Identification of a signal transducer and activator of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression

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Mechanisms of regulation of mouse metallothionein (MT)-I gene expression in response to bacterial endotoxin-lipopolysaccharide (LPS) were examined. Northern blot analysis of hepatic MT-I mRNA in interleukin (IL)-6 or tumour necrosis factor (TNF)-receptor type I knock-out mice demonstrated that IL-6, not TNF-α, is of central importance in mediating hepatic MT-I gene expression *in vivo* after LPS injection. *In vivo* genomic footprinting of the MT-I promoter demonstrated a rapid increase, after LPS injection, in the protection of several guanine residues in the -250 to -300 bp region of the MT-I promoter. The protected bases were within sequences which resemble binding sites for the signal transducers and activators of transcription (STAT) transcription factor family. Electrophoretic mobility-shift assays using oligonucleotides from footprinted MT-I promoter regions showed that injection of LPS resulted in a rapid increase in the specific, high-affinity, *in vitro* binding of STAT1 and STAT3 to a binding site at -297 bp (TTCTCGTAA). Western blotting of hepatic nuclear proteins showed that the time-course for changes of total nuclear STAT1 and STAT3 after LPS injection paralleled the increased complex formation *in vitro* using this oligonucleotide, and binding was specifically competed for by a functional STAT-binding site from the rat α_2-macroglobulin promoter. Furthermore, the MT-I promoter -297 bp STAT-binding site conferred IL-6 responsiveness in the context of a minimal promoter in transient transfection assays using HepG2 cells. This study suggests that the effects of LPS on hepatic MT-I gene expression are mediated by IL-6 and involve the activation of STAT-binding to the proximal promoter.

INTRODUCTION

The acute-phase response is elicited in the host in response to infection, tissue injury or inflammation [1]. One of the major components of this response is the coordinate stimulation of hepatic synthesis of the acute-phase plasma proteins (APPs) [2]. Interleukin-6 (IL-6) is considered to be the principal regulator of most APP genes, but other cytokines including IL-1 and tumour necrosis factor-α (TNF-α) also contribute to stimulation of type-1 APP genes. Glucocorticoids also participate, in a poorly defined way, in the induction of several APPs [1].

The Jak/STAT and the mitogen-activated protein kinase signal transduction pathways play an important role in mediating transcriptional activation by extracellular signals from cytokines and growth factors [3]. The binding of IL-6 to its receptor complex leads to phosphorylation of Jak kinases and subsequent phosphorylation, dimerization and nuclear translocation of STAT proteins. In particular, it is thought that STAT3 plays a central role in the response to IL-6 [4–6]. STAT3 binding to specific sequences in the promoter regions of cytokine-responsive genes leads to activation of transcription [7,8]. The interaction of STAT3 with *cis*-acting promoter elements has been shown to participate in the activation of several APP genes, such as α_2-macroglobulin (α_2MG); [9–11], γ-fibrinogen [12] and C-reactive protein [13].

In addition to the activation of expression of hepatic APPs, inflammation also leads to increased expression of several predominantly intracellular proteins, including haem oxygenase I (HO-I) [14], manganese superoxide dismutase [15] and the metallothioneins (MTs). MTs are cysteine-rich, heavy-metal-binding proteins that have been highly conserved during evolution [16]. Of the four mouse MT genes, the MT-I and MT-II genes are the most widely expressed. These genes are constitutively expressed in the liver and are highly induced in response to a variety of stresses including inflammation [17]. Bacterial endotoxin [lipopolysaccharide (LPS)] is a potent inducer of hepatic MT gene expression [18,19]. Studies using LPS-resistant C3H/HeJ mice suggest that LPS indirectly affects MT gene expression in the liver by activation of inflammatory cytokines [18]. Injection of a variety of cytokines, such as IL-1, IL-6, TNF-α and interferon (IFN), can induce hepatic MT gene expression *in vivo* [18,20–22]. IL-6 has been suggested to be a direct mediator of hepatic MT gene expression in mice [18,22–24]. Studies of rat hepatocytes cultured *in vitro* support the concept that IL-6, in combination with zinc and glucocorticoids, directly mediates MT gene expression during inflammation [25,26]. However, a direct role for IL-6 in mediating MT-I gene expression *in vivo* has not been directly demonstrated.

Studies of the mouse MT-I promoter in transgenic mice indicate that the LPS-responsive region is located between -185 bp and -350 bp of the proximal promoter [19], but the transcription factors involved have yet to be identified. The present study used IL-6 and TNF-α receptor type 1 knockout mice to demonstrate that IL-6 plays a key role in LPS induction

Abbreviations used: APPs, acute-phase plasma proteins; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; βGal, β-galactosidase; HO-I, haem oxygenase I; IFN, interferon; IL, interleukin; LM-PCR, ligation-mediated PCR; LPS, lipopolysaccharide; α_2MG, α_2-macroglobulin; MRE, metal regulatory elements; MT, metallothionein; NP40, Nonidet P40; SAA, serum amyloid A; TNF, tumour necrosis factor; USF/ARE, composite upstream stimulating factor/antioxidant response element.

