Regulation of the Human *MAT2B* Gene Encoding the Regulatory ß Subunit of Methionine Adenosyltransferase, MAT II

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Running Title: The human MAT2B gene and its promoter

Key Words: S-adenosylmethionine, Methionine adenosyltransferase, S-adenosylmethionine synthetase regulatory subunit, promoter activity, transcriptional regulation.

ABBREVIATIONS

The abbreviations used are: MAT, methionine adenosyltransferase; AdoMet, S-adenosylmethionine; PAGE, polyacrylamide gel electrophoresis
Abstract

Methionine adenosyltransferase (MAT) catalyzes the biosynthesis of S-adenosylmethionine (AdoMet), a key molecule in transmethylation reactions and polyamine biosynthesis. The MAT II isozyme consists of a catalytic α2 and a regulatory β subunit. Downregulation of MAT II β subunit expression causes a 6-10 fold increase in intracellular AdoMet levels. To understand the mechanism by which the β subunit expression is regulated, we cloned the \textit{MAT2B} gene, determined its organization, characterized its 5’flanking sequences, and elucidated the in vitro and in vivo regulation of its promoter. Transcription of the \textit{MAT2B} gene initiates at position –203 relative to the translation start site. Promoter deletion analysis defined a minimal promoter between positions +52 and +93 bp, a GC rich region. Inclusion of sequences between –4 and +52 enhanced promoter activity; this was primarily due to a Sp1 recognition site at +9/+15. Inclusion of sequences up to position -115 provided full activity; this was attributed to a TATA at -32. The Sp1 site at position +9 was key for the formation of protein:DNA complexes. Mutation of both the Sp1 site at +9 and the TATA at –32 reduced promoter activity to its minimal level. Supershift assays showed no effect of the anti-Sp1 antibody on complex formation; whereas the anti-Sp3 antibody had a strong effect on protein:DNA complex formation, suggesting that Sp3 is one of the main factors binding to this Sp1 site. Chromatin immunoprecipitation assays supported the involvement of both Sp1 and Sp3 in complexes formed on the \textit{MAT2B} promoter. The data show that the 5’ untranslated sequences play an important role in regulating \textit{MAT2B} gene, and identifies the Sp1 site at +9 as a potential target for modulating \textit{MAT2B} expression, a process that can have a major effect on intracellular AdoMet levels.
Introduction

Methionine adenosyltransferase (MAT) (ATP:L-methionine S-adenosyltransferase) (EC 2.5.1.6) catalyzes the biosynthesis of S-adenosylmethionine (AdoMet); (1,2). AdoMet the major methyl donor in transmethylation reactions, and the propylamine donor in the biosynthesis of polyamines (3-5). Furthermore, AdoMet participates as a co-factor in key metabolic pathways (3-5). Most species studied to date have more than one MAT isozyme (6). In mammals, the two major MAT isozymes are designated MAT I/III and MAT II (7-10). The MAT I/III isozymes are, respectively, a tetramer and dimer of a catalytic α1 subunit that is expressed only in liver (7,11-13). The MAT II isozyme is expressed in all tissues, including the liver (9,14-21). Previous studies from our group have determined that MAT II from human leukemic T and B cells, as well from activated human lymphocytes, is a hetero-oligomer that consists of α2 (53 kDa), α2´ (51 kDa) and β (38 kDa) subunits (15,22). The α2/β hetero-oligomeric composition of MAT II was also determined in bovine brain, Ehrlich's ascites tumor and calf thymus (15,19). The α2 subunit is responsible for the enzyme catalytic activity and is post-translationally modified to generate α2´ (15,22-26). Recently, Halim and LeGros et al. (27) reported the characterization and regulation of the human MAT2A gene, and found it to be remarkably similar to that of the rat and mouse MAT2A genes (28,29) as well as the human MAT1A gene (30), which encodes the liver specific α1 subunit.

In contrast to MAT α subunits, which are highly conserved throughout evolution, the β subunit of MAT II appears to be only present in the mammalian species (15,19). Recently, we cloned and characterized the human MAT II β subunit (26,31), found that it has no catalytic activity, and confirmed that it acts as a regulatory subunit for the enzyme. When β associates with the α subunit it alters its kinetic properties and renders MAT II more susceptible to product inhibition by AdoMet.
Interestingly, the human \( \beta \) subunit can also interact with \( \alpha_1 \) subunit of MAT I/III and the \( E. coli \) \( \alpha \) MAT subunit and alter their kinetic properties as well (26,31).

