Nutritional Modulation of Gene Expression and Homocysteine Utilization by Vitamin B₁₂*

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Vitamins B₁₂, B₆, and folic acid converge at the homocysteine metabolic junction where they support the activities of two key enzymes involved in intracellular homocysteine management, methionine synthase (MS) and cystathionine β-synthase. The molecular mechanism for the regulation of homocysteine metabolism by B₁₂ supplementation has been investigated in this study. B₁₂ supplementation does not alter mRNA or protein turnover rates but induces translational up-regulation of MS by shifting the mRNA from the ribonucleoprotein to the polysome pool. The B₁₂-responsive element has been localized by deletion analysis using a reporter gene assay to a 70-bp region located at the 3′ end of the 5′-untranslated region of the MS mRNA. The cellular consequence of the B₁₂ response is a 2- and 3.5-fold increase in the flux of homocysteine through the MS-dependent transmethylation pathway in HepG2 and 293 cells, respectively. It is speculated that B₁₂-induced up-regulation of MS may have evolved as an adaptive strategy for rapidly sequestering an essential and rare nutrient whose availability may have been limited in the evolutionary history of mammals, a problem that is exacerbated by the absence of this vitamin from the plant kingdom.

Vitamins B₁₂, folic acid, and B₆ converge at a single metabolic junction in mammals that is involved in homocysteine detoxification. Elevated levels of homocysteine are a risk factor for cardiovascular diseases (1), neural tube defects (2), and Alzheimer’s disease (3). It is estimated that up to 40% of the general population at risk for heart diseases may have elevated levels of homocysteine. Despite these significant statistics, our understanding of homocysteine regulation and how nutrients (viz., vitamins) can modulate flux of homocysteine through competing pathways is poor.

Of the three vitamins important for homocysteine metabolism, B₁₂ has the most limited distribution in nature; it is biosynthesized by some bacteria and is absent from the plant kingdom. In humans, it supports the activities of two known enzymes, the cytoplasmic methionine synthase (MS)³ and the mitochondrial methylmalonyl-CoA mutase (4, 5). MS is a housekeeping enzyme that plays an essential function, and knockout of this gene in mice results in embryonic lethality in the homozygous state (6).

The ability of vitamin B₁₂ added to the cell culture medium to activate MS was first reported more than three decades ago (7, 8). It is important to emphasize that in these studies cells were exposed to B₁₂ supplementation after having been cultured in “normal” rather than B₁₂-depleted media. Thus, the induction of MS activity by B₁₂ was elicited by vitamin supplementation rather than a transition from B₁₂-deplete to B₁₂-replete medium. Although the molecular basis for this induction was not described, it was suggested that it resulted from conversion of apoenzyme to holoenzyme in the presence of exogenous B₁₂ (9). More recently, we have published evidence that ruled out apoenzyme to holoenzyme conversion as a plausible mechanism for B₁₂-induced activation of MS and demonstrated that although the steady-state levels of mRNA were unaffected, the MS protein levels increased 2–3-fold (10). These results ruled out transcriptional regulation and suggested that the B₁₂ effect may be exerted at a translational level.

In principle, the effect of B₁₂ at a posttranscriptional level could occur by enhanced mRNA or protein stability or by increased translational efficiency. This study was designed to distinguish between these possibilities and to determine whether modulation of MS translation efficiency by B₁₂ is mediated by an element in the 5′-UTR of the mRNA. The physiological relevance of B₁₂-dependent gene regulation was demonstrated by the observation that B₁₂ supplementation resulted in enhanced flux of homocysteine through the MS-dependent transmethylation pathway in cultured cells.

EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimum essential medium, Dulbecco’s modified Eagle’s medium, hydroxocobalamin, purumycin, actinomycin D, aprotinin, leupeptin, pepstatin, and anti-rabbit IgG (alkaline phosphatase conjugate) were purchased from Sigma. Fetal bovine serum was from HyClone. 293 cells (transformed human epithelial kidney cells) and HepG2 cells (human hepatocellular carcinoma) were from American Type Culture Collection. COS-1 (monkey kidney fibroblast) cells were obtained from Dr. Charles Wood at the University of Nebraska, Lincoln, NE. Restriction enzymes were from Invitrogen or New England Biolabs. [¹⁴C]H₄folate (barium salt, 57 mCi/mmol) was from Amersham Biosciences.

