The intracellular ratio between methionine and its activated form S-adenosylmethionine (AdoMet) is of crucial importance for the one-carbon metabolism. AdoMet recycling into methionine was believed to be largely achieved through the methyl and the thiomethyladenosine cycles. We show here that in yeast, AdoMet recycling actually occurs mainly through the direct AdoMet-dependent remethylation of homocysteine. Compelling evidences supporting this result were obtained owing to the identification and functional characterization of two new genes, SAM4 and MHT1, that encode the yeast AdoMet-homocysteine methyltransferase and S-methylmethionine-homocysteine methyltransferase, respectively. Homologs of the Sam4 and Mht1 proteins exist in other eucaryotes, indicating that such enzymes would be universal and not restricted to the bacterial or fungal kingdoms. New pathways for AdoMet or S-methionine-dependent methionine synthesis are presented.

The sulfur amino acid methionine is a key player of intermediary metabolism; it is not only involved in protein synthesis but is also an essential determinant of the one-carbon metabolism. Indeed, under its activated form, S-adenosylmethionine (AdoMet), it is the methyl donor in hundreds of transmethylation reactions of nucleic acids, proteins, or lipids. Furthermore, AdoMet serves as a precursor for biosynthesis of polyamines and is the substrate used for numerous reactions, including vitamin biosyntheses and nucleotide modifications. Therefore AdoMet is believed to be next to ATP for the number of reactions in which a biological compound is used (1).

Giving such ubiquitous functions, the equilibrium between methionine and AdoMet is thus expected to be of crucial importance for the overall cellular homeostasis. Accordingly it has been known for a long time, owing to both in vitro and in vivo studies, that numerous transformed cells and tumors exhibit a methionine-dependence phenotype (2). In eucaryotic cells, the methionine/AdoMet ratio was thought to be largely maintained owing to the identification and functional characterization of two new genes, SAM4 and MHT1, that encode the yeast AdoMet-homocysteine methyltransferase and S-methylmethionine-homocysteine methyltransferase, respectively. Homologs of the Sam4 and Mht1 proteins exist in other eucaryotes, indicating that such enzymes would be universal and not restricted to the bacterial or fungal kingdoms. New pathways for AdoMet or S-methionine-dependent methionine synthesis are presented.

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MATERIALS AND METHODS

Microbiological Techniques—Yeast strains used in this work are listed in Table I. Standard yeast media were prepared as described in Chrest and Sridin-Kerjan (7) and Sherman et al. (8). S. cerevisiae was transformed after lithium acetate treatment as described in Gietz et al. (9).

Recombinant DNA Methods—Plasmids pRS313 and 314 were used as shuttle vectors between S. cerevisiae and Escherichia coli (10). To disrupt the YPL273w open reading frame, the UR3 gene from Kluyveromyces lactis was used to replace the open reading frame and to disrupt the YLL062c open reading frame, the HIS3 gene from Saccharomyces kluyveri was used. Wild-type cells (strain W303-1A) were transformed with the amount of DNA generated by one polymerase chain reaction, and uracile (YPL273w) or histidine (YLL062c) prototrophs were selected as described in Lorenz et al. (11). Correct replacement of the targeted gene was verified by polymerase chain reaction using DNA extracted from one transformant for each gene. The resulting strains CD216 (yl062Δ) and CD219 (ypl273Δ) were then back-crossed to the parental wild-type strain W303-1A, yielding the CY51 (yl062Δ/YLL062) and CY49 (ypl273Δ/YPL273) diploid strains, respectively. The YLL062c gene was cloned by gap repair as described in Mallet and Jacques (12) using plasmid pRS314, yielding plasmid pLP062. The YPL273 gene was cloned by polymerase chain reaction and introduced in plasmid pRS313, yielding plasmid pLP273.

Isolation of Mutants Able to Transport MTA—To isolate strains per-
measurable to purine nucleosides, 6 × 10^9 cells from strain EMY60 (ade2, ade3) were plated on minimal medium containing 1 m M adenine. 13 spontaneous mutants capable of growing on this medium were isolated. After purification, two mutants able to use adenine as an adenine source were studied. Genetic analysis showed that they carried different mutations that we called ado8-1 and ado12-1. Strains bearing ado8-1 and ado12-1 mutations were crossed to a strain bearing a meu1 disruption, and the phenotype of ado8-1, meu1Δ and of ado12-1, meu1Δ strains was studied.

