Induction of Human Methionine Adenosyltransferase 2A Expression by Tumor Necrosis Factor α

ROLE OF NF-κB AND AP-1

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Two genes (MAT1A and MAT2A) encode for methionine adenosyltransferase (MAT), an essential cellular enzyme responsible for S-adenosylmethionine biosynthesis. MAT1A is expressed mostly in the liver, whereas MAT2A is widely distributed. We showed a switch from MAT1A to MAT2A expression in human hepatocellular carcinoma (HCC), which facilitates cancer cell growth. Using DNase I footprinting analysis, we previously identified a region in the MAT2A promoter protected from DNase I digestion in HCC. This region contains NF-κB and AP-1 elements, and the present study examined whether they regulate MAT2A promoter activity. We found nuclear binding of NF-κB and AP-1 to the MAT2A promoter increases in HCC. Tumor necrosis factor α (TNFα), which activates both NF-κB and AP-1, increased MAT2A expression in a dose- and time-dependent manner, binding of both NF-κB and AP-1 to the MAT2A promoter and MAT2A promoter activity, with the latter effect blocked by site-directed mutagenesis of the NF-κB and AP-1 binding sites. Blocking NF-κB with IκB super-repressor or AP-1 with dominant-negative c-Jun led to decreased basal MAT2A expression and prevented the TNFα-induced increase in MAT2A expression. Although blocking NF-κB had no influence on the ability of TNFα to increase AP-1 nuclear binding, blocking AP-1 with dominant-negative c-Jun prevented the TNFα-mediated increase in NF-κB binding. In conclusion, both NF-κB and AP-1 are required for basal MAT2A expression in HepG2 cells and mediate the increase in MAT2A expression in response to TNFα treatment. Increased trans-activation of these two sites also contributes to MAT2A up-regulation in HCC.

Methionine adenosyltransferase (MAT) is an essential cellular enzyme that catalyzes the formation of S-adenosylmethionine (SAMe), the principal biological methyl donor and the ultimate source of the propylamine moiety used in polyamine biosynthesis (1, 2). In mammals, two different genes, MAT1A and MAT2A, encode for two homologous MAT catalytic subunits, α1 and α2 (3–5). MAT1A is expressed mostly in liver, and it encodes the α1 subunit found in two native MAT isozymes, which are either a dimer (MAT III) or tetramer (MAT I) of this single subunit (5). MAT2A encodes for a catalytic subunit (α2) found in a native MAT isozyme (MAT II), which is associated with a catalytically inactive regulatory subunit (β) in lymphocytes encoded by yet a third gene (5, 6). MAT2A is widely distributed (3–5). MAT2A also predominates in the fetal liver and is progressively replaced by MAT1A during liver development (7, 8). In adult liver, increased expression of MAT2A is associated with rapid growth or de-differentiation of the liver (9–11). Using a cell line model that differs only in the type of MAT expressed, we demonstrated that a switch in MAT expression in liver cancer (from MAT1A to MAT2A) plays an important pathogenetic role by facilitating liver cancer growth (12). The influence of MAT expression on liver growth and injury was further demonstrated using a MAT1A knockout mouse model (13, 14). In this model, absence of hepatic MAT1A is compensated by induction of MAT2A. These animals exhibit chronic hepatic SAMe deficiency, are prone to liver injury and develop spontaneous hepatocellular carcinoma (HCC) (13, 14).

Given the importance of MAT expression in liver disease and cancer, we have been interested in understanding transcriptional regulation of MAT genes. We characterized the promoter region of both human MAT genes (15, 16) and showed previously that in human HCC, both promoter hypomethylation (17) and increased expression of c-Myb and Sp1 with subsequent trans-activation of the MAT2A promoter contribute to transcriptional up-regulation of MAT2A in HCC (18). In the latter work we described increased protein binding to the MAT2A promoter region (~354 to ~312) in HCC as compared with normal liver (18). Two other consensus elements in this DNase I protected region are nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1). We had previously observed that nuclear binding of NF-κB and AP-1 to the promoter of human glutamate-cysteine ligase catalytic subunit was increased in HCC (19). In the current study, we examined whether there is increased nuclear binding of NF-κB and AP-1 to the MAT2A promoter in HCC and whether these two transcription factors regulate MAT2A expression by using tumor necrosis factor alpha (TNFα) as an inducing agent.

IκB super-repressor; NF1, nuclear factor 1; Nrf-2, nuclear factor-erythroid 2 related factor.

