The role of c-Myb in the up-regulation of methionine adenosyltransferase 2A expression in activated Jurkat cells

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INTRODUCTION

Methionine adenosyltransferase (MAT) is a critical cellular enzyme that catalyses the formation of S-adenosylmethionine (SAM), the principal methyl donor. In mammals, two different genes, MAT1A and MAT2A, encode liver-specific and non-liver-specific MATs, respectively. SAM level increases during T-lymphocyte activation and is required for proliferation. A major mechanism for the increase in SAM level is increased MAT2A transcription. In the current work we examined the molecular mechanism of increased MAT2A expression in activated Jurkat cells. Treatment of Jurkat cells with interleukin-2 (IL-2), PMA or PMA plus phytohaemagglutinin (PHA) resulted in a 2-fold increase in MAT2A mRNA levels and a 2-fold increase in luciferase activity driven by the transfected human MAT2A promoter construct. -571/+60 but not -270/+60. The region -571 to -270 of the human MAT2A contains a c-Myb consensus binding site. c-Myb is known to be induced during T-lymphocyte activation and its mRNA level was increased after treatment of Jurkat cells with IL-2, PMA or PMA plus PHA. Increased nuclear binding to the MAT2A c-Myb site was confirmed on electrophoretic mobility-shift and supershift analyses. Mutation of the MAT2A c-Myb site abolished the stimulatory effect of these agents on c-Myb nuclear binding and MAT2A promoter activities. Overexpression of c-Myb increased MAT2A promoter activity by 2-fold. Dexamethasone, a known inhibitor of lymphocyte activation, blocked the effect of these agents on MAT2A expression by preventing the increase in c-Myb expression.

Key words: S-adenosylmethionine, dexamethasone, interleukin-2, phorbol 12-myristate 13-acetate, transcriptional regulation.

MATERIALS AND METHODS

Materials

Cell-culture media, fetal bovine serum and primers were obtained from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.). PMA, PHA and dexamethasone were obtained from Sigma (St. Louis, MO, U.S.A.). IL-2 was obtained from Calbiochem (San Diego, CA, U.S.A.). The Luciferase Assay System and the β-Galactosidase Enzyme Assay System were obtained from Promega (Madison, WI, U.S.A.). All restriction endonucleases were obtained from either Promega or Gibco-BRL. [32P]dCTP (3000 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA, U.S.A.). Anti-c-Myb antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). All other reagents were of analytical grade and were obtained from commercial sources.

Cell culture

Acute T-cell leukaemia cell line Jurkat (ATCC no. TIB-152) was obtained from the Cell Culture Core of the USC Liver Disease
Research Center and grown according to instructions provided by the ATCC (Rockville, MD, U.S.A.). Jurkat cells were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin mixture.

**Recombinant plasmids**

The human MAT2A promoter constructs containing 5' deletions as described in [12] were subcloned in the sense orientation upstream of the luciferase coding sequence of the pGL-3 enhancer vector (Promega). MAT2A promoter constructs mutated in the putative c-Myb site (−350 to −333; from 5'-CGGCCAAC-GGTCCGGAAGG-3' to 5'-CGGTCGTCTAGCAGGGGCT-3', with the mutated bases underlined) was generated by using PCR as follows. (i) Upper fragment clone: forward primer (5'-ATCCCCGGCGCGAAGG-3', −571 to −554 bp) and reverse primer (5'-GGGATAGCTTTCCCGGAGG-3', −349 to −369 bp) were used to amplify the −571 to −349 bp fragment from the MAT2A −571/+60 promoter construct [12]. PCR cycle parameters were 94°C for 30 s, 58°C for 40 s and 72°C for 1 min, for 30 cycles. This PCR fragment was subcloned into the pGL-3 enhancer vector by the ATCC (Rockville, MD, U.S.A.). Jurkat cells were cultured in 4 ml of medium plus penicillin mixture.

