTTGGCTG-3', hybridized to the noncoding strand, and yqeVCtoA2, 5'-H11032-GACACGCACGAGGTTTAAGAAGACAAACGCATGTGTTCAGTTATTTC-3', hybridized to the coding strand; Cdkal1CtoA1, 5'-CCATCAACACGGGGGCTTCTAATGCTGTACCTACGCGAAACACTAAAACAC-3', hybridized to the noncoding strand, and Cdkal1CtoA2, 5'-GTGTTTAGTTTTGGCGTAGGTAGCAGCATTGAGAGCCCCGTGTTGATGG-3', hybridized to the coding strand.

Mutagenesis was carried out on plasmid pT7-mtab and pGEX6P-emtab with QuikChange™ site-directed mutagenesis kits from Stratagene according to the manufacturer’s protocol as described previously (22). Mutations were confirmed by DNA sequencing.

Preparation and Analysis of tRNA from B. subtilis—MGNA-001 (wild type) and MGNA-C496 (yqeV/H11002) strains were grown in LB medium at 37 °C until the A600 reached 1.0. The cells were harvested, and cell-free extract was obtained as described previously (23). Bulk tRNAs were isolated as described previously (24), and 100–200 μg of purified tRNAs were digested to nucleosides by nuclease P1 and bacterial alkaline phosphatase treatment. The resulting hydrolysate was analyzed by HPLC as described previously (24). For the complementation experiment, MGNA-C496 strain lacking the yqeV gene was transformed with the expressing plasmid pDG148-yqeV coding MtaB protein and grown in LB medium as described previously (20).

Overexpression and Purification of YqeV (MtaB) Protein—The pT7-mtab plasmid was used to transform E.coli BL21CodonPlus(DE3)-RIIL™, which was grown at 37 °C in Luria Broth supplemented with 100 mg/liter ampicillin. When the A600 reached 0.6, the production of the MtaB was induced by addition of 100 μM isopropyl 1-thio-β-D-galactopyranoside, and the incubation was continued for 2 h at 30 °C. The MtaB protein was purified aerobically at 4 °C as follows. The frozen cells were thawed, broken by sonication, and centrifuged at 220,000 × g at 4 °C for 1 h. The proteins of the cell-free extract

### TABLE 1

| Genotype Source | E. coli strains | B. subtilis strains | Plasmids |
|----------------|----------------|-------------------|----------|
|                 |                 |                   | pT7-7    | CoE1 origin bla, T7 promoter | 30 |
|                 |                 |                   | pGEX6P-1 | Bla | GE Healthcare |
|                 |                 |                   | TOPO | Bla | Invitrogen |
|                 |                 |                   | pDG148-Stu | Lac Pspac ble bla kan | 20 |
|                 |                 |                   | pDG148-mtab | Fermentas |
|                 |                 |                   | pDG148-yqeV | Invitrogen |
|                 |                 |                   | pDG148-yqeV | 29 |

#### MATERIALS AND METHODS

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were precipitated with ammonium sulfate between 25 and 55% saturation and dialyzed two times against 60 volumes of 10 mM Tris-HCl, pH 7.5, containing 100 mM KCl (buffer A). The colored solution was then loaded on top of a 30-ml Blue-Sepharose column equilibrated with buffer A. The column was washed with 5 column volumes of buffer A and then eluted with a linear gradient of KCl (0.1–1 M) in buffer A. Fractions containing the MtaB protein were pooled, brought to 1 M ammonium sulfate in Tris buffer, and loaded to a 25-ml butyl-Sepharose FF (GE Healthcare) equilibrated in that buffer. The column was washed with 3 column volumes of buffer A, and the pure protein was eluted with a descending gradient (1–0 M) of ammonium sulfate buffer A. The fractions containing MtaB were pooled, concentrated by ultrafiltration using an Amicon cell fitted with a YM30 (Diaflo), and submitted to a final purification on an analytical Superdex-75 gel filtration column using Tris-HCl, pH 8.0, containing 0.25 M KCl and 5 mM DTT. The fractions were analyzed by SDS-PAGE (12%), and the most pure were pooled, concentrated, and stored at 77 K.

