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DNA methylation and histone acetylation of rat methionine adenosyltransferase 1A and 2A genes is tissue-specific ☆

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Abstract

Methionine adenosyltransferase (MAT) catalyzes the biosynthesis of *S*-adenosylmethionine (AdoMet). In mammals MAT activity derives from two separate genes which display a tissue-specific pattern of expression. While *MAT1A* is expressed only in the adult liver, *MAT2A* is expressed in non-hepatic tissues. The mechanisms behind the selective expression of these two genes are not fully understood. In the present report we have evaluated *MAT1A* and *MAT2A* methylation in liver and in other tissues, such as kidney, by methylation-sensitive restriction enzyme digestion of genomic DNA. Our data indicate that *MAT1A* is hypomethylated in liver and hypermethylated in non-expressing tissues. The opposite situation is found for *MAT2A*. Additionally, histones associated to *MAT1A* and *MAT2A* genes showed enhanced levels of acetylation in expressing tissues (two-fold for *MAT1A* and 3.5-fold for *MAT2A* liver and kidney respectively). These observations support a role for chromatin structure and its modification in the tissue-specific expression of both MAT genes.

Keywords: Methionine adenosyltransferase; Gene expression; DNA methylation; Histone acetylation; Anti-acetylated H4

1. Introduction

Methionine adenosyltransferase (MAT; ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) catalyzes the biosynthesis of AdoMet from meth-

☆ LT and GLR made equal contribution to this work.

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ionine and ATP [1]. AdoMet is the major methyl group donor in transmethylation reactions and serves as the propylamino source in the biosynthesis of polyamines [2]. In mammals, this essential enzyme is the product of two different genes, *MAT1A* and *MAT2A*, which display a distinct pattern of expression among different tissues [2,3]. *MAT1A* is the predominant enzyme in liver parenchymal cells, while *MAT2A* is expressed in all other tissues, in the fetal liver and replaces *MAT1A* in hepatocarcinoma cells [4–6]. The existence of a liver-specific enzyme can be explained from a kinetic and regulatory perspective by the main role played by this organ in dietary methionine metabolism, a function for which the product of *MAT1A* is better suited [2,4,7].

Rat *MAT1A* and *MAT2A* promoters have been cloned, sequenced, and functionally characterized [8,9]. However the mechanisms responsible for the tissue-specific expression of these two genes cannot be completely explained by the presence of cell-type-specific promoter elements, or by the action of tissue-specific transcription factors [8,9]. An additional level of information, which can influence gene expression is conveyed by DNA methylation at CpG sites [10–12]. Gene-specific methylation patterns have been inversely correlated with gene activity. In this regard, DNA methylation may interfere with the binding of transcription factors and has been associated with the induction of a repressed state of the chromatin [10,12]. In addition to DNA methylation, core histone acetylation, a reversible post-translational modification, has been associated with the transcriptional competence of the gene [13,14]. Furthermore, the discovery that transcriptional activators have histone acetyl transferase activity stimulated renewed attention to the role of chromatin structure and histone acetylation in gene regulation [15,16]. Recently these two mechanisms, DNA methylation and chromatin acetylation, have been linked together by the demonstration that genomic methylation patterns can influence histone acetylation, and that the degree of histone acetylation can change the structure of the nucleosome and thus modulate chromatin compaction [14,17,18]. In order to gain more insight into the mechanisms behind *MAT1A* and *MAT2A* tissue-specific expression we have analyzed the methylation pattern of both genes and the acetylation status of histones associated with these two loci in different rat tissues.

2. Materials and methods

2.1. Northern blot analysis

Total RNA from liver, kidney, spleen and heart tissues was obtained by the guanidinium thiocyanate method [19]. Aliquots (20 μg) of total RNA were size fractionated by electrophoresis in agarose gels under denaturing conditions, blotted and fixed on Nytran (Schleicher and Schuell) membranes using standard procedures. Blots were prehybridized and hybridized as described [20]. Rat *MAT1A* full length cDNA [21] and a 1147 base pairs cDNA fragment of rat *MAT2A* [5,22] were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Pharmacia Biotech) by random priming using the Megaprime DNA labeling system (Amersham). The amount and quality of the loaded RNA samples were evaluated by ethidium bromide staining of the gels. Membranes were then exposed to X-ray films.