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of hepatic MT-I gene expression. Analyses of protein interactions *in vivo* and *in vitro* with the MT-I promoter suggest that LPS induces activation of STAT1 and STAT3 binding to a site at −297 bp, and transfection studies demonstrated that this site is IL-6 responsive. Therefore, regulation of the mouse MT-I gene during inflammation is likely to be mediated, in part, by the same signal transduction cascades that regulate expression of genes encoding APPs.

EXPERIMENTAL

Materials and methods

Tissue collection, RNA isolation and Northern blot analysis

All experiments involving animals were conducted in accordance with National Institutes of Health guidelines for the care and use of experimental animals. Two- to three-month old C57BL/6, 129/Sv and F1 (C57BL/6 × 129/Sv) wild-type (non-transgenic) and homozygous IL-6 (IL-6$^{-/-}$) knockout mice [27] and homozygous TNF-receptor type I (TNFR1$^{-/-}$) knockout mice [28] were injected with LPS (0.5 mg/ml, *Escherichia coli* serotype 0127:B8; Sigma Chemical Co, St. Louis, MO, U.S.A.) intraperitoneally at a dosage of 100 μg/mouse. Livers were collected at 0, 1, 3.5 and 8 h after treatment and frozen immediately in liquid nitrogen. RNA was extracted from frozen liver as described previously [29], separated by 2.2 M formaldehyde/1 % agarose gel electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Keene, NJ, U.S.A.) and cross-linked by UV irradiation as described [29,30]. Blots were prehybridized, hybridized and washed as described [18,30]. Blots were hybridized successively with ^{32}P-labelled cRNA probes for mouse MT-I [18], HO-I [29] and serum amyloid A (SAA) [31]. Membranes were stripped of probe before each rehybridization, as described [32]. Hybrids were detected by autoradiography at −70 °C with intensifying screens. The blot was further exposed to a phosphor screen, scanned with a PhosphorImager SI, and quantified using the ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). In all experiments, duplicate gels were stained with Acridine Orange to verify integrity and equal loading of RNA.

In vivo footprinting

Nuclei were prepared from freshly collected pieces of liver by homogenization, with a glass–Teflon homogenizer, in 50 vols. (v/w) of homogenization buffer [10 mM Hepes, pH 7.9/10 mM KCl/1.5 mM $MgCl_2$/0.1 % Nonidet P40 (NP40)/0.5 mM dithiothreitol (DTT)/0.2 mM PMSF/0.7 μg/ml pepstatin A/0.5 μg/ml leupeptin] on ice. Nuclei were collected by centrifugation at 800 ***g*** (2500 rev./min; Sorvall, HB-4) for 5 min at 4 °C, and washed once with homogenization buffer without NP40. Nuclei were suspended in 10 mM sodium phosphate buffer (pH 7.4) and exposed to 0.1 % dimethyl sulphate for 4 min at room temperature. Genomic DNA was purified and subjected to piperidine cleavage at the positions of methylated guanines as described elsewhere [33,34]. Piperidine-cleaved DNA (1 μg) was then amplified by ligation-mediated PCR (LM-PCR), using mouse MT-I promoter-specific primers that have been described previously [34].

Preparation of nuclear extracts

Nuclear extracts were prepared with modifications of the method of Sadowski and Gilman [35] to measure STAT-binding activity. Briefly, a freshly collected piece of liver was homogenized with a glass–Teflon homogenizer at 4 °C in 50 vols. (v/w) of homogenization buffer (10 mM Hepes, pH 7.9/10 mM KCl/1.5 mM $MgCl_2$/0.1 % NP40/1 mM DTT/20 mM NaF/1 mM EDTA/1 mM EGTA/1 mM Na_3VO_4/0.5 mM PMSF/1 μg/ml pepstatin A/1 μg/ml leupeptin/1 μg/ml aprotinin). Nuclei were collected by centrifugation at 800 ***g*** (Sorvall, HB-4) for 5 min at 4 °C, and washed once with homogenization buffer without NP40. The nuclei were suspended in 3 vols. of nuclear extraction buffer [20 mM Hepes, pH 7.9/420 mM KCl/1.5 mM $MgCl_2$/25 % (w/v) glycerol/1 mM DTT/20 mM NaF/1 mM EDTA/1 mM EGTA/1 mM Na_3VO_4/0.5 mM PMSF/1 μg/ml pepstatin A/1 μg/ml leupeptin/1 μg/ml aprotinin]. The nuclear suspension was stirred for 30 min on ice, and then centrifuged at 100000 ***g*** (Beckman, TL-100) for 30 min at 4 °C. The supernatant was collected and concentrated in a Microcon3 microconcentrator (Amicon Inc., Beverly, MA, U.S.A.) by centrifugation at 25000 ***g*** (Sorvall, SS-34) for 3 h at 4 °C. The concentrated extract was diluted with an equal volume of dilution buffer (extraction buffer without KCl) and frozen in aliquots at −80 °C. The concentration of nuclear proteins was determined using the Bradford method with BSA as a standard (Bio-Rad, Hercules, CA, U.S.A.).