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**A. Consensus elements in the region -354 to -312 of MAT2A**

\[
\begin{align*}
&\text{NF1} \quad \text{Nrf-2} \quad \text{AP-1} \\
&\text{ATCC CCGCAACGG TCAGAAGGGA GTGCACCATT GACCGCAC} \\
&\text{c-Myb} \quad \text{NF-κB} \\
&\text{Probe used for EMSA in B} \quad \text{Probe used for EMSA in C}
\end{align*}
\]

**B.**

[HCC + anti-NFkB]

HLH c-Myb NF1 p65 p50 Nrf-2

**C.**

|        | Normal Liver | HCC | HCC+ anti-c-Jun Abs | HCC+ anti-c-Jun Abs | HCC+ anti-c-Jun Abs |
|--------|--------------|-----|---------------------|---------------------|---------------------|
| µg protein | 0 | 1 | 2.5 | 5 | 10 | 0 | 1 | 2.5 | 5 | 10 | 0 | 1 | 2.5 | 5 | 5

**Fig. 1.** A, consensus binding sites present in the DNase I protected region of MAT2A in HCC (18). B, electrophoretic mobility shift and supershift assay for the probe that spans -354 to -326 of the human MAT2A gene. EMSA and supershift were done as described under “Experimental Procedures.” In HCC, there is increased binding (two bands) to the probe as compared with normal liver. Supershift analysis shows that the top band is due to c-Myb binding, whereas the bottom band is likely to be NF-κB binding. C, electrophoretic mobility shift assay for the probe that spans the AP-1 site at -327 of the human MAT2A gene. In HCC, there is increased binding to the AP-1 probe as compared with normal liver, which is confirmed by supershift analysis. Representative EMSAs are shown from at least three experiments.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media, fetal bovine serum, primers, and Superscript II were obtained from Invitrogen (Grand Island, NY). The Luciferase Assay System and recombinant human TNFα were obtained from Promega (Madison, WI). All restriction endonucleases were obtained from either Promega or Invitrogen. [32P]dCTP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences (DuPont, Boston, MA). All other reagents were of analytical grade and were obtained from commercial sources.

**Source of Normal and Cancerous Liver Tissue**—Normal liver tissue was obtained from normal liver included in the resected liver specimens of five patients with metastatic colon or breast carcinoma. Cancerous liver tissue was obtained from five patients undergoing surgical resection for primary HCC. Written informed consent was obtained from each patient. The contamination of HCC samples with noncancerous tissue was less than 5% as determined by histopathology. These tissues were immediately frozen in liquid nitrogen for subsequent isolation of nuclear proteins for electrophoretic mobility shift assay (EMSA) as described below. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Keck School of Medicine University of Southern California’s human research review committee.

**Cell Culture and TNFα Treatment**—HepG2 cells were obtained from the Cell Culture Core of the USC Liver Disease Research Center and grown according to instructions provided by the American Type Culture Collection (Rockville, MD) in Earle’s minimal essential medium supple-
mented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin mixture. Prior to treatment with TNF-α/H9251, medium was changed to withhold serum overnight. Cells were then treated with TNF-α/H9251 (1–25 ng/ml) for 15 min to 16 h for various assays as described below.

**Recombinant Plasmids and Adenoviral Vectors**—The human MAT2A promoter constructs were previously described (15, 17) and subcloned in the sense orientation upstream of the luciferase coding sequence of the pGL-3 enhancer vector (Promega). Recombinant, replication-defective adenovirus expressing dominant-negative c-Jun, TAM67, was kindly provided by Dr. David Brenner (20). Recombinant, replication-defective adenovirus expressing IκB super-repressor (IκBSR), which expresses a mutant IκB that cannot be phosphorylated and therefore irreversibly binds NF-κB and prevents its activation was kindly provided by Ebrahim Zandi (21).

**Infection of HepG2 Cells with Adenoviral Vectors**—Recombinant adenoviruses encoding TAM67, IκBSR, or empty vector were amplified in 293 cells. HepG2 cells were infected with unpurified recombinant adenovirus encoding for TAM67, IκBSR, or empty vector at multiplicity of 20 plaque-forming units/cell for 24 h. After 24 h of infection, the viruses were removed and replaced with fresh medium for transfection analysis as described below.

