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Posttranscriptional Regulation of Mammalian Methionine Synthase by B_{12}[1]

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Methionine synthase is one of two key enzymes involved in the removal of the metabolite, homocysteine. Elevated homocysteine levels constitute a risk factor for cardiovascular diseases and for neural tube defects. In cell culture, the activity of methionine synthase is enhanced several-fold by supplementation with its cofactor, B_{12}. The mechanism of this regulation is unknown, although it has been ascribed to a shift from apoenzyme to holoenzyme. Using sensitive assay techniques as well as a combination of Northern and Western analyses, we demonstrate that the effect of B_{12} on induction of methionine synthase activity is paralleled by an increase in the level of the enzyme. These studies exclude conversion of apoenzyme to holoenzyme as a basis for activation that had been described previously. Since the mRNA levels do not change during the same period that the methionine synthase levels increase, regulation of this protein by its cofactor must be exerted posttranscriptionally.

Methionine synthase is one of two key enzymes at the homocysteine metabolic junction in mammalian cell physiology. Its action converts homocysteine, a toxic metabolite to methionine, and concomitantly releases the circulating form of folic acid, methyltetrahydrofolate (CH_3-H_4folate), to tetrahydrofolate (H_4folate), that is then available for supporting DNA biosynthesis (1,2). Elevated levels of homocysteine constitute an independent and significant risk factor for cardiovascular diseases (3). In addition, elevated levels of homocysteine and low maternal levels of B_{12} are correlated with neural tube defects (4,5). Methionine synthase is thus an important housekeeping enzyme at the intersection of sulfur and one-carbon metabolism, and its regulation influences intracellular homocysteine and folate pools. The consequences of lowering homocysteine with vitamins are being measured in clinical intervention studies. Thus, elucidating the mechanism by which B_{12} induces methionine synthase activity and thereby affects homocysteine pools may have an important bearing on these intervention studies.

Studies by Mangum and North almost thirty years ago on the regulation of mammalian methionine synthase in HEp-2 cells in culture revealed that supplementation of the medium with B_{12} resulted in a thirty-fold elevation of enzyme activity (6). This was later extended to several other cell lines in which the B_{12}-induced activation of methionine synthase ranged from 10- to 30-fold (7). In the initial experiments, homocysteine and vitamin B_{12} replaced methionine and choline in the medium. Homocysteine was however found to have no effect on the activation of methionine synthase, since B_{12} in the presence or absence of homocysteine yielded the same extent of stimulation (7). The activity of the only other mammalian B_{12}-dependent enzyme, methylmalonyl-CoA mutase, was unchanged upon supplementation of the medium with B_{12} (8).

Several alternative hypotheses can be considered to explain induction of methionine synthase activity by its cofactor, B_{12}. First, if intracellular B_{12} concentration is limiting, increase in enzyme activity could be due to conversion of apoenzyme to holoenzyme. Second, B_{12} may exert its regulation at the methionine synthase promoter resulting in increased transcription. Third, B_{12} may enhance translation of the methionine synthase mRNA, either by affecting the stability of the message or by increasing access of the message to the translation apparatus. Fourth, B_{12} may enhance the

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Abbreviations used: CH_3-H_4folate, methyltetrahydrofolate; H_4folate, tetrahydrofolate; SSC, saline sodium citrate; PBS, phosphate buffered saline; EMEM, Eagle's minimum essential medium; DEPC, diethylpyrocarbonate; OHB_{12}, hydroxycob(III)alamin; SDS, sodium dodecyl sulfate; MOPS, *N*-morpholinosulfonic acid.

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stability of methionine synthase thereby affecting its turnover in the cell. Finally, B_{12} may induce one or more genes that in turn increases the level of methionine synthase either at a transcriptional or post-transcriptional level.

The recent cloning of the human methionine synthase cDNA (9–11) and the development of sensitive assays to measure the relative apo- and holoenzyme content of the enzyme (12–14) now permit evaluation of these alternative hypotheses. In this study, we have examined whether the induction of methionine synthase by B_{12} is correlated with changes in the mRNA levels, protein levels or the percent holoenzyme. Our studies clearly point to a post-transcriptional mechanism of activation of methionine synthase that is dependent on the synthesis of new enzyme.