Preparation of the Apoprotein—The apo-form of MtaB protein was prepared as described previously (6).

Reconstitution of ApoMtaB—Fe-S cluster reconstitution into B. subtilis MtaB was carried out under strictly anaerobic conditions into a Jacomex NT glovebox containing less than 2 ppm O₂ as described previously (6).

Analytical Methods—Quantitative amino acid analysis was used to determine extinction coefficients of purified B. subtilis MtaB: ε₁80 = 46.8 mm⁻¹ cm⁻¹ for the apoenzyme; ε₁80 = 87.8 mm⁻¹ cm⁻¹ and ε₄₅₀ = 30.0 mm⁻¹ cm⁻¹ for the holo-enzyme (i.e. an A₄₅₀/A₁₈₀ ratio of 0.34). Iron and inorganic sulfide were quantified as described previously (25). tRNAs were digested and analyzed by HPLC as described previously (24).

Mass Spectrometry Analysis—HPLC-tandem mass spectrometry analyses were performed with a 1100 Agilent chromatographic system coupled with an API 3000 triple quadrupole apparatus (PerkinElmer Life Sciences) using a turbo ion spray electrospray source in the positive mode. HPLC separation was carried out on a 2 x 150-mm column containing 3 μm of octadecylsilyl silica gel (Upishphere, Interchim Montluçon, France) using a gradient of acetonitrile in 5 mM ammonium formate. Acetonitrile content was increased from 0 to 40% over the first 20 min and then held constant for 40 min. The settings of the tandem mass spectrometer were optimized by injection of a pure solution of 1³A to favor loss of the ribose unit upon collision-induced fragmentation. Mass spectrometry detection was carried out in neutral loss mode to obtain a high specificity. In this configuration, pseudo-molecular ions ([M + H]⁺) and fragments ([M – 132 + H]⁺) obtained by collision in the second quadrupole (collision) cell were analyzed in the first and third quadrupoles, respectively. Using this approach, only nucleosides losing their ribose unit were detected. The pseudo-molecular ion of the latter compounds was monitored in a 300–450 mass range.

Light Absorption Spectroscopy—UV-visible absorption spectra were recorded under anaerobic conditions in a glove box on an XL-100 Uvikon spectrophotometer equipped with optical fibers.

RESULTS AND DISCUSSION

Phylogenomic Analysis of Radical AdoMet Methylthio-transferases—Sequence profiling techniques were used to search 474 bacterial genomes and the human genome for families of radical AdoMet MTTase. To ensure full coverage of this enzyme class, iterative expansion of the sequence profile was continued until radical AdoMet enzymes with different overall domain architecture were identified by the profile (see under “Materials and Methods”). Five distinct sequence families with representatives in at least three genomes were identified in this manner (Fig. 1). Proteins in all of these families have an N-terminal UPF0004 domain with three invariant cysteines in the CX₃₄₋₃₆CX₂₈₋₃₇C motif, a central radical AdoMet domain with three invariant cysteines in CX₇₋₉CX₂₋₃ motif, and a C-terminal TRAM domain (Scheme 1) (3, 5).

Two of the five MTTase families identified in our analyses represent the well- characterized MiaB and RimO enzyme families (4–6). The human and B. subtilis genomes both encoded a member of the MiaB family (BSR5RAP1 and YmcB/BSU17010, respectively) in addition to a member of another MTTase family. The third MTTase family identified in our analyses includes yveV (BSU25430), the other MTTase encoded in the B. subtilis genome, whose enzymatic activity was characterized in this study. We have designated this family MtaB, for methylthio-threonylcarbamoyl-adenosine transferase B, because it performs the second step in the biosynthesis of this hypermodified base (Scheme 2). This nomenclature was chosen for consistency with the name of MiaB, which performs the second step in biosynthesis of methylthio-isopentenyl-adenosine. The fourth MTTase family includes enzymes of unknown substrate specificity that are found exclusively in eubacteria, including the pathogens in the Helicobacter and Campylobacter genera. We have designated this family MTL1, for methylthio-transferase-like family-1. The fifth and final MTTase family includes CDKAL1, the other MTTase encoded in the human genome, whose enzymatic activity is also characterized in this study. We have designated this family e-MtaB for gukaryotic methylthio-threonylcarbamoyl-adenosine B, because it performs probably the second step in biosynthesis of methylthio-isopentenyl-adenosine. The fourth MTTase family includes enzymes of unknown substrate specificity that are found exclusively in eubacteria, including the pathogens in the Helicobacter and Campylobacter genera. All five families are approximately equally remote from one another, suggesting that functional differences between these families were established at an early evolutionary time.