**Effect of TNFα on MAT2A, c-Jun, and p65 expression in HepG2 cells.** RNA (25 μg/lane) samples from HepG2 cells treated with TNFα (0–25 ng/ml) for 8 h (A–C, top panels) and 15 ng/ml for 0 to 16 h (A–C, bottom panels) were analyzed by Northern blot analysis with a 32P-labeled MAT2A, c-Jun, or p65 cDNA probes as described under “Experimental Procedures.” The same membranes were then rehybridized with a 32P-labeled β-actin cDNA probe. Representative Northern blots are shown.

**MAT2A Transcriptional Regulation by NF-κB and AP-1**

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**Fig. 2. Effect of TNFα on MAT2A, c-Jun, and p65 expression in HepG2 cells.** RNA (25 μg/lane) samples from HepG2 cells treated with TNFα (0–25 ng/ml) for 8 h (A–C, top panels) and 15 ng/ml for 0 to 16 h (A–C, bottom panels) were analyzed by Northern blot analysis with a 32P-labeled MAT2A, c-Jun, or p65 cDNA probes as described under “Experimental Procedures.” The same membranes were then rehybridized with a 32P-labeled β-actin cDNA probe. Representative Northern blots are shown.
At the end of the TNFα treatment, total RNA was extracted, and Northern hybridization analysis was performed using specific MAT2A cDNA probe as described previously (18). Northern hybridization analysis was also performed using a specific c-Jun cDNA probe as we described (22), and a p65 cDNA probe that corresponds to nucleotides 481–1032 of the published human p65 sequence (23). The p65 cDNA probe was obtained by reverse transcription and PCR using a one step RT-PCR kit (Clontech). To ensure equal loading of RNA samples and transfer in each of the lanes, prior to hybridization, membranes were rinsed with ethidium bromide and photographed, and the same membranes were also rehybridized with a 32P-labeled β-actin cDNA probe as described (18). Autoradiography and densitometry (Gel Documentation System, Scientific Technologies, Carlsbad, CA, and National Institutes of Health Image 1.60 software program) were used to quantitate relative RNA. Results of Northern blot analysis were normalized to β-actin.

**Fig. 3. Effect of TNFα treatment on luciferase activity driven by the human MAT2A promoter.** Progressive 5' deletions of the MAT2A promoter extending from −1529 to +60 bp were generated and fused to the promoterless luciferase pGL-3 enhancer vector as described under "Experimental Procedures." HepG2 cells were transfected transiently with these promoter constructs or pGL-3 enhancer vector alone and treated with TNFα (15 ng/ml for 8 h) or vehicle control. Cells were co-transfected with Renilla luciferase for control of transfection efficiency. Results represent mean ± S.E. from four independent experiments performed in triplicates. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control, which is assigned a value of 1.0. *p < 0.05 versus respective control.

**Fig. 4. Effect of TNFα treatment on DNase I footprinting analysis of the −494 to −152 region of the human MAT2A promoter.** DNA fragment was end-labeled on either strand and digested with DNase I in the absence (0) or presence of 5–15 μg of nuclear protein extracts from HepG2 cells treated with TNFα (15 ng/ml for 8 h) or vehicle control. Position of the protected region is indicated at the right of the figure. Lane G+A represents a Maxam-Gilbert sequencing reaction in the same fragment. Representative DNase I footprinting is shown.
Fig. 5. Effect of TNFα, IxBSR, and dominant-negative c-Jun treatments on NF-κB and AP-1 binding. A, NF-κB binding. Nuclear protein extracts (15 µg) were obtained from HepG2 cells previously infected with IxBSR or adenoviral vector alone (Adv) and subsequently treated with TNFα (15 ng/ml for 8 h). EMSA (to NF-κB probe) with supershift was done using anti-p65 antibodies as described under "Experimental Procedures." B, AP-1 binding. Nuclear protein extracts (15 µg) were obtained from HepG2 cells previously infected with TAM67 or adenoviral vector alone (Adv) and subsequently treated with TNFα (15 ng/ml for 8 h). EMSA (to AP-1 probe) with supershift was done using anti-c-Jun antibodies as described under "Experimental Procedures." The arrows to the right point to specific complexes that were supershifted in the presence of specific antibodies.