MATERIALS AND METHODS

Materials. EMEM and hydroxocobalamin were purchased from Sigma. Fetal calf serum was from Calbiochem. The RNeasy kit was from Qiagen and Hybond N+ membrane was from Amersham. RNase free reagents (MOPS, SSC) were molecular biology grade and were purchased from 5 Prime→3 Prime. DEPC treated water was used for making up solutions for RNA studies. Cell lines, Cos-7 (SV40-transformed African Green Monkey kidney cells), HeLa (human cervical carcinoma cells), HepG2 (human hepatoblastoma cells), and 293 (transformed human kidney cells) cells were from ATCC, and 293 t^+ (derivative of 293 expressing the T antigen) was from Mike Fudos (NIH). The actin probe was from an *in vitro* transcription kit from Ambion.

Cell culture. Cells were cultured in EMEM supplemented with 10% fetal bovine serum and maintained at 37°C, 5% CO_2. The concentration of folic acid and methionine in this medium are 2.3 μM and 100 μM respectively. The estimated concentration of B_{12} in the unsupplemented medium is ~125 pM, and is derived from the serum (15). For studies involving RNA isolation, confluent cells from 4 or 5 petri dishes (150 mm) were harvested and frozen in lysis solution and stored at −80°C until further processing.

For studies involving protein analysis (enzyme assays or Western blots) cell monolayers were washed with PBS, collected by centrifugation and the pellet was washed twice with PBS prior to freezing at −80°C. For B_{12} induction studies, cells were grown to subconfluency before replacement of the medium with fresh EMEM supplemented with OHB_{12} at a concentration of 5 mg/L. The B_{12} concentration was chosen based on the dose response for methionine synthase activity that has been reported previously (15). For experiments in which methionine synthase activity, and protein and RNA levels were being determined simultaneously at fixed time points following B_{12} addition (0, 24, and 48 h), a large number of plates were cultured in parallel. Cells from 3 or 4 petri dishes were used per time point for each of the protein and RNA determinations.

Methionine synthase assay using cell extracts. The cell extracts were prepared and anaerobic assays were performed essentially as described previously (13). The cell extracts for the assays were thawed immediately prior to their use. They were made anaerobic by gently passing N_2 for 30 min over the extracts in 1.5 ml eppendorf tubes sealed with red rubber septa. The amount of protein used in the assays ranged from 0.3 mg to 0.6 mg. Protein concentrations were determined using the Bradford assay (BioRad) with bovine serum albumin as standard. One unit of activity is defined as the amount of protein required to synthesize one pmol of methionine h^{-1} at 37°C.

RNA isolation. RNA was isolated using the RNeasy kit as per the vendor's protocol (Qiagen). Aliquots (20–30 μl) of RNA were stored at −80°C until needed.

Northern analysis. RNA samples (10–15 μg) were heated at 65°C for 5 min and then cooled immediately on ice prior to loading onto a 1% formaldehyde agarose gel. The gel was electrophoresed in RNase free 1X MOPS (25 volts for ~20 h at 4°C), equilibrated with 10X SSC and transferred to a Hybond N+ membrane in the same buffer. After overnight transfer, RNA was UV-linked to the membrane. Prehybridization and hybridizations were performed in Church and Gilbert hybridization buffer. Hybridization was conducted overnight at 65°C for 12 h (with 3×10^6 dpm per ml radiolabeled probe). An NcoI-EcoRI fragment containing the homocysteine-binding domain in plasmid SGHcy-28a (described below) was used as one of the probes. Probes (for methionine synthase and actin) were radiolabeled with ^{32}P (Rediprime kit from Amersham Life Science). Following hybridization, the membrane was washed at 65°C for 3 × 20 min with 2X SSC and 0.1% SDS. The membrane was exposed to a phosphoimager screen for 2-3 days at room temperature and then scanned.