Combining these phylogenomic results with the previously established occurrence of hypermodified adenosines ms²t⁶A and ms²t⁶A in both B. subtilis and human tRNAs (26, 27), we hypothesized that both MtaB and e-MtaB enzyme families are likely to catalyze the methylthiolation of t⁶A to form ms²t⁶A (Scheme 2). Because previously characterized MiaB family members all catalyze biosynthesis of ms²t⁶A (22, 28), the MiaB...
family members encoded in the B. subtilis and human genomes are likely to be responsible for the observed biosynthesis of ms²⁶⁶⁶. A. Because only one additional MTTase family is encoded in each of these genomes, the corresponding MtaB and e-MtaB enzymes are leading candidates to catalyze the biosynthesis of ms²⁶⁶, the only other methylthiolated base observed in these organisms. We therefore undertook experimental studies to critically evaluate this bioinformatics-based inference.

YqeV/MtaB and CDKAL1/e-MtaB Transform t⁶A into ms²⁶⁶⁶—To test the function of the corresponding proteins, we assayed the influence of the yqeV and CDKAL1 genes on tRNA modification in vivo in E. coli strain TX3346, which lacks a functional miaB gene. This strain has the advantage of accumulating t⁶A-37, as a consequence of the inactivation of the miaB gene, and also containing t⁶A-37, because E. coli K12 does not encode any enzyme-catalyzing methylthiolation of this nucleoside. The TX3346 strain was transformed with either plasmid pT7-mtab expressing the B. subtilis yqeV/mtaB family or plasmid pT7-emtab expressing the human CDKAL1/e-MtaB gene. Bulk tRNAs were isolated after growth at 37 °C, hydrolyzed, and processed for HPLC analysis of their modified nucleosides, as described previously (24). Under these conditions, chromatograms of tRNA hydrolysates from the control TX3346 strain showed, as expected, both t⁶A-37 and t⁶A-37
eluting at 41 and 71 min, respectively, with no evidence for the presence of ms2i6A-37 and ms2t6A-37 (Fig. 2A). The identity of i6A and t6A was confirmed first by their chromatographic retention times and UV-visible spectra (Fig. 2D and E) (24) and second by coupled HPLC/mass spectrometry analysis. The latter revealed the presence of a compound eluting at 41 min that could be assigned to t6A on the basis of the m/z ratio of its protonated pseudo-molecular ion (MH⁺/H11001/H11005 413.4) (Fig. 2G), in good agreement with the theoretical value for the unprotonated molecular weight of M/H11005 = 412.4. In contrast, HPLC analysis of tRNA extracted from the E. coli strain complemented with pT7-mtab (B) and pT7-emtab (C) showed no evidence for the presence of ms2t6A in the chromatogram, with the peak corresponding to t6A remaining essentially unchanged in intensity and the presence of a new peak eluting at 52 min (Fig. 2B and C). The elution time (Fig. 2, B and C) and UV-visible spectrum (Fig. 2F) of the new peak are identical to that of ms2t6A (24). By HPLC/MS, the corresponding protonated pseudo-molecular ions MH⁺ was found at m/z = 459.2 (Fig. 2, H and I), in excellent agreement with the theoretical value for the unprotonated molecular weight of M = 458.4. These results demonstrate that B. subtilis YqeV/MtaB and human CDKAL1/e-MtaB proteins are both functional in vivo and selectively involved in the conversion of t6A to ms2t6A.