The effect of TNFα on MAT2A promoter activity was examined by measuring luciferase activity driven by MAT2A promoter luciferase gene constructs in transfected HepG2 cells treated with TNFα (15 ng/ml) during the last 8 h of the transfection. To confirm the importance of NF-κB and AP-1 binding in mediating the effect of TNFα on MAT2A promoter activity, human MAT2A promoter constructs 571/+60-LUC mutated in either the NF-κB and/or AP-1 binding sites were generated using PCR. MAT2A promoter constructs mutated in either the putative NF-κB site (−334 to −326) (from 5′-GGGAGGTGC-3′ to 5′-GGGATA-GTGC-3′), AP-1 site (−328 to −318) (from 5′-TGCCATGTAC-3′ to 5′-TGCCATGTAC-3′), or both were subcloned into the pGL-3 promoter-luciferase vector (Promega). HepG2 cells transfected with either wild type or mutant MAT2A promoter constructs were treated with TNFα (15 ng/ml) during the last 8 h of the transfection.

Dña I Footprinting Analysis—The 32P-end-labeled fragment of the 5′-flanking region of human MAT2A strongly induced by TNFα treatment was generated by digestion with restriction endonucleases. Dña I footprinting analysis was performed with 0–15 µg of nuclear protein from HepG2 cells treated with TNFα (15 ng/ml for 8 h) or vehicle, and labeled double-stranded fragments corresponding to nucleotides −494 to −508 were obtained from HepG2 cells treated with TNFα (15 ng/ml for 8 h). To assess the effect of IxBSR or dominant-negative c-Jun, cells were previously infected with IxBSR, TAM67, or empty viral vector and subsequently treated with TNFα (15 ng/ml for 8 h) and processed for EMSA and supershift analysis.

Measurement of Apoptosis—Apoptosis was assessed by both DNA fragmentation analysis and Hoechst staining. To assess DNA fragment-
**Fig. 6. Effect of TNFα, IκBSR, and dominant negative c-Jun treatments on steady-state mRNA levels of MAT2A.** HepG2 cells were infected with adeno viral vectors encoding IκBSR (A) or dominant-negative c-Jun (B) or adeno viral vectors alone and subsequently treated with TNFα (15 ng/ml for 8 h) or vehicle control. Northern blot analysis was carried out with RNA (25 μg/lane) samples after various treatments using a 32P-labeled MAT2A cDNA probe as described under “Experimental Procedures.” The same membranes were then rehybridized with a 32P-labeled β-actin cDNA probe. Representative Northern blots are shown.

**Experimental Procedures.**

**Statistical Analysis.**—For multiple comparisons, ANOVA followed by Fisher’s protected least significant difference was used. Significance was defined by p < 0.05.

**RESULTS.**

**Nuclear Binding of NF-κB and AP-1 to the MAT2A Promoter in HCC.—**We showed previously that −354 to −312 of the human MAT2A promoter was protected from DNase I digestion in HCC but not in normal liver (18). Consensus elements present in this region include nuclear factor 1 (NF1), c-Myb, nuclear factor-erythroid 2 related factor (Nrf-2), NF-κB, and AP-1 (Fig. 1A). We demonstrated increased expression of c-Myb and trans-activation of its binding site in HCC (18). To see if there is increased binding to the other potential binding sites, EMSA and supershift analysis were performed using specific antibodies against NF1, Nrf-2, NF-κB subunits, and c-Jun. Fig. 1B shows that in addition to c-Myb, there is also increased p65 binding to the EMSA probe −354 to −326 but not NF-1 or Nrf-2. To see if there is increased AP-1 binding, EMSA was done using probe −328 to −316, which span mostly the AP-1 binding site. Fig. 1C shows that there is also increased AP-1 binding to this site in HCC.

**Effect of TNFα on MAT2A Expression and Promoter Activity.—**TNFα is a pleiotropic cytokine that can elicit a diversity of cellular responses, depending on the cell type and concentration used (24, 25). It is well known to induce both AP-1 and NF-κB (24, 25) and can serve as an ideal agent to examine whether these two transcription factors modulate MAT2A promoter activity. We first examined whether TNFα treatment affects MAT2A expression in HepG2 cells. Fig. 2A shows that TNFα increased MAT2A expression in HepG2 cells in a dose- and time-dependent manner. It also induced c-jun and p65 expression in a similar manner (Fig. 2, B and C). Maximal induction of MAT2A was seen with 15 ng/ml TNFα for 8 h (300 ± 34% of control, results represent mean ± S.E. from four experiments, p < 0.05 versus control), the condition used for all subsequent experiments.