The expression of the MAT II \( \alpha_2, \alpha_2' \) and \( \beta \) subunits varies considerably in different tissues. The \( \alpha_2, \alpha_2' \) and \( \beta \) subunits are constitutively expressed at high levels in leukemic cells, and at low levels in normal resting T cells. Stimulation of normal human lymphocytes results in marked changes in the level of expression of these subunits. Non-physiological, polyclonal mitogenic stimulation of human T cells induces an increased expression of the \( \alpha_2/\alpha_2' \) subunits, but not the \( \beta \) subunits (22,25). By contrast, physiological stimulation of T cells by bacterial superantigens induces an upregulation of the \( \alpha_2/\alpha_2' \) subunits and a downregulation of the \( \beta \) subunit (25). This results in the formation of \( \alpha_2 \) and \( \alpha_2' \) homo- and/or hetero-oligomers (no \( \beta \)) with a 3-fold higher \( \text{Km} \) for L-Met. The form of MAT II without \( \beta \) is resistant to product inhibition by AdoMet when compared to the form of MAT II found in resting or leukemic T cells (includes \( \beta \)) (25). Importantly, the downregulation of the \( \beta \) subunit in physiologically stimulated T cells was accompanied by a 6-10 fold increase in intracellular AdoMet levels, presumably due to the loss of product inhibition of the enzyme (25). An increase in AdoMet levels is likely to stimulate certain transmethylation reactions catalyzed by methyltransferases with a relatively high \( \text{Km} \) for AdoMet.

Based on our previous results, we hypothesized that the downregulation of MAT II \( \beta \) subunit may be an important event in physiological stimulation of T cells and we sought to characterize the regulation of expression of the \( MAT2B \) gene. Here we report the chromosomal localization and organization of the \( MAT2B \) gene, as well as provide a detailed characterization of the structure and function of its promoter.
**MATERIALS AND METHODS**

*Isolation and genomic organization of the MAT2B gene—* Based on the sequence of the previously reported MAT II β subunit cDNA (31), forward and reverse primers spanning the entire ORF were designed and used to amplify genomic DNA isolated from normal human lymphocytes. Reactions yielding larger than expected products suggested the presence of introns, and these PCR products were cloned and sequenced to verify the authenticity of introns. Sequences at the intron-exon boundaries of the MAT2B gene were determined by aligning the cDNA sequence of MAT2B cDNA with the genomic sequence. A set of primers, GSF (5'-GATTCCTGAGTCCTGTCTTAG) and GSR (5'-GCACTTTTGGCTTTCACTCAG) that amplified a 79bp product which included the 3'-end of intron 4 and the 5'-end of exon 5, were used to screen a human P1 genomic library (Genome Systems Inc. St. Louis, MO). Positive clones were partially sequenced to ascertain the presence of the MAT2B gene. Clone 22646 was selected for further characterization and used to determine the chromosomal location of the MAT2B gene, determine gene organization, verify intron positions, and characterize MAT2B promoter function.

*Chromosomal localization of the human MAT2B subunit gene—* Highly purified DNA was obtained from the MAT2B P1 genomic clone 22646 using the Wizard PureFection DNA purification system (Promega, Madison, WI). The DNA was labeled with digoxigenin dUTP by nick translation, combined with sheared human DNA, and hybridized to normal metaphase chromosomes derived from PHA-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2X SSC. Specific hybridization signals were detected by using fluorescein-conjugated antidigoxigenin antibodies. The chromosomes were counterstained with propidium iodide (DAPI) and analyzed.
Mapping the MAT2B gene transcription start site—Identification of the transcription start site was done by primer extension analysis using poly(A)⁺ RNA prepared from normal human lymphocytes. The primer extension reaction was conducted using the avian myeloblastosis virus reverse transcriptase (AMV-RT) primer extension system (Promega, Madison, WI). Poly(A)⁺ RNA was isolated from 500 ml of human blood by the PolyATract mRNA isolation system (Promega, Madison, WI). Two primers Bra1 5'-GTTCTTTCTCCCTCCCCACCAT-3' (complementary to positions +22 to +1 of the ORF) and primer Bra2 5'-CAGTTCTTTCTCCCTCCCCACC-3' (complementary to positions +24 to +3 of the ORF) were synthesized and end-labeled with γ-³²P ATP & T4 polynucleotide kinase. Free γ-³²P ATP was removed by the QIAquick Nucleotide Removal Kit (Qiagen). Reverse primer extension was carried out using 3 µg mRNA and 5X10⁵ cpm of the labeled primer. The M13mp18 sequencing ladder was prepared by fmol Sequencing (Promega), and run along side the samples on 8% urea/polyacrylamide gels as a size marker.