**Northern Blot Analyses**—Northern blotting was performed as described by Thomas (13), with total cellular RNA extracted from yeast as described by Schmitt et al. (14) and oligo-labeled probes (15). The final concentration was 0.5 mM for S-methylmethionine (SMM) and 0.01 mM for AdoMet.

### Results

**Ypl273p and Yll062p Are Homologous to Cobalamin-dependent Methionine Synthases**—In *S. cerevisiae*, the methionine synthase is encoded by the *MET6* gene (18, 19). Contrary to its mammalian counterpart, the yeast Met6 protein is thought to be a cobalamin-independent enzyme. Accordingly, *S. cerevisiae* cells do not need vitamin B12 for growth, although they are able to synthesize this compound (19–21). Since there exists some evidence that cobalamin-independent methionine synthases are rather inefficient enzymes (for a review, see Ref. 22), we searched data bases to determine whether the yeast genome might encode more than one methionine synthase, as in *E. coli*. Indeed, *E. coli* possesses two methionine synthases, encoded by the *MetE* and *MetH* genes. We thus made a search against the yeast genomic data base for proteins exhibiting similarities to the *E. coli* cobalamin-dependent MetH enzyme. Results showed that two open reading frames, *YLL062c* and *YPL273w*, encode proteins sharing extensive similarities to the amino-terminal part of MetH (24% and 23% identical residues, respectively, Fig. 1). The Yll062 and Ypl273 proteins (323- and 325-amino acid residues, respectively) are two highly homologous proteins (201 identical residues) of unknown function. The possibility that Yll062 and Ypl273 proteins could be methyltransferases was further supported by the fact that they both exhibit sequence similarities to the *E. coli* YagD gene product, a thiol/selenol methyltransferase (23).

Moreover, a striking observation made the two proteins, Yll062p and Ypl273p, attractive candidates for new methionine metabolism-related enzymes: their encoding genes are, respectively, adjacent to the *MMP1* and *SAM3* genes, which have been demonstrated to encode the SMM and AdoMet high affinity permeases, respectively (6).

**Yll062p and Ypl273p Are Involved in S-Methylmethionine and AdoMet Metabolism**—We have recently shown that *S. cerevisiae* is capable of using SMM as a sulfur source, but no clue as to the utilization pathway was provided (6). The above described results led us to test whether *Yll062p* or *Ypl273p* might be implicated in the utilization of AdoMet and SMM as sulfur sources. We therefore constructed heterozygous diploid cells bearing a deleted allele of either the *YLL062c* or the *YPL273w* gene (see “Material and Methods”). Both diploids were sporulated, and the resulting progenies were analyzed for their abilities of using various sulfur sources. In both cases, all the spores were capable of utilizing sulfate as a sulfur source, demonstrating that neither deletion impaired the assimilative synthesis of methionine from sulfate. In contrast, a perfect 2:12 segregation was observed in the presence of SMM, used as a sulfur source for the progeny of the *yll062ΔYLL062* diploid. All the spores that could not grow on SMM were the *yll062Δ* spores. By contrast, sporulation of the *ypl273ΔYPL273* diploid showed that all *ypl273Δ* cells were capable of growing in the presence of SMM. Therefore, a functional *Yll062p* appears to be specifically required for SMM metabolism.

Analysis of both progenies for growth in the presence of AdoMet showed that, although the *yll062Δ* spores are capable of using this compound, *ypl273Δ* cells exhibit a strong growth defect in the presence of AdoMet. Thus, Ypl273p appears to be specifically involved in AdoMet metabolism. These results were corroborated by analyzing the phenotype of *yll062Δ*, *ypl273Δ* double mutant cells: these are unable to use SMM as a sulfur source. Moreover, the double-disrupted strain is strictly unable to grow in the presence of AdoMet while retaining the capacity of growing in the presence of methionine as a sulfur source (Fig. 2).