Electrophoretic mobility-shift assay (EMSA)

STAT binding activity was detected as follows. Hepatic nuclear proteins (5 μg) were incubated in binding reaction buffer containing 12 mM Hepes (pH 7.9), 60 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 12 % (w/v) glycerol, 5 mM $MgCl_2$, 2 μg of poly(dI)·poly(dC), 0.5 mM PMSF and 2–3 fmol of end-labelled double-stranded oligonucleotide (5000 c.p.m./fmol) in a total volume of 20 μl for 20 min at room temperature. MT-I promoter oligonucleotides and the rat α_2MG promoter functional STAT-binding site [10] oligonucleotide sequences were as follows:

MT-I/−297 5′GATCGAG**TTCTCGTAA**ACTC3′

rat α_2M 5′GATCC**TTCTGGGAA**TTCCTA3′

MT-I/−277 5′GATCGCGAT**AGGCCGTAATATCGGGGAA**AGCACTA3′

In binding-site competition experiments, a 100-fold molar excess of unlabelled double-stranded oligonucleotide was included in the EMSA binding reaction. In antibody supershift assays, the indicated antiserum (1 μl) was added to the binding reaction followed by addition of the labelled oligonucleotide. The entire mixture was incubated for 30 min at room temperature. The antibodies against Sp1 (sc-059x), STAT1 (sc-346x), STAT3 (sc-482x), STAT5b (sc-835x) and glucocorticoid receptor (sc-1002x) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA, U.S.A.). The EMSA gel was polymerized and then pre-run at 4 °C in buffer (pH 8.5) consisting of 25 mM Tris, 0.19 M glycine and 0.5 mM EDTA. Protein–DNA complexes were separated electrophoretically in a 4 % polyacrylamide gel at 15 V/cm at 4 °C. After electrophoresis, the gel was dried and labelled complexes were detected by autoradiography.

Western blotting

Equal amounts (5 μg) of nuclear extract were subjected to SDS/10 % PAGE with the discontinuous buffer formulation of Laemmli [36] and transferred to nitrocellulose membranes (Schleicher and Schuell), using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were incubated in a blocking solution of 5 % non-fat dried milk in TBST buffer (10 mM Tris/HCl, pH 7.4/150 mM NaCl/0.5 % Tween-20) overnight at 4 °C. The membrane was incubated with antiserum in the same blocking solution for 1 h at room temperature and then washed in TBST buffer. The membrane was then incubated in TBST containing goat anti-rabbit immunoglobulin conjugated

with horseradish peroxidase for 1 h at room temperature and washed with TBST buffer. Specific protein complexes were visualized with the enhanced chemiluminescence (ECL) system (Amersham Life Sciences, Arlington Heights, IL, U.S.A.).

Luciferase reporter plasmids

Two tandem copies of the MT-I/−297 oligonucleotide, two tandem copies of the rat α_2MG oligonucleotide or a single copy of an oligonucleotide spanning the −300 bp to −251 bp region of the MT-I promoter were subcloned in front of the MT-I minimal promoter (−42 to +62) [29] in the luciferase reporter (Luc) vector, pGL-2 basic (Promega Biotech, Madison, WI, U.S.A.). The TATA box and transcription start point were provided by the MT promoter in these Luc fusion genes. Oligonucleotides were synthesized by the Biotechnology Support Center (University of Kansas Medical Center, Kansas City, KS, U.S.A.).