2.2. DNA isolation, methylation analysis and Southern blot

Genomic DNA from different rat tissues was isolated as described previously [23]. Aliquots of high molecular weight DNA (20 μg) were digested to completion with 5 U/μg *Msp*I or *Hpa*II restriction enzymes (Boehringer Mannheim) by incubation at 37°C for a total of 6 h (enzyme was added in two aliquots at 3 h intervals). Samples were electrophoresed on 1% agarose gels and then transferred to Nytran membranes. Prehybridization and hybridization with *MAT1A* and *MAT2A* cDNA probes were carried out as described [23].

2.3. Nuclei isolation

Liver and kidney from Wistar rats were placed in buffer A (0.25 M sucrose, 10 mM NaCl, 5 mM

$MgCl_2$, 2 mM EDTA, 0.2% Triton X-100, 2 mM *p*-aminobenzamidine, 1 mM PMSF, 2.5 μg/mL leupeptin, 1.5 μg/mL chymostatin, 50 mM Tris–HCl, pH 7.5) at a ratio of 5 ml buffer per gram of tissue. All the steps were carried out at 4°C. Tissues were homogenized on ice and after filtration of the homogenates through 200 and 100 μm nylon filters, nuclei were collected by centrifugation at 1500 ***g*** for 5 min and the sediment was washed twice in buffer B (buffer A without Triton and EDTA). Nuclear pellets were resuspended in the same buffer and placed on a discontinuous sucrose gradient (0.75 M/1.75 M) in buffer B. After centrifugation at 10,000 ***g*** for 30 min, nuclei were collected from the 1.75 M sucrose cushion and washed twice with buffer B.

2.4. Micrococcal nuclease digestion

Nuclei were resuspended in digestion buffer (0.25 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM sodium butyrate, 2 mM *p*-aminobenzamidine, 1 mM PMSF, 2.5 μg/mL leupeptin, 1.5 μg/mL chymostatin, 50 mM Tris–HCl, pH 7.5) at a concentration of 5 mg DNA per ml. Nuclei from the liver were digested with micrococcal nuclease (Worthington) at 15 U per mg DNA and nuclei from kidney with 20 U per mg DNA for 10 min at 37°C. The digestion was stopped by adding EDTA to a final concentration of 5 mM and the samples were cooled on ice and centrifuged at 12,000 ***g*** for 10 min. The supernatant was saved and the pellet was resuspended in lysis buffer (0.25 mM EDTA, 10 mM sodium butyrate, 2 mM *p*-aminobenzamidine, 1 mM PMSF, 2.5 μg/mL leupeptin, 1.5 μg/mL chymostatin, 50 mM Tris–HCl, pH 7.5) incubated for 10 min on ice and recentrifuged as above. The first and second supernatants were pooled and analyzed on agarose gels to determine the extent of the micrococcal digestion. Typically, mononucleosomes account for 40% of micrococcal digestion products.

2.5. Production of antisera to hyperacetylated histone H4 and antibody purification

Polyclonal antisera to hyperacetylated H4 were raised by rabbit immunization with a synthetic peptide corresponding to residues 3–18 of histone H4 and acetylated in the ε-amino groups of lysines 5, 8, 12 and 16. The acetylated peptide was custom-synthesized by Genosys and coupled to Keyhole Limpet Hemocyanin [24]. The IgG fraction was purified from the serum by HiTrap Protein-A FPLC chromatography according to the manufacturer (Amersham Pharmacia Biotech).

The specific recognition of acetylated histones by this antibody was tested in two different assays. First by the specific interaction of the antibody with in vitro acetylated histones blotted onto nitrocellulose filters, and the lack of reactivity towards the same amounts of non-acetylated histones. Second, by the ability to specifically immunoprecipitate acetylated histones from a mixture of acetylated and non-acetylated histones, under the same conditions used for the immunofractionation of nucleosomes described below.