**Northern-blot analysis**

Northern-blot analysis was performed on total RNA using a specific MAT2A cDNA probe as described in [17]. A 580 bp human c-Myb cDNA probe, corresponding to nucleotides 380–961 of the published human c-Myb sequence [18] was obtained by reverse transcription and subsequent amplification by PCR. Primary PCR (forward and reverse primers were 5'-TCAAAAGATGCTGACGCGAAG-3' and 5'-GGTCTGTG-TTGGTGAGCAGGTGC-3', respectively; cycle parameters were 94°C for 30 s, 56°C for 50 s and 72°C for 1 min, for 25 cycles) and secondary PCR (forward and reverse primers were 5'-GTCGTCAAGGGTCTGTCCTGAC-3' and 5'-GTCTCTGTA-ATGCTCGGCTGCTG-3', respectively; cycle parameters were 94°C for 30 s, 65°C for 1 min and 72°C for 1 min, for 25 cycles) were used to amplify the 580 bp human c-Myb cDNA probe. To ensure equal loading of RNA samples and transfer in each of the lanes, the same membranes were also rehybridized with a labelled β-actin probe as described in [19]. All cDNA probes were labelled with [32P]dCTP using a random-primer kit (Primer-It II Kit; Stratagene, La Jolla, CA, U.S.A.). Autoradiography and densitometry (Gel Documentation System, Scientific Technologies, Carlsbad, CA, U.S.A., and NIH Image 1.60 software) were used to quantify relative RNA. Results of Northern-blot analyses were normalized to β-actin.

**Western-blot analysis of c-Myb**

Western-blot analysis of the c-Myb protein level was done as described in [20] using total cell extracts as well as nuclear protein extracts from Jurkat cells treated with IL-2 (24 units/ml), PMA (50 ng/ml) or PMA (50 ng/ml) plus PHA (1 µg/ml) or vehicle control for 20 h.

**Electrophoretic mobility-shift assay (EMSA) and supershift assay**

EMSAs for the putative binding sites were done as described in [20,21]. Nuclear protein (15 µg) from Jurkat cells treated with IL-
2 (24 units/ml), PMA (50 ng/ml), PMA (50 ng/ml) plus PHA (1 μg/ml) or vehicle control for 20 h were preincubated with 2 μg of poly(dI-dC) in a buffer containing 10 mM Hepes (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂ and 10%, glycerol for 10 min on ice. 

Statistical analysis

Data are given as means ± S.E.M. For changes in mRNA levels, ratios of MAT2A or c-Myb to β-actin densitometric values were compared. For changes in protein levels, ratios of c-Myb to Coomassie Brilliant Blue-staining densitometric values were compared. Statistical analysis was performed using ANOVA followed by Fisher’s test for multiple comparisons. Significance was defined as P < 0.05.

RESULTS

Effect of IL-2, PMA, PMA + PHA on MAT2A expression in Jurkat cells

Treatment of Jurkat cells with IL-2 (24 units/ml), PMA (50 ng/ml), PMA (50 ng/ml) plus PHA (1 μg/ml) increased the steady-state mRNA level of MAT2A by about 2-fold (IL-2 = 190 ± 9%, PMA = 197 ± 6%, and PMA + PHA = 186 ± 6% of the control by densitometric analysis, n = 3, P < 0.05 by ANOVA; Figure 1). Since increased MAT2A expression during T-lymphocyte activation was shown to be mediated largely by increased transcription [14], we next examined the effect of these agents on MAT2A promoter activity. Figure 2 shows that these treatments increased the reporter activity driven by the MAT2A promoter–luciferase construct, which was assigned a value of 1.0. 

![Figure 1](image1.png)

**Figure 1** Effect of IL-2, PMA, PMA plus PHA, dexamethasone (DEX) and PMA plus dexamethasone on steady-state mRNA levels of MAT2A and c-Myb in Jurkat cells

Total RNA samples (15 μg each lane) obtained from Jurkat cells treated with dexamethasone (DEX) (0.1 mM), PMA (50 ng/ml), PMA (50 ng/ml) plus PHA (1 μg/ml), IL-2 (24 units/ml) or vehicle control for 20 h were analysed by Northern-blot hybridization with a 32P-labelled MAT2A cDNA probe as described in the Materials and methods section. The same membranes were then rehybridized sequentially with 32P-labelled c-Myb and β-actin cDNA probes. Cells treated with the PMA/dexamethasone combination were pretreated with dexamethasone for 30 min prior to adding PMA. Representative blots from three experiments are shown.