Cloning and expression of homocysteine binding domain of human MS. The N-terminal region of human methionine synthase extending from amino acids 1 to 368 is homologous to the homocysteine binding domain of the *E. coli* protein ranging from residues 2-353 (16,17). The segment of the human cDNA encoding this fragment was amplified by PCR and cloned using the primers described below. An NcoI site and an EcoRI site were engineered into the 5′ sense and 3′ antisense primers respectively and are indicated in bold letters in the primer sequences shown below. 5′ sense: 5′ CATG**CCATGG**-CTCCGGCGCTGCAGGACCTGTCGC 3′. 3′ antisense: 5′ **GAATTC**-AATGTTAACAAATTACTAGTACGGTCCAATCCT 3′. In the 5′ primer, the *E. coli* codon preference was employed at some locations to potentially enhance translation efficiency. The codon preference for amino acids following the initiator methionine codon located within the NcoI site (underlined in primer sequence) are indicated in parentheses: Met-Ala (*E. coli*)-Pro (*E. coli*)-Ala (human)-Leu (*E. coli*)-Gln (*E. coli*)-Asp (human)-Leu (human)-Ser (human). In the 3′ primer, two tandem stop codons (underlined) were engineered into the sequence after residue 368 encoding tyrosine. A 1.1 kb fragment was amplified by PCR using Klentaq polymerase (Clonetech) and cDNA from human pancreas (Clonetech) as template. The amplified band was initially cloned into the TA vector, PCR 2.1, from Invitrogen, and the ligation mixture was used to transform *E. coli* Top10 F′ cells (Invitrogen). DNA from a single colony was digested with NcoI and EcoRI and the fragment was cloned into the pet 28 a expression vector (Novagen) to create SGHcy-28a, and transformed into BL21 DE3. The DNA sequence of the insert in SGHcy-28a was determined.

Expression of homocysteine binding domain of human methionine synthase. A colony containing plasmid SGHcy-28a with the 1.1 kb insert, was grown overnight in 2 ml LB medium (containing 50 μg/ml kanamycin) which was used to inoculate a 50 ml LB/kanamycin culture the next day. The culture was grown at 37°C to an $O.D_{600}$ of 0.5-0.6 (~3 h) and expression was induced with 0.8 mM IPTG for 3 h. Cells were collected by centrifugation and the pellet was resuspended in 3 ml of 50 mM Tris-HCl pH 8.0, 2 mM EDTA. The soluble and insoluble cell fractions were obtained using a protocol from Novagen. Lysozyme at a concentration of 100 μg/ml and 0.1 volume of 1% Triton X-100 (Bio-Rad) were added to the resuspended pellet and incubated at 30°C for 15 min. This was followed by centrifugation at 12,000 X g for 15 min at 4°C. The soluble (supernatant) and insoluble (pellet) fractions were resuspended in a 1X SDS sample loading buffer (Bio-Rad) containing β-mercaptoethanol, and separated by electrophoreses on a 10% SDS polyacrylamide gel (Figure 3A). The identity of the recombinant 37 kDa band was confirmed by N-terminal amino acid sequence analysis and by Western analysis using antibodies generated against porcine methionine synthase (12). DNA and N-terminal sequence analyses revealed that Glu 12

TABLE I

Induction of Methionine Synthase Activity by B_{12} in Cell Culture

Medium:	EMEM			EMEM + B_{12}			
	Specific activity			Specific activity[a]			
Cell line	$-B_{12}$	$+B_{12}$	%Holo	$-B_{12}$	$+B_{12}$	%Holo	Fold increase[b]
Cos7	48 ± 6	72 ± 5	67	179 ± 28	271 ± 4	66	3.8
HeLa	140 ± 21	163 ± 12	86	237 ± 40	320 ± 6	74	2.0
293t+	52 ± 9	149 ± 1	35	883 ± 1	860 ± 14	103	5.8
HepG2	12 ± 2	15 ± 2	80	167 ± 9	205 ± 6	81	13.7
293	99 ± 6	119 ± 1	83	218 ± 6	299 ± 28	73	2.5

[a] Specific activity is expressed in pmol methionine formed min^{-1} mg^{-1} at 37°C.
[b] The fold increase was estimated by comparing the values for specific activities measured in the presence of added B_{12}.

was deleted. This could have resulted from a polymorphism at this position that was present in the cDNA library or, more likely, from a PCR error.