2.6. Immunofractionation of nucleosomes

Immunofractionation of nucleosomes obtained by micrococcal nuclease digestion of nuclei was performed essentially as previously described [25]. FPLC-purified antibody (500 μg of IgG) dissolved in fractionation buffer (50 mM NaCl, 10 mM sodium butyrate, 1 mM EDTA, 0.2 mM *p*-aminobenzamidine, 0.1 mM PMSF, 2.5 μg/mL leupeptin, 1.5 μg/mL chymostatin, 10 mM Tris–HCl, pH 7.5) was incubated with 100 mg of protein A Sepharose prewashed with fractionation buffer. The incubation of the antibody with protein A Sepharose was carried out at 4°C for 3 h under gentle rotation. The protein A Sepharose bound-antibody was mixed with 2 mg of soluble chromatin in a final volume of 3 ml of fractionation buffer and incubated overnight with gentle rotation at 4°C. The immunocomplexes were collected by centrifugation (6500 ***g***, 1 min) on a 0.65 μm Ultrafree filter unit (Millipore) and the filtered through fraction was removed and stored on ice. Protein A Sepharose beads were washed eight times with 500 μl of fractionation buffer as before and filtrates were pooled together (unbound fraction). Antibody-bound fraction

was eluted from protein A Sepharose by addition of 400 μl of 1.5% SDS in fractionation buffer and rotated for 15 min at room temperature. After centrifugation as before, the eluate was saved and protein A Sepharose was reincubated for another 15 min period with 400 μl of 0.5% SDS in fractionation buffer. The two eluates (bound fraction) were pooled. DNA from all chromatin fractions (input, unbound and bound) was purified and quantitated by fluorescence with PicoGreen dye (Molecular Probes).

2.7. Slot-blot and hybridization analysis

DNA samples were denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min at 37°C followed by 1 min at 95°C and then kept on ice. DNA from each fraction (250 ng) were loaded through a slot–blot manifold (BioRad) on a Biodyne B membrane (Pall) prewetted in 2× SSC. The filters were immersed in denaturation solution for 5 min, neutralized in 1.5 M NaCl, 1 mM EDTA, 0.5 M Tris–HCl, pH 7.5 for 30 s and the membranes were dried by baking at 80°C for 30 min. Hybridizations were carried out using *MAT1A* or *MAT2A* cDNA probes mentioned above. The probes were labeled by random priming. Filters were prehybridized for 2 h and hybrydized overnight using Quick Hyb solution (Stratagene) at 65°C in the presence of 200 μg of heat-denatured salmon sperm DNA. Filters were rinsed in 2× SSC, 0.1% SDS and washed sequentially twice with 2× SSC, 0.1% SDS at 65°C for 5 min, once with 2× SSC, 0.1% SDS at 65°C for 30 min and once with 0.2× SSC, 0.1% SDS at 65°C for 20 min. The radioactivity present in the filters was initially measured with an InstantImager (Packard) and the filters were then autoradiographed.

3. Results and discussion

Digestion of genomic DNA using the methylation-sensitive restriction isoschizomers *Msp*I and *Hpa*II, and subsequent Southern blot analysis allowed us to evaluate the methylation status of *MAT1A* and *MAT2A* in different tissues [26]. Both restriction enzymes recognize the sequence CCGG, but *Hpa*II cannot cleave if the internal C is methylated, whereas *Msp*I is not affected by this modification [27]. In the case of *MAT1A*, shown in Fig. 1A, the restriction pattern observed in the four tissues tested was identical when genomic DNA was digested with *Msp*I. However after complete digestion with *Hpa*II a distinct pattern was obtained in the case of liver DNA. The appearance of new bands (shown by asterisks in Fig. 1A) is consistent with hypomethylation of *MAT1A* in this tissue. These bands were absent in kidney, heart or spleen samples, tissues in which the *MAT1A* gene is silenced as previously reported [2] and as can be observed in the Northern blot shown in the lower panel of Fig. 1A. A large body of experimental evidence supports the existence of a relationship between DNA methylation and gene expression, in the sense that nonmethylated CpG sequences located either in the 5′ regions or in the complete sequence of genes can mark them for expression [10]. Conversely, DNA methylation has been proposed to favor a condensed and inactive state of the chromatin resulting in gene silencing [10–12]. Our finding of *MAT1A* being hypomethylated in the liver contributes to the understanding of the tissue-restricted expression of this gene. Additionally, this situation may explain why the *MAT1A* promoter was able to drive reporter gene expression upon transient transfection not only in liver type cells such as the rat hepatoma H35, but also in the non-hepatic CHO cells, in which the endogenous *MAT1A* gene is silenced [8].