**Both yll062p and ypl273p Catalyze Methyl Transfer to the Homocysteine Acceptor**—Taken together, sequence homologies and phenotype of the mutant cells suggested that the *Yll062* and *Ypl273* proteins would be SMM and AdoMet methyltransferases, respectively. A literature survey revealed that, 36 years ago, a partial purification of a methyltransferase from *S. cerevisiae* capable of using either AdoMet or SMM as methyl donor was reported (24). This enzyme was shown to catalyze the transfer of the methyl group from either AdoMet or SMM to homocysteine. We therefore assayed AdoMet- and SMM-homocysteine methyltransferases activities in wild-type, *yll062Δ*, and *ypl273Δ* cells. Results (see Table III) show that neither AdoMet- nor SMM-homocysteine methyltransferase activities can be detected in extracts of *yll062Δ*, *ypl273Δ* double mutant cells, whereas extracts of wild-type cells do contain high levels of both enzymatic activities. In accord with the phenotype of the single mutant cells, the *ypl273Δ* single mutation (strain CY49-1B) leads to a very low level of AdoMet-homocysteine...
methyltransferase activity, whereas SMM-homocysteine methyltransferase activity is comparable with the activity measured in wild type cells. Conversely, the yll062Δ single mutation (strain CY51-1A) results in a strong decrease of the SMM-homocysteine methyltransferase activity as compared with a wild-type strain. However, the yll062Δ mutation also decreases the AdoMet-homocysteine methyltransferase activity. This latter result suggests that Yll062 protein could function to some extent for AdoMet by using an extract of strain CY49-1B (ypl273Δ). This allowed us to demonstrate that in vitro, Yll062p exhibits an AdoMet-homocysteine methyltransferase activity, but with an apparent $K_m$ for AdoMet 20-fold higher than that of Ypl273p.

To confirm these results, the YLL062 and YPL273 genes were cloned on multicopy plasmids, and the resulting plasmids, pL062 and pP273, were used to transform the mutant strains CY51-1A (yll062Δ) and CY49-1B (ypl273Δ), respectively. As expected, in both cases, the obtained transformants were capable of growing in the presence of either SMM or AdoMet, used as sulfur source (not shown). The SMM- and AdoMet-homocysteine methyltransferase activities were assayed in extracts of the transformants. As shown in Table II, when ypl273Δ mutant cells are transformed with plasmid pP273, AdoMet-homocysteine methyltransferase activity is restored to the wild-type level, whereas when yll062Δ cells are transformed with plasmid pL062, the SMM-homocysteine methyltransferase activity is equivalent to that measured in wild-type cells. Interestingly, in the latter case, the AdoMet-homocysteine methyltransferase activity is increased 2-fold as compared with untransformed yll062Δ cells, a result in accord with the low AdoMet-homocysteine methyltransferase activity displayed by the Yll062 protein.

Taken together, all these results demonstrate that the YPL273e gene encodes an AdoMet-homocysteine methyltransferase and the YLL062c gene encodes a SMM-homocysteine methyltransferase. Although Ypl273p appears to be highly specific for AdoMet, Ypl062p is less specific, capable of transferring the methyl group of SMM to homocysteine but also the methyl group of AdoMet. This allows us to demonstrate that the expression of either enzyme has been induced in wild-type cells by the presence of SMM, whereas when yll062Δ cells are grown on SMM, the expression of Yll062p is increased 2-fold as compared with untransformed yll062Δ cells. In the latter case, the AdoMet-homocysteine methyltransferase activity is increased 2-fold as compared with untransformed yll062Δ cells, a result in accord with the low AdoMet-homocysteine methyltransferase activity displayed by the Yll062 protein.

Expression of the SAM4 Gene Is Induced by High Extracellular Methionine—When yeast cells are grown in the presence of a high concentration of extracellular methionine (1 mM), transcription of most of the genes involved in methionine biosynthesis is repressed (4). This transcriptional regulation is mediated by the SCF$^{Met4p}$ complex, a ubiquitin ligase that triggers the degradation of the transcriptional activator Met4p.
in response to high extracellular methionine (25). The 5′- upstream region of the SAM4 gene lacks the DNA consensus regulatory sequences on which the Met4p activator is recruited to activate the transcription of the MET genes (4). To determine by Northern blot whether SAM4 gene expression is modified by the presence of high concentrations of extracellular methionine, a wild-type strain was first grown in the presence of glutathione, a non-repressive sulfur source and transferred to a medium containing 1 mM of L-methionine, and RNAs were extracted at regular time intervals after the shift. The result shows that, contrary to the methionine biosynthetic genes, transcription of the SAM4 gene increases in response to high extracellular methionine (Fig. 3).