Cloning of the human MAT2B gene promoter—We cloned and sequenced 3.5 kbp of the 5'-flanking DNA of the human MAT2B gene. The promoter was contained within 1.1 kbp. Primers (5'WP; 5'-GCTCGAGTAAGATGATCTTGGC) and 3'WP (5'-GAAGCTTGCCCGCTTCAC) were designed to amplify the region –998/+204 with respect to the transcription start site. The primers introduced a XhoI site at the 5' end and a HindIII site at the 3' end of the cloned promoter. The Pfu-amplified fragment was cloned into the pGEM-TEasy vector (Promega, Madison, WI) and the cloned MAT2B promoter was sequenced in both directions with overlapping segments to verify the sequence and confirm the integrity of the cloned promoter. The cloned promoter DNA was excised from the pGEM-TEasy vector by digestion with XhoI and HindIII (Promega, Madison, WI), purified, and processed as described below.
Generation of Luciferase Reporter Constructs of the MAT2B Promoter— A 1.1-Kbp Xho I/Hind III digested fragment of the MAT2B gene containing the 5’ flanking region starting at position +204 from the transcription start site was cloned upstream of the firefly luciferase reporter gene in the pGL3-Enhancer vector (Promega, Madison, WI). Directional insertion was verified by restriction digestion and by sequencing the clone from both directions. Subsequent deletion constructs were generated by PCR using sequence specific primers (Table I), containing the restriction sites Xho I/Hind III as described above. The purified PCR products were cloned into the pGEM-TEasy vector (Promega, Madison, WI), and sequenced for verification. The cloned deletion constructs were excised from the pGEM-TEasy vector using Xho I/Hind III and then cloned upstream from the firefly luciferase gene into the pGL3-Enhancer vector (Promega, Madison, WI). All constructs were verified by sequence analysis.

In vivo analysis of the MAT2B Promoter Activity—The functional expression of the pGL3-MAT2B promoter deletion constructs was analyzed in Cos-1 and Jurkat T cells as detailed elsewhere (27).

In Vitro Analysis of the MAT2B Promoter Activity By Electrophoretic Mobility Shift (EMSA) And Supershift Assays— Double-stranded oligonucleotide probes were generated by PCR amplification with 32P-end-labeled primers. The amplified DNA representing specific regions of the proximal promoter of the MAT2B gene were generated using the primers listed in Table I. Jurkat cell nuclear extracts were prepared as described previously (27). The binding reactions were performed in 20 µl final volume by incubating 60 fmol (∼ 50,000 cpm) of 32P-labeled probe with 5 µg of crude nuclear extracts from Jurkat cells in the presence of 2 µg of poly(dI-dC), 1 µg salmon sperm DNA in 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol and 6 µg BSA for 40 min on ice. The reactions were conducted in the absence or presence of specific or non-specific competitors, both added at a 100-fold molar excess. Specific competitors were cold probes; non-specific competitors were unlabeled PCR amplification products of an unrelated DNA sequence, free of sites of interest, and similar
in size to specific competitor. An Sp1-specific competitor was purchased Santa Cruz Biotechnology Inc., CA (catalogue # SC 2502). The DNA-protein complexes formed were analyzed by electrophoresis on nondenaturing 4% polyacrylamide gels. The gels were pre-run for 1 h at 100 V, and electrophoresis was conducted at 30 mAmp constant current.

Supershift assays were performed using antibodies (Abs) to specific factors that have corresponding recognition elements within the MAT2B promoter region analyzed. Binding reactions were carried out as described above in the absence, or in the presence of 2 µg Ab specific to one of the transcription factors of interest. The Ab was either premixed with the nuclear extract, incubated for 30 min at room temperature prior to addition of radiolabeled probe, or added after the binding reaction was completed and incubated for 30 min on ice. The following Abs (Santa Cruz Biotechnology Inc., CA) were used in supershift analyses: rabbit polyclonal anti-Sp1 (SC-59X), anti-Sp2 (catalogue # sc-643X), anti-Sp3 (catalogue # sc-644X), anti-Sp4 (catalogue # sc-645X), and anti-NF1 mAb (SC-870X).