![Figure 2](image2.png)

**Figure 2** Effect of IL-2, PMA and PMA/PHA on luciferase expression driven by the human MAT2A promoter

Progressive 5' deletions of the MAT2A promoter extending from −1329 to +60 bp were generated and fused to the promoterless luciferase pGL-3 enhancer vector as described in [12]. The construct −571/+60 was also mutated in the c-Myb site (−250 to −333; labelled as c-Myb mutation). Jurkat cells were transfected with these MAT2A promoter constructs and treated with IL-2 (24 units/ml), PMA (50 ng/ml), PMA (50 ng/ml) plus PHA (1 μg/ml) or vehicle control during the last 20 h of the transfection. Data are expressed as luciferase activity relative to that of the pGL-3 enhancer vector control, which was assigned a value of 1.0. 

Effect of IL-2, PMA and PMA + PHA on c-Myb expression in Jurkat cells

The region between −571 and −270 of the human MAT2A contains numerous consensus binding sites for various transcription factors [12]. One that stands out is c-Myb (−350 to −333), since it is known to be induced during T-lymphocyte activation [14]. Consistent with this, treatment of Jurkat cells with IL-2, PMA and PMA plus PHA all increased the steady-state mRNA level of c-Myb by 2–3-fold (IL-2 = 190 ± 9%, PMA = 197 ± 6%, and PMA + PHA = 186 ± 6% of the control by densitometric analysis, n = 3, P < 0.05; Figure 1). A comparable increase in the c-Myb protein level was observed in the total cell extracts but a much more significant increase was seen in the nuclear protein extracts (≈ 4–5-fold increase; Figure 3).

Role of c-Myb in the up-regulation of MAT2A expression in Jurkat cells

To examine if the c-Myb site was critical for mediating the effect of IL-2, PMA and PMA plus PHA, Jurkat cells were transfected with the MAT2A −571/+60 promoter–luciferase construct.
enhancer vector control, which was assigned a value of 1.0.

adding IL-2 or PMA. Data are expressed as relative luciferase activity to that of the pGL-3

activity was evaluated by pretreating cells with dexamethasone (0.1 nM) for 30 min prior to

20 h. The effect of dexamethasone on IL-2- and PMA-mediated induction of MAT2A promoter

treated with dexamethasone (DEX; 0.1 nM), IL-2 (24 units/ml), PMA (50 ng/ml) or vehicle for

experiments, Jurkat cells were transfected with the MAT2A promoter construct

triplicate. In one series of experiments, Jurkat cells were co-transfected with the c-Myb

expression vector and MAT2A promoter construct, as described in the Materials and methods section. In another series of

expression vector and MAT2A promoter construct by 2-fold but not if the c-Myb site was mutated.

reporter activity driven by the MAT2A

overexpression of c-Myb in quiescent Jurkat cells increased

stimulatory effect of these agents on reporter activity.

Figure 2 shows that mutation of the c-Myb site abolished the

and was shown to block the effect of IL-2 and phorbol ester on

occurs in Jurkat cells and what the mechanism might be. Figure

Table 1 Effect of co-transfection with the c-Myb expression vector, or
dexamethasone (DEX) pretreatment on IL-2- and PMA-mediated induction of
MAT2A promoter activity

Results represent means ± S.E.M. from three to five independent experiments performed in
tripli cate. In one series of experiments, Jurkat cells were co-transfected with the c-Myb
expression vector and MAT2A promoter construct — 571/+ 60; wild-type or mutated in the
c-Myb-binding site, as described in the Materials and methods section. In another series of
experiments, Jurkat cells were transfected with the MAT2A promoter construct — 571/+ 60,
treated with dexamethasone (DEX; 0.1 nM), IL-2 (24 units/ml), PMA (50 ng/ml) or vehicle for
20 h. The effect of dexamethasone on IL-2- and PMA-mediated induction of MAT2A promoter
activity was evaluated by pretreating cells with dexamethasone (0.1 nM) for 30 min prior to
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* P < 0.05 versus MAT2A — 571/+ 60-luciferase
† P < 0.05 versus IL-2- or PMA-treated plasmids (ANOVA followed by Fisher’s test).