We next investigated the effect of TNFα treatment on the promoter activity of MAT2A. Fig. 3 shows that TNFα treatment induced the reporter activity driven by the MAT2A promoter, particularly the promoter construct −571 to +60, where TNFα led to a nearly 2-fold increase in activity. It had no influence on the promoter activity driven by the construct −270 to +60, suggesting that the region responsible for this stimulatory effect lie between −571 and −270.

**DNase I Footprinting Analysis of MAT2A 5′-Flanking Region.—**To delineate the cis-acting elements that may be involved in mediating the effect of TNFα on the MAT2A promoter, DNase I footprint analysis of the −494 to −152 5′-flanking region of the MAT2A gene was carried out. Fig. 4 shows that TNFα treatment led to an increase in nuclear protein-dependent DNase I-protected region from −352 to −314 on both strands. Both NF-κB and AP-1 consensus binding sites are present in this region.

**EMSA and Supershift Analysis.—**We next examined whether TNFα treatment led to an increase in both NF-κB and AP-1 binding to their respective sites on the MAT2A promoter (Fig. 5). As expected, nuclear binding to the NF-κB and AP-1 sites increased following TNFα treatment. Note that cells previously infected with IκBSR had a lower basal NF-κB binding and blunted the increase in NF-κB binding following TNFα treatment (Fig. 5A). Similarly, cells previously infected with TAM67 also had a lower basal AP-1 binding and prevented the increase in AP-1 binding following TNFα treatment (Fig. 5B). These treatments did not induce apoptosis in HepG2 cells as determined by DNA fragmentation and Hoechst staining (data not shown).

**Role of NF-κB and AP-1 in Basal and TNFα-induced Increase in MAT2A Expression.—**To evaluate the importance of NF-κB and AP-1 in both the basal expression and in the TNFα-mediated increase in MAT2A expression, HepG2 cells were infected with IκBSR, TAM67, or empty vector and the effect of blocking NF-κB or AP-1 activity on TNFα-mediated changes was examined. Fig. 6A shows that the basal expression of MAT2A fell when NF-κB was blocked (43% lower), and although the TNFα-mediated induction in MAT2A was lower, it was still much higher than IκBSR alone. This suggests that TNFα-induced MAT2A expression by both NF-κB-dependent and -independent mechanisms. Fig. 6B shows that the basal expression of MAT2A also depends on AP-1, because the expression fell (50% lower) when AP-1 was blocked. Furthermore,
dominant negative c-Jun was able to prevent the increase in MAT2A following TNFα/H9251 treatment more completely than I/H9260 BSR.

The importance of NF-κB and AP-1 binding in mediating the effect of TNFα/H9251 on the MAT2A promoter was confirmed using promoter constructs mutated in these binding sites. We first determined the number of bases that need to be mutated to obliterate nuclear binding completely. Fig. 7A shows that nuclear binding was completely obliterated only when four bases are mutated. These were then used in subsequent transfection experiments. Fig. 7B shows that when the NF-κB or AP-1 site is mutated, the effect of TNFα is significantly blunted, and when both sites are mutated, the effect of TNFα is completely prevented.

**Fig. 7. Effect of mutation of putative AP-1 and NF-κB sites on nuclear binding activity (A) and luciferase expression driven by the human MAT2A promoter construct −571/+60-LUC (B).**

A. EMSA was performed with 10 μg of nuclear protein from control or TNFα-treated HepG2 cells (15 ng/ml for 8 h) using wild type AP-1 and NF-κB probes or probes mutated by 1–4 bp as described under “Experimental Procedures.” Note that nuclear binding was obliterated only when four bases are mutated. B. MAT2A promoter-luciferase construct −571/+60, wild type or mutated in the putative AP-1 site (−328 to −318) (from 5′-TGCCATGCAC-3′ to 5′-TGCTCTACAC-3′), the putative NF-κB site (−334 to −326) (from 5′-GGAGGCTGC-3′ to 5′-GAGAATGC-3′), or both were subcloned into pGL-3 enhancer vector and used for transient transfection analysis in HepG2 cells. Cells were treated with TNFα (15 ng/ml for 8 h) or vehicle control prior to luciferase assays. Results represent mean ± S.E. from three independent experiments performed in triplicates. Data are expressed as relative luciferase activity to that of pGL-3 enhancer vector control, which is assigned a value of 1.0. * p < 0.05 versus −571/+60-LUC construct; † p < 0.05 versus respective mutated −571/+60-LUC constructs; ** p < 0.05 versus −571/+60-LUC construct and constructs mutated in either AP-1 or NF-κB binding sites.