Cell Culture Conditions—Cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum and maintained at 37 °C, 5% CO₂. B₁₂ derived from fetal bovine serum is present at an estimated final concentration of ~125 pg in this medium (9). For B₁₂ induction studies, the cells were grown to 60–80% confluency, and fresh Eagle’s minimum essential medium supplemented with 5 ng/liter hydroxocobalamin (or 3.6 μM final concentration) was added. For Western and Northern analyses of MS and for the luciferase reporter studies, cells were harvested from 100-mm plates. [¹⁴C]H₄folate incorporation was examined in cells grown in 60-mm plates. Protein and mRNA turnover experiments were performed in the presence of purumycin and actinomycin, respectively (at final concentrations of 10 μg/ml each), added to the cell culture medium.

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¹ The abbreviations used are: MS, methionine synthase; UTR, untranslated region; CAT, chloramphenicol acetyltransferase; H₄folate, tetrahydrofolate.

20778
Western blot Analysis of MS—Western blot analysis was performed as described previously (10). The bands were quantitated by densitometry using NIH image software. To ensure equal loading, the membranes were also probed with an antibodies against Propionibacterium shermanii methylmalonyl-CoA mutase.

Northern Blot Analysis of MS mRNA—Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Individual blots were performed as described previously (10). The membrane was then exposed to Biomax film (Eastman Kodak Co.) at –70 °C and developed, and the bands were quantitated by densitometry using NIH image software. The band intensities were normalized versus 18S ribosomal RNA in the same samples.

Polysome Analysis—Polysome analysis was performed essentially as described (11). Briefly, four 100-mm dishes of COS-1 cells at subconfluent density were used for each polysome isolation. Cells were washed 3 times with ice-cold phosphate-buffered saline containing 100 μg/ml cycloheximide, removed using a cell scraper, and lysed in polysome lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.4), 100 μg/ml cycloheximide, 0.5% Nonidet P-40, 20 units/ml RNasin (Promega). Nuclei were pelleted by centrifugation at 12,000 × g at 4 °C for 5 min. Equivalent amounts of RNA were loaded onto each 15%–7.4, 100/11032/H9262, 100/11032/H11032/H11032 range of amplification by examining the dependence of the product concentration, 53/14CH3-H4folate incorporation into proteins was measured as previously described (10). The bands were quantitated by densitometry using Quantity One (Bio-Rad) software. The band intensities were normalized versus 18S ribosomal RNA. The bands in each sample were used for correct for variations in loading quantities.

Measurement of 14CH3-H4folate Incorporation into Proteins—Cells were grown to 60–80% confluency on 60-mm plates, and B12 (5 μg/liter) was added to half of the plates for 24 h. After B12 induction, incorporation of 14CH3-H4folate into proteins was measured as previously described (12). Cells were incubated for 30 min in the presence of media (Dulbecco’s modified Eagle’s medium lacking methionine and supplemented with 100 μM homocysteine and 9 μM folate). 5 μCi of 14CH3-H4folate (Amersham Biosciences, 57 mCi/mmol; final medium concentration, 53 μM) was added, and incubation was continued for an additional 2 h. Cells were then washed three times with phosphate-buffered saline and suspended in 1 ml of phosphate-buffered saline. 100 μl from each sample was used for determination of radioactivity. The radioactive content was normalized to total protein concentration in each sample.

Statistical Analysis—Each experiment was repeated at least three times. Statistical analysis was performed using one-way analysis of variance (Microcal Origin software), and results were considered significant if the p value was < 0.05. Each of the methods employed in this study, viz., densitometric analysis of Western and Northern blots and luciferase reporter assays, have errors that are inherently associated with them, which renders measurement of 2-fold effects of the kind reported in this study challenging. However, it should be noted that in all cases we have examined the relative effect of the variable, i.e., B12 supplementation, on experiments run in parallel using unamplified media.