containing a mutated putative c-Myb site (5'-CGGCCAAC-CCGCGGCT-3') to 5'-CGGCGTCTAGCCGCGGCT-3'). Figure 2 shows that mutation of the c-Myb site abolished the stimulatory effect of these agents on reporter activity.

The role of c-Myb was confirmed further by co-transfecting Jurkat cells with a c-Myb expression vector. Table 1 shows that overexpression of c-Myb in quiescent Jurkat cells increased reporter activity driven by the MAT2A — 571/+ 60 promoter construct by 2-fold but not if the c-Myb site was mutated.

Finally, EMSA and supershift analyses were used to demon-

strated increased nuclear-binding activity to the MAT2A c-

Myb site after treatment of Jurkat cells with IL-2, PMA or PMA plus PHA. Figure 4(A) shows increased binding to the c-Myb site after treatment of Jurkat cells with these agents, which was

abolished if a mutant oligonucleotide probe was used. The identity of the protein was confirmed by supershift analysis.

Dexamethasone prevents IL-2-, PMA- and PMA plus
PHA-mediated up-regulation of MAT2A expression

Dexamethasone is known to inhibit T-lymphocyte activation and was shown to block the effect of IL-2 and phorbol ester on MAT2A expression [14]. We next examined whether the same occurs in Jurkat cells and what the mechanism might be. Figure 1 shows that dexamethasone treatment alone had no effect on the basal mRNA level of MAT2A but blocked the increase in MAT2A mRNA level after PMA treatment. Table 1 shows that

Figure 3 Effect of IL-2, PMA and PMA/PHA on c-Myb protein levels in total cell extracts or nuclear protein fraction

Cell extracts (50 μg of protein/lane) and nuclear protein (10 μg/lane) obtained from Jurkat cells treated with IL-2 (24 units/ml), PMA (50 ng/ml), PMA (50 ng/ml) plus PHA (1 μg/ml) or vehicle control (Con) for 20 h were analysed by Western-blot analysis using anti-c-Myb antibodies as described in the Materials and methods section. Equivalent protein loading was assured by Coomassie Brilliant Blue staining of gels after transblotting (results not shown). Representative blots from n = 3 are shown.

Table 1 Effect of co-transfection with the c-Myb expression vector, or
dexamethasone (DEX) pretreatment on IL-2- and PMA-mediated induction of
MAT2A promoter activity

Results represent means ± S.E.M. from three to five independent experiments performed in
tripli cate. In one series of experiments, Jurkat cells were co-transfected with the c-Myb
expression vector and MAT2A promoter construct — 571/+ 60; wild-type or mutated in the
c-Myb-binding site, as described in the Materials and methods section. In another series of
experiments, Jurkat cells were transfected with the MAT2A promoter construct — 571/+ 60,
treated with dexamethasone (DEX; 0.1 nM), IL-2 (24 units/ml), PMA (50 ng/ml) or vehicle for
20 h. The effect of dexamethasone on IL-2- and PMA-mediated induction of MAT2A promoter
activity was evaluated by pretreating cells with dexamethasone (0.1 nM) for 30 min prior to
adding IL-2 or PMA. Data are expressed as relative luciferase activity to that of the pGL-3 enhancer vector control, which was assigned a value of 1.0.

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* P < 0.05 versus MAT2A — 571/+ 60-luciferase
† P < 0.05 versus IL-2- or PMA-treated plasmids (ANOVA followed by Fisher’s test).