**Chromatin Immunoprecipitation**— Cross-linking between transcription factors and chromatin was achieved in Jurkat cells following the method described by Yang et al. (32). Briefly, formaldehyde was added to cells at a final concentration of 1% for 10 min, and 0.125 M glycine was used to stop the reaction. The cells were washed three times with cold PBS, once with PBS containing 1 mM phenylmethylsulfonyl fluoride, then lysed in 2 ml of cell lysis buffer (5 mM Pipes-KOH, pH 8.0, 85 mM KCl, 0.5% (v/v) Nonidet P-40) in the presence of protease inhibitors (100 ng/ml leupeptin, 100 ng/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were homogenized, and the nuclei recovered by centrifugation at 250 xg for 10 min and resuspended in 0.2 ml of nuclear lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% (w/v) SDS plus the protease inhibitors. The lysate was sonicated to shear the chromatin to an average length of < 2 kb. Samples were diluted 10-fold with the immunoprecipitation dilution buffer (1% v/v Triton X-100, 16.7 mM Tris, pH 8, 1.2 mM EDTA, 167 mM NaCl plus the protease inhibitors). A 240 µl slurry of salmon sperm DNA/protein A-agarose
(Upstate Biotechnology, Inc., Lake Placid, NY) was added to reduce nonspecific binding, and the mixture was rotated for 1 h at 4 °C, then centrifuged at 500 x g for 1 min. Precleared chromatin solutions were incubated with antibody to Sp1 (10 µg), Sp3 (10 µg) (Santa Cruz Biotechnology) or with no antibody (negative control) and rotated at 4 °C for 12 h. Immune complexes were collected by adding 80 µl of salmon sperm DNA/protein A-agarose slurry for 4 h with rotation. Samples were washed four times with 1 ml of wash buffer (0.1% (v/v) Triton X-100, 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA) and the immunoprecipitated material was eluted by three successive 5 min incubations with 150 µl of elution buffer (1% (w/v) SDS, 50 mM NaHCO3). To reverse the formaldehyde-induced cross-linking, the eluates were pooled, NaCl was added at a final concentration of 0.3 M and the samples were incubated at 65 °C for 4 h. This was followed by digestion in 10 µl of 2 M Tris, pH 6.8, 10 µl of 0.5 M EDTA, 2 µl of proteinase K (20 mg/ml). The samples were incubated for 2 h at 45 °C and then the DNA was extracted with phenol/CHCl3 followed by ethanol precipitation, resuspension in 50 µl of sterile H2O. Five ul were used as a template in PCR analysis. Primers were designed to amplify the regions from -174 or -4 to + 52. Primer 5’BP8 (5’-ACTCGAGAAACTCAAGGCGATCCACTT) or primer 5’ BP9 (5’-CCTCGAGACCGCGTGACC) were paired with primer 3’BP8 (5’-TCTGCCCCCAGCCCACG). The PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining.

**In vivo and in vitro Analysis of the Effect of MAT2B Promoter Mutations**—Several mutations were introduced into clones pGL3-MAT2B(-174)-LUC pGL3-MAT2B(-115)-LUC and pGL3-MAT2B(-4)-LUC according to a modification of the method of Wang et al. (33) that targets putative factor recognition sites. Mutation of the TBF site at -111/-105 was generated using primers 5’MU1 (5’-GCTCGAGAATAAACAAGCACTCAAATAAAATCTCC) and 3’MU1 (5’-GAGATTTATTTTGAGTTGCTTGTTATTTCTCGAGCC). Another possible TBF site at -95/-91 was mutated using primers 5’MU2 (5’-GCACCTCAAATACCATCTCCGAAACAAAACCTG) and 3’MU2 (5’-CAGGTTTTGTTTCGGAGATGTTATTTGAGTC). The TATA box at -32/-28 was mutated with
oligos 5'MU3 (5'-CTTTTGTGTGTCGCTTTTTGCATCGGCGCGTG) and 3'MU3 (5'-CACGCGCCGATGCAAAAAGCGACACACACAAAAAG). Mutation of the Sp1 site at position +9/+15 was generated with primers 5'MU2 (5'-CAGACCGCGCGTACCTTACCTCTTCTTCTTG) and 3'MU2 (5'-CCAGAAAGAGGTACGCGCGGTCTG). The second Sp1 site at +23/+28 was mutated using primers 5'MU5 (5'-CCTCTTTCTGGGTTTTCGGCGGAGCGTGGC) and 3'MU5 (5'-GCCACGCTCCGGCAGAAAAACCAGAAAGAGG).

Site directed mutagenesis was performed on pGL3-\textit{MAT2B} promoter constructs by PCR using \textit{pfu Turbo} DNA polymerase (Stratagene) and the oligos listed above, following the method of Wang et al. (33). Briefly, following the PCR reaction, the product was incubated at 37\textdegree C with \textit{Dpn} I (Promega) to remove the methylated template DNA. A portion of the digested reaction mix was analyzed on a 1\% agarose gel to verify the PCR product size. \textit{E. coli} strain JM109 was transformed using 5µl of the PCR-amplified vector and six clones of each transformation were sequenced to confirm the presence of the desired mutations. The mutated DNA products were excised from the pGL3 vector using \textit{Xho} I and \textit{Hind} III, and individually re-cloned into an unamplified pGL3-Enhancer vector (Promega) to ensure that the vector itself was not modified during the mutagenesis reaction. The in vitro and in vivo activity of each mutant construct was determined as described above. EMSA probes –115/-4 and -4/+52 were generated using the mutated pGL3-\textit{MAT2B}(115)-LUC clones as templates and external primers 3'BP8 (5'-TCTGCCCCCAGGCCACG) and 3'BP9 (5'-GTTGATTGGCCACGCCTCC). Results from only those mutations that affected promoter activity are described below.
RESULTS