RESULTS

Effect of B12 on MS mRNA Stability—The influence of B12 on MS mRNA stability was assessed in 293 cells treated with the transcription inhibitor actinomycin D. The relative steady-state MS mRNA levels were followed by Northern blot analysis as shown in Fig. 1. Two messages corresponding in size to ~7 and 10 kb are seen in all human tissues that have been examined so far (13). Our results show that rate of MS mRNA turnover is unaffected by B12 supplementation of the medium and that the half-life of the message is ~19 h. These results indicate the B12 induction of MS activity is not exerted at the level of increased mRNA stability.
Effect of B12 on MS Stability—It is not uncommon for protein stability to be enhanced by the binding of its cofactor, and in fact rat MS has been shown to be stabilized by B12 (14). To determine whether B12 supplementation affected the steady-state turnover of MS, we examined the relative levels of this protein by Western blot analysis in 293 cells treated with the translation inhibitor puromycin (Fig. 2A). The turnover rate of MS in the presence and absence of exogenous B12 was the same (half-life \(t_1/2\) 12 h) within experimental error excluding a role for B12 in modulating MS stability under these conditions (Fig. 2B). This is consistent with our earlier observations that MS is present predominantly (>80%) in the holoenzyme form in 293 cells grown in the presence of normal Eagle’s minimum essential medium (10). In addition, we have reported that the relative proportion of the holoenzyme form is unchanged under conditions of B12 supplementation, although the enzyme activity is increased 2.5-fold under these conditions (10).

Effect of B12 on MS Polysomes—We analyzed the influence of B12 on translation of MS by examining the polysomal association of mRNAs in the presence and absence of B12. As shown in Fig. 3, B12 supplementation elicits a 2.5–3-fold increase in the amplitude of MS polysomes and a corresponding decrease in the proportion of MS mRNA associated with the ribonucleoprotein fraction. These results are consistent with activation of MS translation by B12 supplementation. The magnitude of the increase in MS polysomes is consistent with the previously reported 3.8-fold increase in MS activity induced by B12 in COS-1 cells (10). As a control, the distribution of methylmalonyl-CoA mutase, whose activity is not affected by B12 supplementation, was determined under the same conditions. The amplitude of methylmalonyl-CoA mutase message in the polysomes and ribonucleoprotein pools was unchanged, revealing a specific effect of B12 supplementation on MS mRNA.

Changes in the polysome density (i.e. number of ribosomes per mRNA) or amplitude (i.e. number of actively translated mRNAs) are diagnostic of the status of translation from a given message. Activation of protein synthesis can occur via increased recruitment of ribosomes to the mRNA being translated. Alternatively, activation can result due to an increase in the number of mRNA molecules undergoing translation due to a shift in the equilibrium between the inactive (translation suppressed, ribonucleoprotein) and active (translation active, polysome) pools (15).

Role of the 5′-UTR in Mediating B12-dependent Increase in Translational Efficiency—The potential role of B12 in increasing the translational efficiency of MS mRNA was probed with two reporter gene assays. The 5′-UTR of the MS mRNA was chosen for these initial studies because it is unusually long (394
Reporter constructs were initially generated with either the structures, suggestive of a role in regulation of translation. bases) and displays a high potential for formation of secondary bases) of the 5'-reporter gene (Fig. 4).

293 cells ing frame increased the translational efficiency of luciferase in /H11032 set of controls. Both the full-length 5'-UTR of the MS mRNA levels. The presence of the full-length 5'-UTR served as one set of controls. The respective vectors lacking the 5'-UTR of the MS-UTR and the 18 S -UTR of MS and the mRNAs were normalized to the luciferase and CAT mRNA levels, respectively, as normalized to the luciferase mRNA levels determined by Northern blot analysis, and equal loading of RNA was confirmed by quantitation of the 18S rRNA in these samples (Fig. 4). In both 293 (Fig. 4A) and HepG2 (not shown) cells, the presence of B12 induced an increase in luciferase activity with the full-length and second half of the leader sequence. Similarly, in COS-1 cells, a 1.2- and 2.9-fold enhancement of CAT reporter activity was observed with B12 when the full length and the second half of the 5'-UTR respectively, were present (Fig. 4B).

In all three cell lines the increase in reporter activity induced by B12 was lower than the increase in MS activity (2.5-, 3.8-, and 13.7-fold increase in 293, COS-1, and HepG2 cells, respectively) reported previously (10). The difference may be attributed to the use of transient transfections in which the full extent of induction is missed or to the boundaries of the B12-responsive element extending beyond those of the 5' leader used in the present constructs or to long distance interactions with elements that are missing in the reporter constructs. These will be explored in future investigations.