We have also examined the *MAT2A* methylation status in the liver and non-hepatic tissues using the same approach described for *MAT1A*. The results shown in Fig. 1B indicate that *MAT2A*, which is widely expressed outside the liver (as shown in the lower panel of Fig. 1B), is hypomethylated in all expressing tissues so far examined, while hypermethylated in liver. This is supported by the appearance or increased representation of two bands in the *Hpa*II treated DNA samples from kidney, heart or spleen (indicated by asterisks), and by the disappearance of another band present in *Hpa*II treated DNA

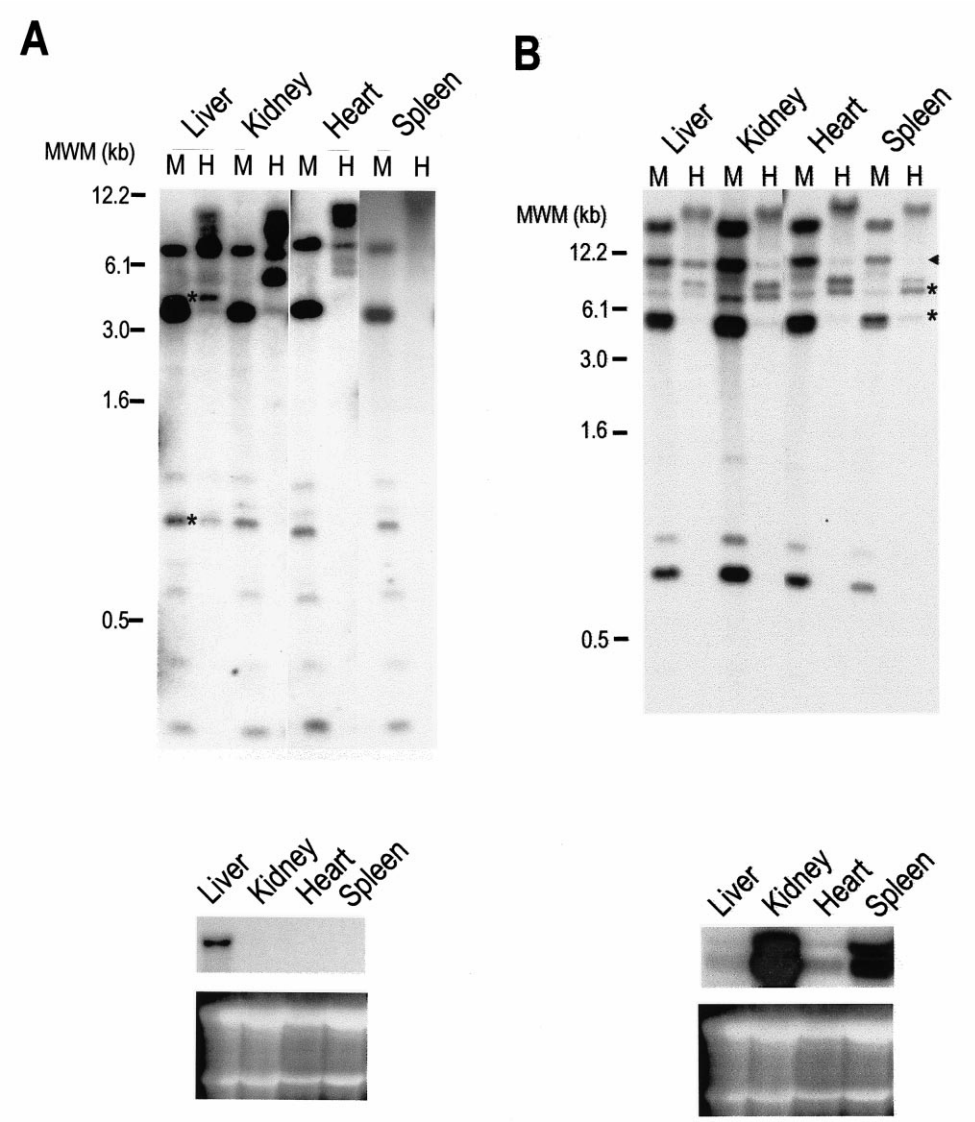

Fig. 1. Methylation status of *MAT1A* (A) and *MAT2A* (B) in rat tissues. DNA was extracted from the indicated rat tissues and digested to completion with either *Msp*I (M) or *Hpa*II (H) and analyzed by Southern blotting as described in Section 2. Asterisks indicate the location of new or more represented bands detected after digestion with *Hpa*II, indicating hypomethylation at the internal C of CCGG sites. Arrowhead in part B indicates a band present in the *Hpa*II treated DNA from liver which is lost upon digestion with this enzyme in all other tissues examined, supporting the hypomethylation of *MAT2A* outside the liver. Lower panels show the expression of either *MAT1A* (A) or *MAT2A* (B) in the different rat tissues as analyzed by Northern blotting.

from liver (marked with an arrowhead). The *MAT2A* promoter contains a large G+C rich domain characteristic of housekeeping genes [9,10]. These CpG islands are usually free of methylated cytosines, but if methylated the gene is rendered inactive [10,28]. Our observations demonstrate that *MAT1A* and *MAT2A* display a methylation pattern which closely correlates with their expression profile. Although these findings alone cannot explain the tissue distribution of

both MAT genes it is known that the pattern of DNA methylation can influence the chromatin structure formed around a gene [10,11,28] and that the inactive chromatin structure associated with DNA methylation is important for the loss of transcriptional activity [12].

Histone acetylation is a reversible covalent modification which has been associated with transcriptional activation [15,16,29,30]. Immunoprecipitation experiments using antibodies specific for acetylated histones have demonstrated that these modified histones are present on potentially and transcriptionally active chromatin, but not on permanently repressed chromatin [31]. We wanted to know the degree of acetylation displayed in vivo by histones associated with *MAT1A* and *MAT2A* genes in liver and kidney, and whether this covalent modification correlated with the degree of methylation. For this purpose nucleosomes obtained from both tissues were