**Genomic organization of the MAT2B gene**— Human genomic clone 22646 was determined to harbor the MAT2B gene and a significant portion of its 5’ flanking sequence. A series of primers were designed, based on the known MAT2B cDNA sequence, to determine the structure of the MAT2B gene. The gene consisted of seven exons interrupted by six introns spanning ~6.8kbp of genomic DNA (Fig. 1). The size and locations of the various exon and introns as well as the donor and acceptor sequence are summarized in the inset Table. All boundaries were found to conform to the GT-AG rule (34). Exon 1 contained 203 bp of 5' noncoding region and 63 bp of coding sequence. Exon seven contained 171 bp of coding sequence and 802 bp of 3' untranslated sequence.

**Chromosomal localization of the human MAT2B subunit gene**—A total of 80 metaphase cells were analyzed as detailed in Methods; 69 of those exhibited specific labeling with DNA from clone 22646 on chromosome 5. An anonymous genomic probe, previously mapped to 5q22 and confirmed by cohybridization with a probe from the cri du chat locus, hybridized to the same chromosome as clone 22646, confirming the location as the long arm of chromosome 5. Ten individual measurements of specifically labeled chromosome 5 demonstrated that the 22646 clone hybridized to a position 89% of the distance from the centromere to the telomere of 5q, an area which corresponded to the interface between bands 5q34 and 5q35.1 (data not shown).

**Mapping the MAT2B gene transcription start site**—The transcription start site was identified by primer extension analysis. Two primers, Bra1 and Bra2, corresponding to +22 and +24 of the MAT2B ORF were annealed to mRNA prepared from normal human lymphocytes and extended in the presence of AMV reverse transcriptase as described in Materials and Methods. Primer Bra 2 yielded
a 227bp product and Bra1 a 225bp product (Fig. 2). Therefore, transcription was shown to start 203bp upstream of the *MAT2B* translation start site.

Cloning and sequencing of the human *MAT2B* gene promoter — The sequence of ~1.1 kbp of the 5’ flanking region of the *MAT2B* gene is shown in Fig. 3. The sequence from -15 to +203 in the 5’ flanking region is high in GC content with clusters of overlapping Sp1 sites (Fig. 3). A TATA box is located 32 bp upstream from the transcription start site.

In vivo analysis of the *MAT2B* Promoter Activity — Series of promoter deletion constructs coupled to a firefly luciferase reporter gene in a pGL3 Enhancer vector were generated as described in Materials and Methods and used to analyze functional expression in Cos-1 and Jurkat human leukemic T cells. There was little difference in the pattern of expression of pGL3-*MAT2B* promoter constructs in both types of cells (data not shown). Successive deletions from –998 to –115 of the *MAT2B* promoter had little effect on promoter activity; however, further deletions resulted in a gradual decrease of promoter activity (Fig. 4). Little to no promoter activity was seen when only the region from +93 to +204 was included in the construct. The region from +52 to +93 provided minimal promoter activity and the inclusion of sequences between –4 and +52 significantly increased activity. This indicated that the 5’noncoding sequences of *MAT2B* are contributing to promoter function. Another significant enhancement in promoter activity was seen when sequences between position –115 and –4 were also included. Functional studies described below indicated that this enhancement was conferred by the inclusion of a TATA sequence at position –32/-28. No further enhancement was observed when additional upstream sequences were included. Together the data indicate that the sequences between +52 and +93 provide minimal promoter activity, whereas sequences between –115 to +93 provide the full promoter activity.
Identification of functional sites in the MAT2B Promoter Activity By Electrophoretic Mobility Shift (EMSA), Supershift Assays—Analysis of the proximal MAT2B promoter sequence identified several putative recognition sites for known transcription factors. These regions were subjected to further functional analysis using EMSA, supershift assays, and mutation of specific sites. Competition experiments showed that the complexes which formed on a probe representing the region from –115 to –4 were non-specific (Fig. 5B). By contrast, strong and specific complexes were formed on a probe representing the region from –4 to +52 (Fig. 5C). Strong complexes were also formed on the region from +52 to +93 which is high GC rich. These complexes were partially competed off with the non-radioactive probe covering the same sequence (Fig. 5D).