In Vivo Experimental Validation of the Function of YqeV/MtaB by Using B. subtilis MGNA-C496 Strain—To obtain further evidence that MtaB from B. subtilis is the enzyme that is

In Vivo Experimental Validation of the Function of YqeV/MtaB by Using B. subtilis MGNA-C496 Strain—To obtain further evidence that MtaB from B. subtilis is the enzyme that is
involved in the transformation of t6A into ms2t6A, we analyzed bulk tRNA extracted from the following: (i) *B. subtilis* wild-type strain (MGNA-A001); (ii) yqeV-strain (MGNA-C496) lacking the mtab gene; and (iii), MGNA-C496 complemented with a plasmid containing the yqeV (mtab) gene. Bulk tRNAs were isolated after growth at 37 °C, hydrolyzed, and processed for HPLC analysis of their modified nucleosides, as described previously (24). Under these conditions, chromatograms of tRNA hydrolysates from the *B. subtilis* wild-type MGNA-A001 strain showed, as expected, in the 45–90-min region the presence of t6A, ms2t6A, t6A, and ms2t6A that elutes at 42, 53, 71, and 85 min, respectively (Fig. 3A). The identity of the four modified nucleosides was established by their chromatographic retention times and UV-visible spectra (Fig. 3, A and D–G) (24). In Fig. 3B is shown the HPLC of tRNA hydrolysates obtained from MGNA-C496 strain. The analysis revealed that the 53-min peak detected on the UV trace in the parental strain (MGNA-A001), corresponding to the ms2t6A, disappeared in the MGNA-C496 strain, and the t6A peak increased in intensity. The ms2t6A peak was recovered when this strain was transformed with a plasmid carrying the wild-type *yqeV/mtaB* gene (plasmid pDB148-mtaB) confirming that the absence of ms2t6A modification in tRNAs extracted from the MGNA-C496 was due to the loss of *yqeV/mtaB* gene.

**Fig. 3.** HPLC and UV-visible detection of t6A, ms2t6A, t6A, and ms2t6A modified nucleosides using *B. subtilis*. The chromatograms correspond to the analysis (45–90-min region) of bulk tRNA from the following: MGNA-001 *B. subtilis* wild-type strain (A); MGNA-C496 *B. subtilis yqeV* strain (B); MGNA-C496 *B. subtilis yqeV* strain complemented with the pDB148-yqeV plasmid (C). The UV-visible spectra of the t6A (D), ms2t6A (E), t6A (F), and ms2t6A (G) are shown. The experiments have been run in triplicate, and the areas have been found to be reproducible within a 5% margin error. mAU, milli-arbitrary units.

In Vitro Characterization of the *B. subtilis* MtaB (*YqeV*) Protein—We concentrated initially on purification and *in vitro* characterization of the *B. subtilis YqeV/MtaB* protein because of the generally greater difficulty of purifying human proteins in functional form. Induction of *YqeV/MtaB* expression from plasmid *pT*17-mtab in *E. coli* strain BL21(DE3)RIL resulted in the overproduction of a protein migrating at ~50,000 Da on SDS gels, in good agreement with the molecular mass deduced from amino acid sequence (51,657 Da). The expressed protein was found in the soluble fraction of cell-free extracts. After the final step of purification, the purity was evaluated by SDS-PAGE to be over 95% (Fig. 4A). The as-purified protein was light brown in color and found to contain low amounts of both iron and sulfur atoms (<0.2 iron, sulfur per monomer). This suggested the presence of a protein-bound iron-sulfur cluster, however, in substoichiometric amounts, probably as a consequence of cluster losses during aerobic purification. Anaerobic treatment of the protein solution with an excess of ferrous iron and enzymatically produced sulfide generated a form of *YqeV/MtaB* protein, which after desalting contained up to 7.5 ± 0.2 iron and 7.5 ± 0.5 sulfur atoms per polypeptide chain, suggesting the presence of two [4Fe-4S] clusters.