Figure 4 EMSA and supershift assay for the c-Myb site of the human
MAT2A gene

(A) Jurkat cells were treated with IL-2 (24 units/ml), PMA (50 ng/ml), PMA (50 ng/ml) plus
PHA (1 μg/ml) or vehicle control for 20 h, and nuclear proteins were obtained for EMSA and
supershift analysis as described in the Materials and methods section. Arrow on the left shows
c-Myb binding as confirmed by disappearance of the signal when a mutant oligonucleotide
probe was used and when the signal supershifted in the presence of antibodies against c-Myb.
There was increased c-Myb-binding activity after treatment of Jurkat cells with IL-2, PMA and
PHA plus PHA. (B) Jurkat cells were treated with PMA/PHA or vehicle control for 20 h, or
pretreated with dexamethasone (DEX) for 30 min and then treated with PMA/PHA for 20 h.
Arrow on the left shows c-Myb binding, which was increased after treatment with PMA and
PHA. This is confirmed by disappearance of the signal in the presence of 100 x specific
competitor. Note that dexamethasone pretreatment prevented the increase in c-Myb nuclear-
binding activity.
dexamethasone pretreatment prevented the IL-2- and PMA-mediated increase in reporter activity driven by the MAT2A –571/+60 promoter–luciferase construct.

To see if dexamethasone’s effect is mediated by c-Myb, we examined c-Myb mRNA level and c-Myb EMSA after dexamethasone treatment. Figure 1 shows that dexamethasone treatment alone had no effect on the basal mRNA level of c-Myb but blocked the increase in c-Myb mRNA level after PMA treatment. Finally, Figure 4(B) shows that dexamethasone pretreatment prevented the increase in c-Myb nuclear binding after PMA/PHA treatment.

DISCUSSION
MAT is a critical cellular enzyme because it catalyses the only reaction that generates SAM. The MAT gene is one of 482 genes absolutely required for survival of an organism [1]. In mammals, two distinct genes code for the enzyme MAT [3–5]. MAT1A is a liver-specific gene that is expressed in the liver shortly before birth and becomes the major form of MAT as the liver matures [22,23]. It is a marker for the differentiated or mature liver phenotype. In contrast, MAT2A is expressed in all non-hepatic tissues as well as during periods of rapid liver growth [3,6,17,22–26]. Despite the importance of MAT2A, little is known about its transcriptional regulation. MAT2A gene expression appears to be influenced by the cell cycle, as evident from its induction during liver regeneration, malignant liver transformation and T-lymphocyte activation [10,11,17,24–27]. In all cases the mechanism involved in part increased transcription [11,24,27]. It has been speculated that the induction of MAT2A and MAT II may be a mechanism for the cell to provide an increased supply of SAM, the precursor to polyamine synthesis, which is required for cell growth [11]. Consistent with this notion, inhibition of SAM synthesis blocked T-lymphocyte proliferation [9]. Our laboratory has been interested in studying transcriptional regulation of MAT2A and has cloned a 1.3 kb 5′-flanking region of the human MAT2A gene [12]. In the current work we examined the molecular mechanism for the transcriptional up-regulation of MAT2A in Jurkat cells after treatment with IL-2, PMA or PMA plus PHA.

Using doses of IL-2, PMA or PMA/PHA similar to those that other investigators have reported either induce MAT2A expression in rat lymphoblasts [11] or activate Jurkat cells [14], we first confirmed increased MAT2A expression in Jurkat cells. Analysis of reporter activity driven by the MAT2A promoter constructs revealed that the region between –571 and –270 of MAT2A is crucial for the stimulatory effect to occur. Of the multiple consensus binding sites for transcription factors, we focused on a putative c-Myb site (–350 to –333) because it is known that c-Myb expression is induced during T-lymphocyte activation [14]. A number of different approaches were then taken to critically examine the role of c-Myb in the IL-2-, PMA- and PMA/PHA-mediated up-regulation of MAT2A expression. Specifically, nuclear binding to the c-Myb site of the MAT2A is increased after treatment of Jurkat cells with these agents. If the c-Myb site is mutated, both induction of the MAT2A promoter activity and c-Myb nuclear-binding activity were abolished. Overexpression of c-Myb in otherwise quiescent Jurkat cells also led to increased MAT2A promoter activity, an effect that was blocked if the c-Myb site was mutated. Finally, dexamethasone, a known inhibitor of T-lymphocyte activation, blocked the effect of these agents on MAT2A expression, MAT2A promoter activity, c-Myb nuclear binding and c-Myb expression. Collectively, these results demonstrate clearly that these agents modulate MAT2A expression in Jurkat cells via c-Myb.