The region from –4 to +93 has several Sp1 and NF1 sites; however, neither the Sp1 nor the NF1 antibodies caused supershift of the complexes. Although the anti-Sp2 antibody induced a slight supershift, the anti-Sp3 antibody had the strongest effect causing the complete disappearance of complex II (Fig. 6A and B). The data suggest that Sp3 is one of the main factors involved in complex formation in this region; other members of the Sp1 family may also be involved in protein:DNA interaction on this region of the MAT2B promoter.

Chromatin immunoprecipitation studies showed that both the anti-Sp1 and anti-Sp3 antibodies were independently able to pull down the MAT2B promoter because PCR products were obtained in reactions with primers covering the regions from –174/-4 and –4/+52 (Fig. 6C). Thus, even though Sp3 appears to be a major factor that binds to the proximal MAT2B promoter, the binding of Sp1 to this site in vivo cannot be ruled out.

Effect of Mutating Specific Sites in the MAT2B Promoter on the In Vitro Promoter Activity—Several mutations were also made to probes covering the region from –115/+93. Mutation of the Sp1 site at position +9 completely abolished protein:DNA complex formation on probes representing sequences
from –115 to +52 or from –4 to +52 (Fig. 7). By contrast mutation of other Sp1 sites in the region from –115 to +52 had no effect on complex formation. Similarly, mutation of the three TBF sites located between –115 and –4 had no effect on complex formation. Together the data suggest that the Sp1 site at +9 is key for protein:DNA interaction on this region of the promoter. The sequence from +52 to +93 is too heavily GC rich to mutate in a meaningful way.

**Effect of Mutating Specific Sites in the MAT2B Promoter on the In Vivo Promoter Activity**—The effect of mutating several putative factor recognition sites located in the region between –115 and +52 on the in vivo activity of the MAT2B promoter was tested; however, only two mutations affected promoter activity (Fig. 8). The TATA at –32 and the Sp1 site at +9 were individually or simultaneously mutated on the pGL3-MAT2B(-115)-Luc or the pGL3-MAT2B(-4)-Luc reporter construct. Mutation of the Sp1 site at position +9 reduced promoter activity by 35-50%; whereas mutation of the other Sp1 sites located in the region from –115/+52 had no effect on activity. Mutation of the TATA at –32 reduced in vivo activity of the MAT2B promoter by only 25%. However, when both the TATA at –32 and the Sp1 site at +9 were simultaneously mutated, promoter activity was reduced by almost 60%, reaching a level that is comparable to that driven by the GC rich sequence from +52 to +93. Together the data indicate that GC rich sequences in the region from +52 to +93 can drive MAT2B gene expression up to 25-30% of it full activity, while the presence of the Sp1 site at +9/+15 and the TATA sequence at –32/-28 are required for 100% activity.
DISCUSSION

The MAT II isozyme is expressed in all tissues where it is found as an oligomer that comprises catalytic α2 (53kDa) and α2` (53kDa) subunits complexed with the β regulatory subunit (38-kDa) (15,22,31). The β subunit lowers the K_m of the enzyme for L-Met and confers susceptibility to product inhibition by AdoMet (26,31). Interestingly, we have not been able to detect the β protein in *E.coli* or yeast extracts (26,35), despite the presence of high level of homology between this protein and enzymes that catalyze the reduction of TDP-linked sugars in bacteria (36). Therefore, the unique role and significance of the β protein in mammalian cells represents an intriguing area of study, particularly since β is differentially expressed in normal and leukemic T cells and subsequently affects AdoMet levels.

In leukemic T cells both the α2 and β subunits of MAT II are constitutively expressed at a high level. Non-physiological, polyclonal mitogenic stimulation of primary human lymphocytes induces an increased expression of MAT II α2 subunit only; whereas physiological stimulation via the T cell receptor results in a downregulation of the β subunit (25). This is accompanied by 6-10 fold increase in AdoMet levels (25). Thus the pattern of expression of the *MAT2B* gene may be an important mechanism for regulating intracellular levels of AdoMet.

To shed light on the mechanisms underlying differences in MAT II β subunit expression in different cells, we cloned and characterized the *MAT2B* gene and its 5' flanking sequence. Promoter activity was very similar in Cos-1 cells and Jurkat human leukemic cells. This is consistent with our previous observations that the β subunit is constitutively expressed in both cell types (37). Minimal promoter activity is contained between position +52 and +93 which is rich in GC content. However, full promoter activity was achieved when sequences from –115 to +52 were included. The Sp1 site
located within the 5’noncoding region of the gene (+9/+15) appears to play a key role in this
enhancement inasmuch as mutation of this site abolished DNA:protein interactions and significantly
reduced promoter activity in vivo. It is possible that the GC rich region of the proximal promoter
drive the residual activity when this Sp1 site is mutated. Further enhancement of \textit{MAT2B} promoter
activity is conferred by the TATA at –32/-28. When the Sp1 at +9 and the TATA at –32 are
simultaneously mutated, promoter activity is reduced by 60-70%.