Generation of antibodies against the human methionine synthase. The pellet containing the insoluble N-terminal domain of human methionine synthase was washed extensively with 50 mM Tris, pH 8.0 containing 2 mM EDTA. The washed pellet (~1.5 mg) was separated on a preparative 7% SDS polyacrylamide gel and the band of interest was excised after staining with Coommasie brilliant blue R-250 (Bio-Rad) followed by extensive destaining. The excised band was sent to a commercial antibody facility (Alpha Diagnostics, Texas) for generation of polyclonal antibodies in rabbits.

Western analysis of human methionine synthase in cells cultured in medium ± B_{12}. Extracts (50 μg protein), from cells grown in EMEM medium supplemented with B_{12} for 0, 24 and 48 h, were separated on a 5% SDS polyacrylamide gel. The proteins were transferred to a PVDF membranes using a tank electroblotter for 6 h at 100 V. Western analysis was performed using a 1:100 dilution of the first antibody and a 1:5000 dilution of the second antibody, anti-rabbit IgG/alkaline phosphatase conjugate (BioRad), followed by chemiluminescent detection using the CDP-Starr kit (Tropix). Chemiluminescent signals were quantitated using a Molecular Dynamics densitometer.

RESULTS

Induction of methionine synthase activity by B_{12} is not due to apoenzyme to holoenzyme conversion. In order to examine whether or not activation by B_{12} is due to conversion of apoenzyme to holoenzyme, the effect of B_{12} addition to the cell culture medium was examined in five different cell lines (Table I). Enzyme activity was measured in the anaerobic titanium citrate assay either in the presence or absence of exogenous B_{12}. Activity determined in the presence of B_{12} represents total methionine synthase activity. In the absence of B_{12}, only holoenzyme activity is measured. Thus the ratio of activity observed in the absence to that in the presence of additional cofactor represents the proportion of holoenzyme present. As shown in Table I, all five cells lines showed stimulation of methionine synthase activity when the medium was supplemented with B_{12}. The fold increase varied with the cell line, ranging from 2-fold in HeLa cells to ~14-fold in HepG2 cells. Increasing the concentration of the cofactor ten-fold did not further increase the fold-induction (data not shown). Comparison of enzyme activity from cells that had been supplemented with B_{12} with those grown in unsupplemented media show that the proportion of holoenzyme remained the same, within experimental error. The only exception was $293t^+$ cells where the holoenzyme content increased from 34% to 100%. However, the 3-fold increase in percent holoenzyme does not account for the almost 6-fold increase in methionine synthase activity resulting from B_{12} supplementation. 293 cells were employed for the remainder of the studies described below because of their high holoenzyme content and rapid growth rate.

Kinetics of methionine synthase induction by B_{12}. The time course for the activation of methionine synthase was examined next. As shown in Figure 1, increase in methionine synthase activity could be observed as early as two hours following B_{12} addition. The activity increased rapidly during the first six hours reaching a plateau in ~24 to 48 h and was sustained until 72 h.

B_{12} supplementation does not change methionine synthase RNA levels. To examine whether the B_{12} effect is due to transcriptional activation of the methionine synthase gene, mRNA levels were analyzed 0, 24 and 48 h after addition of the cofactor (Figure 2A). Two methionine synthase mRNA's of ~10 and 7.5 kb sizes were observed by Northern analysis as has been reported previously (9,11). However, the level of methionine synthase mRNA remained unchanged during the same period that a 2.5-fold increase in enzyme activity was observed. Equal loading of RNA in the three lanes was confirmed by probing for the actin message (Figure 2B). Northern analysis of three other cell lines (Cos7, HepG2 and $293t^+$) also revealed that the methionine synthase mRNA levels do not change upon B_{12} supplementation (data not shown).