**Interactions between NF-κB and AP-1**—Previous studies show that the NF-κB p65 can physically interact with c-Fos and c-Jun and potentiate each other’s biological function (26). To see if p65 and c-Jun physically interact in the modulation of MAT2A promoter activity, we first examined whether p65 and c-Jun can bind to each other’s binding sites. Fig. 8A shows that c-Jun does not bind to the NF-κB site, and p65 does not bind to the AP-1 site. We next examined the effect of blocking either NF-κB or AP-1 on each other’s nuclear binding activity. Although blocking NF-κB had no influence on the ability of TNFα to induce AP-1 binding to the MAT2A promoter (Fig. 8B), blocking AP-1 with dominant negative c-Jun abolished the ability of TNFα to induce NF-κB binding (Fig. 8C).
**DISCUSSION**

MAT is a critical cellular enzyme because it catalyzes the only reaction that generates SAMe. The MAT gene is one of 482 genes absolutely required for survival of an organism (1). In mammalian liver, MAT1A is a marker for the differentiated or mature liver phenotype, whereas MAT2A is a marker for rapid growth and de-differentiation (27). Although the two MAT genes are highly homologous, the enzymes they encode for are different in the kinetic profiles and regulatory properties (27). Due to these differences, the type of MAT expressed by a cell can influence the steady-state SAMe level of the cell and methylation status. Using a cell line model that differs only in the type of MAT expressed, cells that expressed MAT1A had the highest intracellular SAMe level and DNA methylation, whereas cells that expressed MAT2A had the opposite (12). Interestingly, cells that expressed MAT2A grew faster than cells that expressed MAT1A (12). Thus, the switch in MAT expression in liver cancer is pathogenetically important, because it offered the cancerous cell a growth advantage. The importance of MAT expression on SAMe level and liver phenotype was recently confirmed in the MAT1A knockout mouse model where replacement of MAT1A with MAT2A resulted in chronic hepatic SAMe depletion and eventual development of HCC (13, 14).

Despite the importance of MAT2A, studies addressing its transcriptional regulation have only begun to appear recently. MAT2A gene expression is influenced by the cell cycle, as evident by its induction during liver regeneration, malignant liver transformation and T-lymphocyte activation (10, 17, 28). It has been speculated that the induction in MAT2A and MAT II may be a mechanism for the cell to provide an increased supply of SAMe, the precursor to polyamine synthesis that is required for cell growth (28). Our laboratory has been interested in studying transcriptional regulation of MAT2A and, in particular, the molecular mechanism for its up-regulation in human liver cancer. Our previous studies (17, 18) described promoter hypomethylation and increased trans-activation of the MAT2A promoter by c-Myb and Sp1 as mechanisms that contribute to its up-regulation in HCC. Two other consensus elements present in the DNase I-protected region of the

**FIG. 8. Interactions between NF-κB and AP-1 in the TNFα-mediated increase in nuclear binding to MAT2A promoter.** A, nuclear protein extracts (15 μg) were obtained from HepG2 cells treated with TNFα (15 ng/ml for 8 h) or vehicle control, and EMSA with supershift was done as described under “Experimental Procedures” using both NF-κB and AP-1 probes. Note that supershift occurred only with anti-p65 antibodies to the NF-κB probe and anti-c-Jun antibodies to the AP-1 probe. In B, binding to the AP-1 probe was assessed in cells previously infected with IxBSR and subsequently treated with TNFα (15 ng/ml for 8 h). In C, binding to the NF-κB probe was assessed in cells previously infected with TAM67 and subsequently treated with TNFα (15 ng/ml for 8 h). Note that TAM67 treatment blocked the increase in NF-κB binding following TNFα treatment. The arrows to the right point to specific complexes that were supershifted in the presence of specific antibodies.
MAT2A promoter in HCC are NF-κB and AP-1. In the current work we tested the hypothesis that MAT2A is also regulated by NF-κB and AP-1.