The transcription factor Sp3 seems to bind to the Sp1 site at +9/+15, although we cannot rule out that
Sp1 and Sp2 are also part of the complexes that form on this site. That Sp3 is involved in regulating
\textit{MAT2B} promoter activity is particularly interesting in light of our recent studies that showed that this
transcription factor plays a key role in regulating the \textit{MAT2A} gene, which encodes the regulatory
subunit of the same enzyme. As mentioned above, stimulation of T cells with a physiological
stimulus induces the expression of \(\alpha 2\) and downregulates the expression of the \(\beta\) subunit; whereas in
leukemic T cells both subunits are expressed at a high level (22,37). Sp3 is a bifunctional protein that
can both activate and repress transcription of genes (38,39). Internal isofroms of this protein
containing activation and/or repressor domains have been described (40). It is conceivable that Sp3
may enhance \textit{MAT2A} and \textit{MAT2B} gene expression in leukemic T cells while enhancing \textit{MAT2A} and
suppressing \textit{MAT2B} in normal T cells. Studies of the role of Sp1 family of transcription factors in
regulating MAT II \(\alpha 2\) and \(\beta\) subunits expression in normal and leukemic T cells are ongoing in our
laboratory. The identification of an Sp1 site on the promoter for both subunits that is key for driving
promoter activity puts us closer to our goal to elucidate the differential regulation of MAT II subunits
in normal and leukemic T cells. Achieving this goal will allow us to design targeted therapeutic
strategies for potentiating intracellular AdoMet levels that may lead to the control of malignant
growth.
FOOTNOTES

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Figure Legends

Fig. 1. Structural organization of the human MAT2B gene. The MAT2A gene consists of seven exons and six introns. Shaded boxes represent the open reading frame of the MAT II ß subunit protein. The length of each exon in nucleotide pairs is denoted by the numbers in the boxes. Asterisks mark the translation initiation and termination sites. The inset table indicated exact location of exons and introns and the sequence at each Exon/Intron junction.

Fig. 2. Transcription initiation of the human MAT2B gene as determined by primer extension analysis. Two primers starting at MAT2B cDNA sequence +22 (Bra-1) and +24 (Bra-2) with respect to the translation initiation site were annealed to human lymphocyte poly (A)* RNA and extended in the presence of AMV reverse transcriptase. Extension products of 225 and 227 bases in length were observed, indicating that transcription starts 203 bases upstream of the translation start site. An M13mp18 sequencing reaction was used as a size marker.

Fig. 3. Sequence of the 5′ flanking region of the human MAT2B gene. 1.1 Kb of the MAT2B gene 5′ flanking sequence is depicted. The transcription start site is marked with an arrow. Putative sites for known transcription factors are underlined.

Fig. 4. In vivo activity of the human MAT2B gene promoter constructs. A series of MAT2B promoter deletion constructs fused to the firefly luciferase reporter were transfected into Cos-1 cells and luciferase activity was assayed by the dual luciferase reporter assay system as described in Materials and Methods. The data was calculated in RLU and expressed as percentage of the pGL3-MAT2B(-998) construct, which was set at 100% activity. Data presented are mean ± SEM of at least 4 separate experiments, each
performed in triplicate. An almost identical pattern was seen when the pGL3-MAT2B constructs were transfected into the Jurkat human leukemia cell line by the method previously described (27).

**Fig. 5.** In vitro analysis of the MAT2B proximal promoter by EMSA. Probes used for EMSA and supershift analysis with Jurkat nuclear cell extracts are indicated and only those that show relevant patterns are shown. For each autoradiogram, lane 1 is probe alone, lane 2 is probe plus Jurkat nuclear cell extract, and lane 3 is probe and Jurkat nuclear cell extract plus 100-fold molar excess of the relevant nonradioactive probe.

**Fig. 6.** Involvement of different members of the Sp1 family of transcription factors in protein:DNA complexes formed on the MAT2B promoter. Panels A and B show the pattern of supershift with antibodies to NF1 and different members of the Sp1 family of transcription factors. Lane 1 is probe alone, lane 2 is probe plus Jurkat nuclear cell extract, and lanes 3-6 are supershift with anti-Sp1, -Sp2, -Sp3, and -Sp4 Abs, respectively. Lane 7 is supershift with anti-NF1 antibody. Complexes are designated I and II in order of mobility.