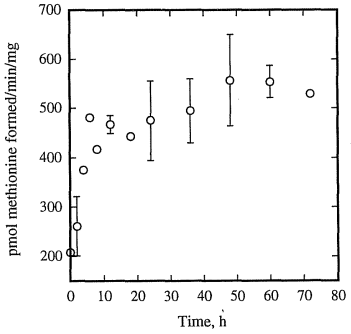

FIG. 1. Kinetics of methionine synthase induction in 293 cells by B_{12}. Cells were grown to subconfluency in 150 mm petridishes as described under Materials and Methods. The medium was aspirated off and replaced with fresh EMEM with either ± B_{12}. The cells were harvested by scraping at the indicated time points and frozen until further analysis. Methionine synthase activity was determined in the anaerobic titanium citrate assay in the absence of additional cobalamin. The time points were repeated in either duplicate or triplicate, and standard errors are indicated.

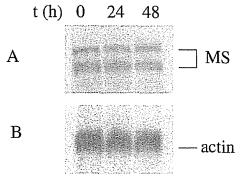

FIG. 2. Northern blot analysis of RNA from 293 cells, 0, 24, and 48 h after addition of B_{12}. RNA was isolated and detected by Northern blot analysis as described under Materials and Methods. (A) Two bands corresponding to the ~10 kb and 7.5 kb methionine synthase cDNA forms were observed. (B) To confirm equal loading of RNA in the lanes, the blot was later probed for the actin message.

Western analysis of methionine synthase levels in cell cultures exposed to B_{12}. Cell extracts from the 0, 24 and 48 h time points were analyzed by Western blotting. As shown in Figure 3B, an increase in methionine synthase level is observed between the 0 and 24 h time points, while the difference between the 24 and 48 h time points is negligible. To ensure equal loading, the membrane was also exposed to antibody generated against the *P. shermanii* methylmalonyl-CoA mutase. As expected, the levels of this protein did not exhibit a time dependent change (Figure 3B lower panel). An additional lower band also crossreacted with the mutase antibody. Although its identity is unknown (it may be a proteolytic form), it serves as an additional control to demonstrate that a specific increase in methionine synthase levels is observed on supplementation of the medium with B_{12}.

DISCUSSION

Elevated concentrations of homocysteine are correlated with risk for two apparently unrelated pathologies: cardiovascular diseases (3) and neural tube defects (5). In mammalian cells, two major enzymatic routes detoxify homocysteine. Transmethylation, catalyzed by either methionine synthase or betaine homocysteine methyltransferase, salvages homocysteine to the AdoMet-dependent methylation cycle. Transsulfuration, catalyzed by cystathionine β-synthase converts homocysteine to cystathionine, and represents the first step in its catabolic removal. Due to the limited tissue distribution of betaine homocysteine methyltransferase in the liver and kidney (18), methionine synthase is the major transmethylase. A number of nutrients function as cofactors or substrates at the homocysteine metabolic junction. Cystathionine β-synthase is a vitamin B_6- and heme-dependent enzyme whereas methionine synthase requires both B_{12} and a folate derivative, CH_3-H_4folate, for activity. This has recently led to the promotion of a multivitamin tablet containing B_6, B_{12}, and folic acid as a "homocysteine defense"

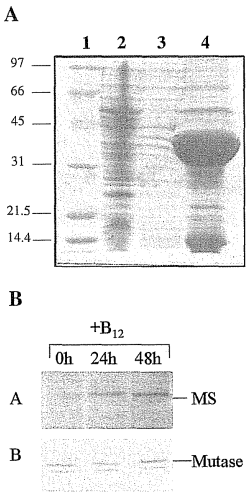

FIG. 3. Overexpression of human methionine synthase fragment and Western analysis of methionine synthase at various time points following addition of B_{12}. (A) Overexpression of the homocysteine-binding domain of human methionine synthase. Cell extracts containing SGHcy-28a were prepared as described under Materials and Methods. Lane 1 has low molecular weight markers, lane 2 has cell extract after sonication, lanes 3 and 4 have the soluble and insoluble fractions respectively separated from the cell extract. (B) Western analysis of methionine synthase levels in the 293 cell extracts at the indicated time points. As a control, the membrane was also probed with antibodies generated against methylmalonyl-CoA mutase from *Propionibacterium shermanii.* The band indicated by an arrow corresponds to the human mutase. MS refers to methionine synthase. The sizes of the molecular weight markers (in kDa) are indicated on the left.

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