The UV-visible spectrum of reconstituted MtaB (Fig. 4B trace 2) displays a broad absorption band centered at around
420 nm, which is assigned to sulfur-to-iron charge transfer transitions characteristic of a $[4\text{Fe}-4\text{S}]^{2+}$ cluster. This absorption band has an $A_{420}/A_{280}$ ratio of 0.34 $\pm$ 0.02 and a molar extinction coefficient at 400 nm of 30,000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ in the lower range of most biological $[4\text{Fe}-4\text{S}]^{2+}$ centers ($\epsilon_{420} = 15$–17 $\text{M}^{-1} \cdot \text{cm}^{-1}$ on a per cluster basis) suggesting that the reconstituted protein contains slightly less than two $[4\text{Fe}-4\text{S}]$ clusters per polypeptide chain. Upon addition of dithionite, the absorption decreased over the entire 350–700 nm range, as expected upon reduction of the chromophore to the $[4\text{Fe}-4\text{S}]^{+}$ state (data not shown). EPR analysis of the resulting reduced protein confirmed the presence of $S = \frac{1}{2}$, $[4\text{Fe}-4\text{S}]^{1+}$ centers (Fig. 4C).

**Conclusion**—The physiological role of CDKAL1/e-MtaB gene in eukaryotic cells remains to be elucidated. However, its similarity to CDKSRAP1 led to the speculation that it might play an important role in insulin production under glucotoxic conditions through interaction with CDK5 that belongs to the well-known large family of cyclin-dependent kinases (16). Our *in vivo* studies now indicate that it encodes a radical AdoMet MTTase that is involved in biosynthesis of 2-methylthio-$N^6$-threonylcarbamoyladenosine ($\text{ms}^2\text{t}^6\text{A}$) in tRNA. We have also shown that *B. subtilis* *yqeV/mtaB* encodes a protein with equivalent biochemical activity, even though it belongs to a different MTTase sequence family. Thus, $\text{ms}^2\text{t}^6\text{A}$ is synthesized through the conversion of $A$ to $\text{t}^6\text{A}$ by the action of YrdC/Sua5 enzymes, as recently shown (15), followed by the transformation of $\text{t}^6\text{A}$ to $\text{ms}^2\text{t}^6\text{A}$ by the action of either CDKAL1/e-MtaB or YqeV/MtaB.

It is now well established that the MTTase enzymes that catalyze the methylthiolation reactions belong to a class of the radical AdoMet iron-sulfur enzyme family containing two $[4\text{Fe}-4\text{S}]^{2+}$ clusters. The enzymatic reaction proceeds through the following steps: (i) AdoMet reductive cleavage promoted by the radical AdoMet $[4\text{Fe}-4\text{S}]^{1+/-2+}$ cluster to generate a 5'-deoxyadenosyl radical, Ado'; (ii) selective H atom abstraction at the substrate by Ado'; (iii) reaction of the resulting intermediate radical with a second $[4\text{Fe}-4\text{S}]^{2+}$ cluster in the N-terminal UPF0004 domain, by an unknown mechanism, to generate a thiolated intermediate; and (iv) methylation at the introduced sulfur atom most probably through the reaction with the electrophilic methyl group of a second AdoMet molecule. The observation that YqeV/MtaB is able to bind two redox-active $[4\text{Fe}-4\text{S}]^{1+/-2+}$ clusters is thus in full agreement with its involvement in a methylthiolation reaction.

To our knowledge five naturally occurring methylthio modifications have been reported so far; one of these occurs on a aspartic acid residue in ribosomal protein S12 and is
Enzymatic Function of Two Methylthiotransferase Families

Jonathan D. Luff and John Rohde

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Addendum—During the submission process of this study, a closely related paper appeared in the literature (Anton, B. P., Russell, S. P., Vertrees, J., Kasif, S., Raleigh, E. A., Limbach, P. A., and Roberts, R. J. (2010) Nucleic Acids Res., in press) that presents some results similar to ours on the B. subtilis YmcB/YqeV enzymes, in general agreement with our data. However, our study is substantially broader in the scope of both its bioinformatic and experimental analyses.

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