We first established that in HCC there is also increased binding of NF-κB and AP-1 to the MAT2A promoter. On the other hand, two other transcription factors, NF1 and Nrf-2, did not bind to this region. We next used TNFα as an inducing agent to see if it affects MAT2A expression. TNFα was chosen for several reasons. First is the fact that it is well known to induce both NF-κB and Jun kinase (24, 25). Second, MAT2A is expressed in many conditions, where TNFα is also known to be induced. Specifically, liver regeneration after partial hepatectomy and after ethanol feeding (10, 29). Finally, TNFα is known to act as a mitogen to stimulate cell proliferation (24) and MAT2A is known to be induced under a proliferative state (9, 10, 27). In addition to being a mitogen, TNFα can also elicit a cytotoxic response (25, 30). Hepatocytes are normally resistant to TNFα cytotoxicity but undergoes cell death in the setting of transcriptional or translational arrest, or inactivation of NF-κB (25, 30). Thus, NF-κB activation is thought to turn on several antiapoptotic proteins to resist TNFα toxicity. The role of Jun kinase activation in TNFα cytotoxicity has been more controversial, because both antiapoptotic and proapoptotic effects have been described (25, 30). In our studies we have utilized adenoviral vectors that block either NF-κB or AP-1. We have made certain that under our experimental conditions, there was no difference in apoptosis to complicate interpretation of the results.

TNFα induced MAT2A expression in a time- and dose-dependent manner and binding of NF-κB and AP-1 to the MAT2A promoter. Early induction of NF-κB and AP-1 is likely to occur via activation of existing proteins. However, TNFα treatment also induced the gene expression of both p65 and c-Jun after 2 h. TNFα treatment induced maximally the reporter activity driven by MAT2A promoter construct −571/+60 but not −270/ +60, thus narrows the site of action to −571 to −270 of MAT2A. DNase I footprinting analysis further narrowed this to −352 to −314. The obvious candidates here are NF-κB and AP-1.

To establish an essential role for NF-κB and AP-1 in modulating MAT2A expression, two approaches were taken. First, we blocked their nuclear binding activity by adenoviral vectors that express either IκB or dominant negative c-Jun. To our surprise, we found the basal MAT2A expression lower when NF-κB or AP-1 was blocked. This suggests that the basal expression requires these transcription factors. TNFα was still able to increase MAT2A expression and promoter activity in cells pre-infected with IκB. This suggests that both NF-κB-dependent and -independent mechanisms contribute to TNFα-mediated MAT2A induction. TNFα was not able to increase MAT2A expression in cells pre-infected with dominant negative c-Jun, which suggests that AP-1 trans-activation is the other major mechanism that is responsible for TNFα-mediated MAT2A induction. The second approach was site-directed mutagenesis, which confirmed the importance of these two binding sites in mediating the effect of TNFα on the MAT2A promoter. Consistent with results obtained with the dominant negative constructs, mutation of either of these binding sites lowered the basal promoter activity and blunted the TNFα-mediated increase in promoter activity. When both sites are mutated, the stimulatory effect of TNFα is completely prevented. At the present time we cannot exclude the possibility that there may be other unidentified transcription factors involved in this region. However, taken together, our data show that both AP-1 and NF-κB sites are functional in this promoter and are largely responsible for the TNFα-mediated induction in MAT2A expression.

Why would blocking AP-1 prevent TNFα-mediated MAT2A induction almost completely if NF-κB was important? Gel shift analysis provided a major clue to its mechanism. TNFα treatment was not able to induce NF-κB nuclear binding in cells pre-infected with dominant negative c-Jun. On the other hand, blocking NF-κB with IκB was shown to synergize and potentiate each other's biological function (26). However, a recent study failed to show physical interaction between these two proteins and in fact showed a reciprocal relationship presumably due to competition for co-activator p900 (31). To complicate this further, another recent study in a rat hepatocyte cell line showed inhibition of NF-κB led to a sustained induction of AP-1 (30). In our studies, we did not find binding of p65 to the AP-1 site or c-Jun to the NF-κB site. We also did not observe enhanced AP-1 binding activity when NF-κB is blocked. However, the fact that dominant negative c-Jun was able to block NF-κB activation suggests they do interact in HepG2 cells. One possibility is that the dominant negative c-Jun can bind to p65 and prevent it from binding to NF-κB binding sites. This remains to be examined.

In summary, we have identified NF-κB and AP-1 to be essential for the basal expression of MAT2A and to mediate the stimulatory effect of TNFα on MAT2A promoter activity and expression. This may be one of the key mechanisms for MAT2A up-regulation during rapid growth. In HCC, there is increased nuclear binding of both NF-κB and AP-1 to the MAT2A promoter, and it is likely that increased trans-activation by these two transcription factors also contribute to the transcriptional up-regulation of MAT2A in HCC.

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