In contrast to the SAM4 gene, the 5′-upstream region of the MHT1 gene does contain the AAACGGTGG motif (at position −179), the DNA binding site of Met4-Met28-Met31(Met32) complexes. These complexes are part of the high molecular weight complexes that are assembled in the 5′-upstream regions of the methionine biosynthetic genes and are responsible for their transcriptional activation in the absence of high extracellular methionine. To determine whether MHT1 gene expression is indeed regulated as the “classical” MET genes, Northern blots used in the above-described experiment were analyzed with a MHT1 probe. The results (Fig. 3) show that the expression of the MHT1 gene is indeed repressed when cells are grown in the presence of a high concentration of extracellular methionine. To further confirm the involvement of the transcriptional regulator Met4p in MHT1 gene expression, we assayed met4Δ cells for their capacities of using SMM as a sulfur source. As shown in Fig. 4, met4Δ cells do not grow in the presence of SMM, whereas they do grow in the presence of AdoMet.

Induced AdoMet-dependent Methionine Synthesis—As noted in the introduction, this study was aimed to decipher which pathways allow yeast cells to synthesize methionine from AdoMet in the absence of the methionine synthase encoded by the MET6 gene. Given the above-reported identification and characterization of the SAM4 and MHT1 genes, we wondered whether their encoded products might be responsible for methionine synthesis in met6Δ cells. We therefore recombined the met6Δ mutation with the sam4Δ and mht1Δ mutations. Phenotypic analyses showed that, in contrast to the parental met6Δ mutant cells, the met6Δ, sam4Δ, mht1Δ triple mutant cells are unable to use AdoMet as a methionine source. To further confirm this result, we next used cells in which both methionine synthase, Met6p, and AdoMet synthases, Sam1p and Sam2p, were lacking (26). As met6Δ cells, the met6Δ, sam1Δ, sam2Δ cells are capable of using AdoMet to synthesize methionine. In contrast, the met6Δ, sam1Δ, sam2Δ, sam4Δ, mht1Δ quintuple mutant requires both methionine and AdoMet to grow. Therefore, in the absence of the MET6-encoded methionine synthase or in the presence of extracellular AdoMet, S. cerevisiae cells synthesize methionine from AdoMet due to the Sam4p and Mht1p activities (Table III).

The Methylthioadenosine Salvage Pathway Is Functional in Yeast—One additional outcome of the above experiments was that, if the MTA salvage pathway exists in yeast, it is not capable by itself of providing sufficient methionine for the cell growth whatever the concentration of AdoMet used. To address this issue, we next performed two successive sets of experiments.

First, we tried to determine whether S. cerevisiae cells can use MTA as the methionine source. Since wild type S. cerevisiae cells do not normally transport nucleosides, we searched for mutations that render the cells capable of transporting these compounds. This was done by searching for cells capable of using adenosine as the adenine source. Two mutations, called ado8-1 and ado12-1, were selected (see “Materials and Methods”). The resulting strains were shown to be able both to use adenosine as a source of adenine and to use MTA as a source of both methionine and cysteine (Table IV). This result thus suggested that a MTA salvage pathway may exist in yeast. However, it must be noted that the concentration of MTA required to sustain the cell growth was very high (5 mM), leaving the possibility that the observed growth may be due to MTA contamination. A second genetic approach was therefore used to strengthen these results.

In mammalian cells, the first committed step of the MTA salvage pathway is the cleavage of MTA by the MTA phosphorlyase, yielding 5-methylthioribose-1-phosphate and adenosine (27, 28). We thus searched the yeast genomic data base for proteins exhibiting similarities to the human MTA phosphorlyase. Results showed that the yeast genome comprises one gene, called MEU1, whose product is highly related to the human MTA phosphorlyase. The MEU1 gene had been first isolated by Donoviel and Young (29) as a gene whose mutation impairs the

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**TABLE III**

**Growth requirements of different mutant strains**

| Strain   | Relevant genotype | Addition to YNB complete medium* | Metb AdoMetb Met + AdoMetb |
|----------|-------------------|---------------------------------|-----------------------------|
| CY61–1A  | met6Δ             | +                               | +                           |
| CY61–1D  | met6Δ, sam4Δ, mht1Δ| +                               | +                           |
| W744–1A  | sam1Δ, sam2Δ      | -                               | +                           |
| CC974–5C | met6Δ, sam1Δ, sam2Δ| -                               | +                           |
| CY69–14C | met6Δ, sam1Δ, sam2Δ, sam4Δ, mht1Δ | - | - |