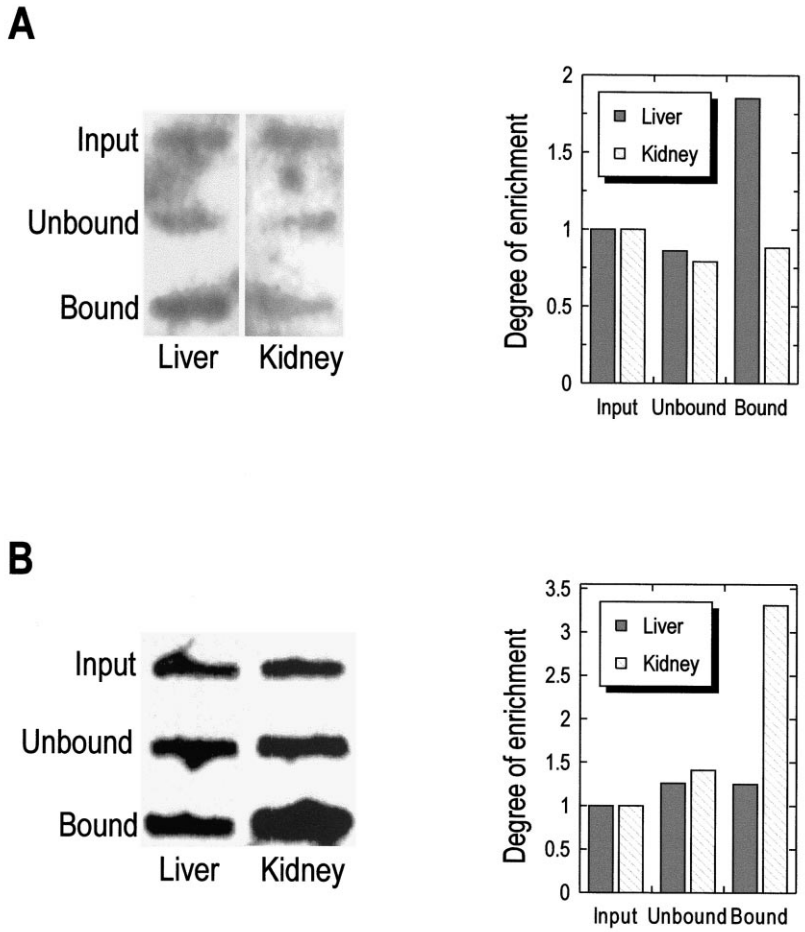

Fig. 2. Histone acetylation of *MAT1A* (A) and *MAT2A* (B) chromatin in rat liver and kidney. Nucleosomes from liver and kidney were obtained and immunoprecipitated with antibodies specific to acetylated histone H4. Equal amounts of DNA from input, unbound and bound fractions were slot–blotted and filters were hybridized with *MAT1A* (autoradiograph in panel A) or *MAT2A* (autoradiograph in panel B) probes as described in Section 2. Graphics show the degree of enrichment obtained by quantitation of the radioactivity present in each fraction relative to the signal obtained in the input fraction for each gene in the two tissues examined.

immunoprecipitated with an antibody mainly specific to hyperacetylated histone H4 as described in Materials and Methods. DNA was extracted from the "input", "unbound" and "bound" fractions, equal amounts were slot–blotted onto nylon membranes and hybridized with *MAT1A* or *MAT2A* cDNA probes. The intensity of the signal obtained from the antibody-bound fraction relative to the signal from the input fraction gives the degree of enrichment generated by the antibody selection. In the case of *MAT1A* a two-fold enrichment was observed in the acetylated fraction (bound fraction) relative to input fraction (bulk chromatin) in liver tissue, while no enrichment was found in the case of kidney (Fig. 2A). Conversely, *MAT2A* was preferentially associated to acetylated histones in kidney (3.5-fold enrichment) and showed no enrichment in liver tissue (Fig. 2B). Hence, *MAT1A* and *MAT2A* display a pattern of tissue-specific histone acetylation which closely correlates with their expression profiles. This is consistent with previous observations showing that transcriptionally active chromatin is associated with enhanced histone acetylation levels [12,31].

Until recently it was not completely understood how cytosine methylation patterns could perform their proposed function controlling transcriptional activity. Several mechanisms have been proposed, they included the direct modulation of chromatin structure by DNA methylation [28], the interference with binding of transcription factors [10], and the implication of methylated-DNA binding proteins that would induce the formation of an inactive chromatin structure [10,11]. However the molecular link between methyl groups on the DNA and the modification of chromatin remained elusive. This mechanism has been recently exposed and appears to involve the recruitment of histone deacetylases by methylated-DNA binding proteins such as MeCP2 [14,17]. Histone acetylation patterns would then be modeled by the underlying DNA methylation profile [18]. It has been proposed that deacetylated histones can establish closer interactions with DNA and between adjacent nucleosomes, thus favoring a condensed and inactive chromatin [32]. Our present findings demonstrate that both MAT genes possess a distinct methylation profile, which probably dictates the accompanying histone acetylation pattern found in expressing and non expressing tissues for each gene. These structural differences may be of importance in determining the tissue-specific expression of *MAT1A* and *MAT2A*. In addition, changes in the DNA methylation and histone acetylation pattern of both genes could participate in the developmental regulation of *MAT1A* and *MAT2A* expression in liver, as well as in their abnormal expression observed in hepatocarcinoma. Furthermore, we have recently provided evidence supporting a functional role for DNA methylation and histone acetylation in the case of *MAT1A* expression, by showing that 5-aza-2′ deoxycytidine (a DNA demethylating agent) and trichostatin A (an inhibitor of histone deacetylases) were able to restore *MAT1A* expression in hepatoma cells [33].

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