Localization of B12-responsive Element—A set of nested deletions extending from the 3' end of the 5'-UTR were constructed, and their ability to confer responsiveness to B12 in the luciferase reporter construct was tested in COS-1 cells (Fig. 5). With each deletion construct, the luciferase activity was compared in the presence and absence of B12 supplementation to the medium. These experiments localized the B12-responsive element to a 70-bp region extending from the 3' end of the 5'-UTR. It is interesting to note that deletion of the upstream sequences in the 5'-UTR resulted in a small increase in the magnitude of the B12 effect, as seen in the constructs starting from bp 254 and 324.

B12 Supplementation Enhances Flux Through the Transmethylation Pathway—The effect of B12-induced increase in MS levels on the flux of homocysteine through the transmethylation pathway was examined by measuring incorporation of [14CH3]-H4folate into proteins. CH3-H4folate is a substrate for only one known enzyme, B12-dependent MS, which transfers the methyl group to homocysteine to give methionine (Fig. 6A). B12 supplementation resulted in a 2–3.5-fold increase in incorporation of radiolabel into proteins in HepG2 and 293 cell lines (Fig. 6B). Three other B12 forms, cyano-, deoxyadenosyl- and methylcobalamin, were equally efficacious in eliciting enhanced flux through the MS-dependent transmethylation pathway (not shown). These results support a physiological relevance for the role of B12 supplementation in increasing flux through the transmethylation pathway and, therefore, in intracellular homocysteine clearance.

DISCUSSION

Translational regulation affords a relatively rapid response to external stimuli, bypassing the steps of transcription, splicing, and translation. This mode of regulation can lead to both global and selective control of gene expression. Well studied examples of global control include changes that occur in eggs immediately after fertilization and in reticulocytes in response
to heme levels. Specific translational control may be achieved by one of a variety of mechanisms including (i) autoregulation via binding of the encoded protein to its mRNA as in thymidylate synthase and dihydrofolate reductase (16–18), (ii) by trans-regulatory factors such as the iron-responsive element-binding protein involved in ferritin expression (19) or a redox-sensitive protein that binds to a translational enhancer in the 3′-UTR of manganese superoxide dismutase (20), or (iii) by a short upstream open reading frame in the 5′-UTR as in ornithine and S-adenosylmethionine decarboxylases (21).

The response of human MS mRNA to the presence of a nutritional cofactor, B12, appears to be a novel example of specific translational control, since the other B12 enzyme found in humans, methylmalonyl-CoA mutase, is unaffected (10). The results reported here rule out a role for B12 in influencing the rate of MS mRNA or protein turnover (Figs. 1 and 2), and together with our earlier results, which excluded conversion of preexisting apoenzyme to holoenzyme as a plausible mechanism (10), implicate translational regulation. This model is further supported by a B12-induced increase in the amplitude of MS mRNA polysomes with a corresponding decrease in the ribonucleoprotein fraction, which suggests alleviation of translational inhibition or “unmasking” of existing MS mRNAs by the cofactor.

Fig. 5. Localization of the B12-responsive element. The B12 effect in COS-1 cells was probed with a set of nested deletions containing variable lengths of the 5′-UTR upstream of the luciferase open reading frame. The experimental details are the same as described in the legend to Fig. 4. The boundaries of the 5′-UTR inserted in the reporter constructs are shown on the left. Luciferase/mRNA activity for each deletion construct in the absence of B12 supplementation was measured and served as a control (denoted as having a value of 100). The ratio of the luciferase/mRNA activity in the presence and absence of B12 supplementation is plotted for each construct. Asterisks denote a significant difference (p < 0.05) compared with controls not treated with B12.