* Complete YNB medium contains histidine, leucine, uracil, adenine, and tryptophan.

b Met, 0.1 mM L-methionine; AdoMet, 0.1 mM AdoMet.
regulation of the ADH2 gene expression, but no information was further gained about the exact function of its encoded product. A meu1Δ strain was then crossed with an ado8–1 or an ado12–1 mutant. Phenotype analysis of the resulting recombinant mutant cells showed that, in contrast to their parental strains, both meu1Δ, ado8–1 and meu1Δ, ado12–1 were unable to use MTA as sulfur source. As expected, the two strains retained the ability to use adenosine as a source of adenine (Table IV).

Taken together, all our results strongly suggest that, although the MTA cycle is functional in S. cerevisiae, its activity cannot recycle sufficient AdoMet into methionine to sustain growth of the cells when the methionine synthase Met6p is lacking.

**DISCUSSION**

The here reported identification and functional characterization of the two new genes SAM4 and MHT1 shed new light on the methionine biosynthetic pathways in the yeast S. cerevisiae. The SAM4 and MHT1 genes were identified through the sequence homology of their encoded products to the cobalamin-dependent methionine synthase from E. coli. Both genes were highly similar, sharing around 60% of identical residues. Biochemical assays performed with cells that do not express the SAM4 and/or MHT1 proteins conclusively demonstrate that both proteins are capable of catalyzing the direct methylation of homocysteine. Although Sam4p appears to function exclusively with AdoMet as a substrate, Mht1p is less specific, capable of transferring to homocysteine the methyl group of either SMM or AdoMet, but with a high apparent Km for the latter. In accord with these functional assignments, the Sam4Δ cells are specifically impaired in AdoMet utilization, whereas the Mht1Δ cells are unable to grow on SMM used as sulfur source.

The identification of Mht1p as the yeast SMM-homocysteine methyltransferase as well as the phenotype of the mht1Δ cells demonstrate that in yeast, SMM catabolism occurs through only one pathway that is identical to the one found in bacteria, plants, and mammals (16, 23). To date, SMM synthesis is known to exist in plants only, where it is synthesized from methionine and AdoMet and where it serves as a precursor for the biosynthesis of the osmoregulator, dimethylsulfoniopropionate (30–32). Since the natural biotope of yeasts is leaves and fruits, it is therefore not unexpected that yeast cells have evolved a system that allows them to utilize SMM of plant origin as an alternative methionine source. Strikingly, on the yeast genome, the MHT1 gene is clustered with the MMP1 gene, which encodes the high affinity SMM permease. The two genes are divergent, separated by a short intergenic region of 343 base pairs, which contains one of the two cis-acting sequences regulating the MET gene network. Accordingly, Northern blot assays demonstrated that MHT1 gene is regulated as the other MET genes, repressed when the cells are grown in the presence of high extracellular methionine. Furthermore, transcription activation of both the MHT1 and MMP1 genes appears to require the Met4p activator as do most of the MET genes, a result corroborated by the inability of met4Δ cells to grow on SMM as a sulfur source.

The identification of the SAM4 gene, encoding the AdoMet-homocysteine methyltransferase, uncovered that the genes specific for AdoMet utilization are also clustered in the S. cerevisiae genome. The SAM4 gene is adjacent to the SAM3 gene, which has been shown to encode the high affinity AdoMet permease. Since the permease-encoding genes (MMP1 and SAM3), on one hand, and the homocysteine methyltransferase-encoding genes (MHT1 and SAM4), on the other, form two pairs of highly similar genes, it is tempting to postulate that the two clusters have arisen through the duplication of an ancestral cluster. However, this hypothetical duplication event would have been followed by one or several recombination events, since, contrary to the MHT1 and MMP1 genes, which are divergent, the SAM3 and the SAM4 genes are transcribed in the same direction.