c-Myb is a transcription factor encoded by the c-Myb proto-oncogene and is best known as a regulator of cell growth and differentiation in haematopoietic cells [28]. c-Myb is highly expressed in immature haematopoietic cells, and the expression is strongly down-regulated during terminal differentiation [28]. Overexpression of c-Myb inhibits differentiation of haematopoietic precursor cells, while antisense-mediated ablation of c-Myb inhibits their proliferation [29]. Despite the strong link between c-Myb and proliferation, there remains a large void in understanding the relevant target genes for c-Myb. Binding sites for c-Myb have been identified in an increasing number of gene promoters, including those of mim1, cde-2, c-myc, bcl-2, T-cell receptor δ and γ chains, CD4 and Ick [30]. However, establishing a direct link between candidate genes and growth control has been difficult. MAT2A is a gene that is linked directly to cell growth in both lymphocytes and hepatocytes. Thus it represents an important target gene for c-Myb in the regulation of cell growth.

IL-2 is the major mitogenic cytokine for T-cells [31]. IL-2 stimulation activates a number of secondary messengers, including tyrosine kinases, Ser/Thr kinases, adaptor proteins and small G-proteins. These secondary messengers, in turn, activate inducible transactivating factors including STATs (signal transduction and activators of transcription), NF-kB (nuclear factor κB) and CREB (cAMP-response-element-binding protein) [31]. Some of the genes that are IL-2-responsive may be regulated by these transactivating factors. IL-2 has also been shown to relieve transcriptional attenuation of c-Myb in human thymic blast cells [31]. Whatever the mechanism, it is well established that IL-2 induces c-Myb expression.

The effect of phorbol ester on lymphocyte proliferation and c-Myb expression is more controversial. Farrar and Anderson suggested that activation of protein kinase C (PKC) by IL-2 is a crucial step in the signal transduction elicited by IL-2 [32]. This idea is supported by the findings that phorbol esters activate PKC and mimic some of the IL-2 actions, such as the induction of some genes and T-cell proliferation [33]. However, phorbol esters induce proliferation in some T-cell clones but not in others [34], whereas IL-2 induces proliferation in all. Redondo and co-workers showed that there are two different pathways for T-cell proliferation, one PKC-dependent and the other PKC-independent [34]. Our study shows that, in Jurkat cells, the two pathways probably converge to result in activation of c-Myb and subsequently MAT2A expression.

Glucocorticoids are known inhibitors of lymphocyte activation [11]. Dexamethasone blocked the IL-2- and phorbol ester-mediated induction of MAT2A expression in lymphoblasts [11]. We have confirmed this in Jurkat cells as well. The molecular events that mediate dexamethasone’s inhibitory effect on lymphocyte proliferation is unclear. Our data suggest the inhibitory effect of dexamethasone on MAT2A expression may be mediated by c-Myb. However, how dexamethasone blocks the IL-2- and PMA-mediated increase in c-Myb is unclear. It is likely that the effect is indirect, as dexamethasone by itself had no influence on c-Myb expression.

In summary, the present work demonstrates that IL-2, PMA and PMA/PHA induced MAT2A expression in Jurkat cells by increasing c-Myb expression, which *trans*-activates the MAT2A promoter by binding to the c-Myb site located at –350 to –333 of MAT2A. Dexamethasone blocked the effect of IL-2, PMA and PMA plus PHA on MAT2A expression by blocking the increase in c-Myb. Since MAT is the only enzyme that catalyses the synthesis of SAM, which is required for growth, we have identified MAT2A as an important target for c-Myb in the regulation of cell growth.
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