Fig. 6. Effect of B12 supplementation on intracellular transmethylation flux. A, pathway leading to incorporation of radiolabel from CH3-H4folate into protein. B, increase in incorporation of radioactivity into protein induced by hydroxocobalamin in two cell lines. The data on 293 and HepG2 cells represent the average of 7 and 2 independent determinations, respectively.
we have tested (10) and leads to an up-regulation rather than repression, which is more commonly observed in examples of translational control of gene expression. Luciferase and CAT reporter constructs implicate the importance of sequences in the 3' end of the 5'-UTR in modulating the B\textsubscript{12} response (Fig. 4). This stretch of the human MS mRNA is predicted to form extensive secondary structures (Fig. 7). The ~2–3-fold enhancement of MS translational efficiency by B\textsubscript{12}, albeit modest, is not unexpected for MS, which is a housekeeping enzyme. In another folate cycle enzyme, serine hydroxymethyltransferase, ferritin was reported to increase translation efficiency 1.14–2.16-fold in reporter constructs containing the 5'-UTR (24). The magnitude of changes for most genes involved in energy metabolism and biosynthesis appears to be ~2-fold, as revealed by a global analysis of gene profiles associated with aging (23).

Recent reports reveal inhibition of translation of genomic RNA in the hepatitis C virus by vitamin B\textsubscript{12} (25). Toe-printing studies reveal that the vitamin stalls initiation by trapping the 80 S ribosomal complexes on the internal ribosome entry site element (26). Translational regulation by B\textsubscript{12} has also been documented in prokaryotes, where it represses the *btuB* gene involved in B\textsubscript{12} transport in *Escherichia coli* and *Salmonella typhimurium* and the *cob* biosynthesis operon in *S. typhimurium* (for review, see Ref. 27). In contrast to the situation observed with the human gene, the bacterial response is specific for adenosylcobalamin and represents classic feedback regulation for both the transport and synthesis pathways when exogenous B\textsubscript{12} levels are high. The proposed model involves long range interactions between a translational enhancer in the 5'-UTR and the region preceding the Shine-Dalgarno sequence, which promotes initiation in the absence of B\textsubscript{12} (28). In the presence of B\textsubscript{12}, the conserved "B\textsubscript{12} box" interacts with the translational enhancer, relieving the long distance interactions and promoting secondary structure formation in the region encompassing the Shine-Dalgarno sequence, thereby inhibiting initiation. Adenosylcobalamin has been shown to specifically inhibit ribosome binding to the *btuB* RNA (29). A similar mode of regulation has been proposed for other bacterial vitamin biosynthesis genes including thiamin (30) and riboflavin (31). Regulatory proteins that bind to adenosylcobalamin have not been identified in prokaryotes despite intensive efforts, raising the possibility that elements in the mRNAs are the direct sensors of this nutrient. Although this issue has not yet been explored in the eukaryotic system reported in this study, it is
interesting to note that RNA aptamers selected for very high and specific binding of cyanocobalamin are known (32). The structure of one of these aptamers has revealed the basis of the specificity that underlies the high affinity \( K_d = 90 \text{ nM} \) and represents one of the tightest aptamer-small molecule interactions that has been characterized (32).

Although the existence of B\(_{12}\) -induced translational repression in prokaryotes can be rationalized from the standpoint of metabolic economy, the advantage of translational up-regulation in mammals is less obvious. One possibility is that the availability of B\(_{12}\) has been limited through much of our evolutionary history, a problem exacerbated by the absence of this vitamin from the plant kingdom. MS is an essential gene, as evidenced by the embryonic lethal phenotype of MS null mice (6), and is absolutely dependent on B\(_{12}\) for its activity. The B\(_{12}\)-independent MS found in some bacteria and in plants is in animals. It is striking that the known B\(_{12}\)-binding proteins and B\(_{12}\)-dependent enzymes found in mammals bind their cofactor avidly, in contrast to many prokaryotic enzymes such as glutamate mutase (33). Indeed, one of the tightest protein-ligand interactions that have been measured is for such a reaction (6), and is absolutely dependent on B\(_{12}\) for its activity. The demonstration that B\(_{12}\) added to cells grown in normal medium leads to enhanced flux through the transmethylation pathway suggests a beneficial role for B\(_{12}\) supplementation. The increased incorporation of radioactivity from \(^{14}\text{CH}_3\)-H\(_4\)folate to proteins (Fig. 6B) in B\(_{12}\)-supplemented medium suggests the potential value of such an intervention in homocysteine-lowering clinical trials by a molecular mechanism that has previously not been recognized.

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