In contrast to the MHT1 gene, transcription of the SAM4

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**TABLE IV**

**Utilization of various sulfur and adenine source by different mutant cells**

| Strain          | Relevant genotype          | addition to B medium<sup>a</sup>   |
|-----------------|----------------------------|-----------------------------------|
| W303–1A         | MEU1, ADOS, ADO12          | ade + HC  ade + HC  ade + MTA  HC + MTA |
| MDY14           | meu1Δ ADOS, ADO12          | +                   -         -          -          |
| CY85–3C         | MEU1, ADOS, ado12–1        | +                   +         +          +          |
| CY85–5B         | meu1Δ, ADOS, ado12–1       | +                   +         +          +          |
| CY92–5A         | MEU1, ado8–1, ADO12        | +                   +         +          +          |
| CY92–2A         | meu1Δ ado8–1, ADO12        | +                   -         +          -          |

<sup>a</sup> B medium (7) contains histidine, leucine, uracile, and tryptophan. ade, adenine (0.3 mM); ado, adenosine (1 mM); HC, dl-homocysteine (0.2 mM); MTA, methylthioadenosine (5 mM).
gene is induced by growth in the presence of high extracellular methionine. Accordingly, neither the 5′-upstream region of SAM4 contains the cis-acting regulatory MET sequences nor the growth of the met4Δ cells is impaired on AdoMet. At first view, it might appear surprising that, in the presence of high extracellular methionine, yeast cells repress SAMM but not AdoMet utilization, as the remethylation of homocysteine by SMM releases two methionine molecules, whereas only one is formed when AdoMet is used. However, it must be stressed that homocysteine is only needed at a catalytic level for AdoMet-dependent methylation. Indeed the reaction also produces homocysteine from sulfate, with the reaction producing homocysteine as a consequence of the sulfate assimilation pathway repression, the use of SMM would be only achieved through the expensive and complete ATP dephosphorylation required for the de novo AdoMet-dependent synthesis of homocysteine (Fig. 5A).

Identification of the AdoMet-homocysteine methyltransferase-encoding gene led to the unexpected finding that, contrary to what was previously thought, neither the methyl cycle nor the MTA salvage pathways account for the majority of AdoMet recycling into methionine in yeast cells. Compelling evidences supporting this view are provided by the phenotypes of the sam4Δ, mht1Δ cells that are unable to grow on AdoMet as well as by the fact that, contrary to met6Δ cells that lack methionine synthase, recombinant met6Δ, sam4Δ, mht1Δ mutants lose the faculty of using AdoMet as methionine source. Moreover, the observed induction of the SAM4 gene expression in response to high extracellular methionine lends further support to the notion that the Sam4p AdoMet-homocysteine methyltransferase plays an essential role in the control of the equilibrium between methionine and AdoMet in yeast cells. AdoMet-dependent homocysteine methylation appears to be a propitious means to get such control, since this reaction is of low energy cost, uses catalytic amounts of homocysteine, and is, propitious to get such control, since this reaction is of low energy cost, uses catalytic amounts of homocysteine, and is, independent of the folate biosynthetic pathway (Fig. 5C).

As noted in the Introduction, it is well established that about 50% of the human tumor cells exhibit a methionine-dependent phenotype growth. Contrary to normal cells, which are capable of using homocysteine or methionine to meet their requirement in sulfur amino acids, these tumor cells only grow in the presence of methionine. Accordingly, it was shown that athymic mice that are grafted with human cancers and fed a methionine-free diet are greatly reduced in their tumor burden (33). The metabolic basis for this phenotype is not understood and appears not to result from alterations in the cobalamin-depend-ent methionine synthase that catalyze the folate-dependent methylation of homocysteine (34). Regarding the here-reported role of the AdoMet-homocysteine methyltransferases in yeast cells, it would be of great interest to determine the status of these enzymes in human tumor cells. A Blast search against the Drosophila genome revealed that it contains two genes whose products are highly similar to the Sam4 and Mht1 proteins (Fig. 6), a result supporting the notion that such enzymes would be universal and not restricted to the bacterial or fungal kingdoms. Surprisingly, the Drosophila genome, under its published form, does not comprise a gene whose product is homologous to either the cobalamin-dependent synthase from human and E. coli cells or to the cobalamin-independent methionine synthase from yeast. The diversity of the enzymes providing cells with methionine could be therefore much more larger than previously anticipated.

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Reverse Methionine Biosynthesis from AdoMet

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