Panel C shows the in vivo interaction of Sp1 and Sp3 with the MAT2B promoter using chromatin immunoprecipitation. Shown is an ethidium bromide-stained 1.5% agarose gel showing PCR analysis of complexes immunoprecipitated with Sp1 or Sp3 antibodies. PCR was performed with the immunoprecipitates as described in Materials and Methods. Lane a is a positive control for PCR reaction and represents amplification with primers for the region from –174 to –4, using the pGL3-MAT2B plasmid as template; lane b is a negative control consisting of samples processed through the CHIP protocol, without the primary antibody; lanes c and d are samples processed through the CHIP protocol, with either an Sp1 or an Sp3 antibody. Primers were designed to amplify the regions from –174 or -4 to + 52.
Fig. 7. Effect of mutating specific factor recognition sites on MAT2B promoter on EMSA. The position of each mutation is indicated by underlines and the resultant sequence is shown in the inset table. Indicated wild type(WT) or mutated (Mu) probes were incubated with Jurkat nuclear cell extract and subjected to EMSA analysis as detailed in Fig. 5. The data show the dramatic effect of mutating the Sp1 site at +9/+15.

Fig. 8. Effect of mutating specific factor recognition sites on MAT2B promoter on in vivo activity. The position of each mutation is indicated in the line diagram. Reporter pGL3-MAT2B(-115)-Luc or pGL3-MAT2B(-4)-Luc constructs with and without TATA and/or Sp1 mutations were transfected into Cos-1 cells and assayed for luciferase activity as described in the legend to Fig. 4. The activity of the indicated wild type(WT) or mutated (Mu) constructs is expressed as percent of respective WT construct activity. The data are from at least three experiments, performed in triplicate.
Human Lymphocyte $MAT2B$ Gene

Exons and Introns

| Exon | Number | Size (bp)          | Intron | Number | Size (bp) | Amino Acid Interrupted | Type |
|------|--------|-------------------|--------|--------|-----------|------------------------|------|
| 1    | 5’NC(205) + 63 |                      | I      | 619    | Glu/Glu22     | 0          |
| 2    | 189    |                      | II     | 1068   | Gln/Pro85     | 0          |
| 3    | 115    |                      | III    | 155    | Ala123        | 1          |
| 4    | 153    |                      | IV     | 1730   | Gly174        | 1          |
| 5    | 194    |                      | V      | 753    | Leu/Asp239    | 0          |
| 6    | 114    |                      | VI     | 437    | Pro/Ile277    | 0          |
| 7    | 171 + 3’NC (802) |                  |        |        |             |            |

Sequence at Exon/Intron Junction

| 5’ Donor Exon | Intron | 3’ Acceptor Exon |
|---------------|--------|------------------|
| TGGTGGAG      | gtgaggga---ccttttag | GAGGAAGT |
| ATTTTCAG      | gtattgat---actcttag | CCCCATGT |
| GGAAGCAG      | gtaatgat---tttttgtag | CTGCTGTT |
| CAATCTAG      | gttaagacc---ttctctag | GAGCTGCT |
| GAATGCTG      | gttaagaag---gttcttag | GATCCATC |
| TAAGACCT      | gttaagtac---cttttaag | ATTACTGA |
Probe (-4/+52)  

Probe (+52/+93)  

PCR Analysis of CHIP
LeGros/Halim et al. _____

-115  AGAATAAAAAGCACTCAAATAAAATCTCCGAAACAAAAACCTGAATTCACTGCCTAAGG

TCAGGGCCTTTTTTTTGTTGTCGCTTTTAAAGCATCGGCCTGCGCTGGGCTGGGGGCAGACCCG

Sp1  Sp1
CGTACCCGCCCCTTTTTCTGGGGCGTGGCCGGAGCTGGCAGCCAATCAACG  +52

| SITE | FORM | LOCATION/SEQUENCE |
|------|------|-------------------|
| TATA | WT   | -32 TTTAA -28     |
|      | Mu   | -32 TTTTT -28     |
| Sp1  | WT   | +9 CCCGCC +15      |
|      | Mu   | +9 CCTTACC +15     |
| Sp1  | WT   | +23 GGGGCG +28     |
|      | Mu   | +23 GGGTTT +28     |

A

B

WT  Sp1  TATA
P NE  P NE  P NE

WT  Sp1  Sp1
P NE  P NE  P NE

-115/+52

-4/+52
Regulation of the human MAT2B gene encoding the regulatory beta subunit of methionine adenosyltransferase, MAT II
Leighton LeGros, Abdel-Baset Halim, Margaret E. Chamberlin, Arthur Geller and Malak Kotb

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