Transient transfection assay

Human HepG2 cells were obtained from the American Type Culture Collection (Rockville Pike, MD, U.S.A.). HepG2 cells were cultured in complete medium (Dulbecco's modified Eagle's medium)-rich glucose supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin/100 μg/ml streptomycin. Cells were seeded at a density of 70000 per well in 24-well plates and incubated for 36 h at 37 °C in a humid atmosphere of 5% CO_2 in air. Cells were transfected using LipofectAMINE reagent according to the manufacturer's suggestions (Life Technologies, GIBCO BRL, Gaithersburg, MD, U.S.A.). Each well was incubated for 5 h at 37 °C in 0.25 ml of Dulbecco's modified Eagle's medium containing 2 μl of LipofectAMINE (2 mg/ml), 200 ng of SV-βGal (Promega Biotech) transfection control plasmid and 300 ng of Luc reporter plasmid. Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (0.25 ml) was added to each well and the incubation was continued for 18 h. This medium was replaced with 0.5 ml of complete medium and, after 8 h, human recombinant IL-6 (Preprotech Inc., Rockhill, NJ, U.S.A.) was added (2 ng/ml or 20 ng/ml). The cells were treated with IL-6 for 18 h and then lysed and assayed for Luc and β-galactosidase (βGal) activities using the luminescence assays, as described [37]. βGal activity was used to correct for transfection efficiency. Each experimental condition tested was replicated six times within each experiment, and the entire experiment was repeated at least twice. Statistical significance was determined using analysis of variance and Student's t-test. Differences were considered significant when the P value was < 0.001. Values are given as means ± S.E.M.

RESULTS

LPS-induced hepatic MT-I gene expression is severely attenuated in IL-6$^{-/-}$ mice, but not in TNFR1$^{-/-}$ mice

The effects of LPS injection on hepatic MT-I mRNA levels was examined in non-transgenic mice and in IL-6$^{-/-}$ and TNFR1$^{-/-}$ mice. In non-transgenic mice, a dramatic (> 60-fold) induction of MT-I mRNA was noted at 8 h after injection. Similar results were obtained using three strains of mice as wild-type (non-transgenic) controls: C57BL/6, 129/Sv and F1. C57BL/6 × 129Sv F1 mice served as non-transgenic controls in the experiments shown (Figure 1).

In the IL-6$^{-/-}$ mice, LPS induction of MT-I mRNA was attenuated to only 14% of that found in the non-transgenic mice at 8 h (Figure 1A). In sharp contrast, the time-course and extent of induction of MT-I mRNA in TNFR1$^{-/-}$ mice was indistinguishable from that in the non-transgenic controls (Figure 1A). Changes in SAA mRNA were monitored as a positive control for the acute-phase response [38]. In non-transgenic mice, a dramatic induction (> 80-fold) of hepatic SAA mRNA was noted by 8 h after LPS injection. LPS induction of this mRNA was severely attenuated in both the IL-6$^{-/-}$ and the TNFR1$^{-/-}$ mice and the effect of IL-6 deficiency (24% of non-

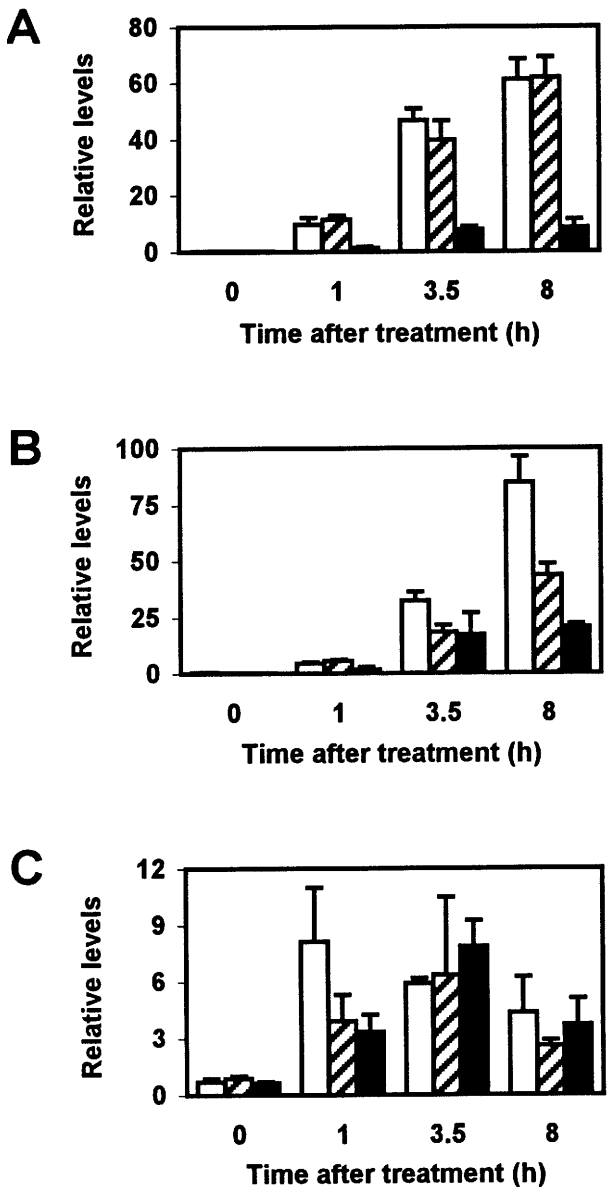

Figure 1 Northern blot analysis of hepatic MT-I, SAA and HO-1 mRNA levels in IL-6 and TNFR1 knockout mice after LPS injection

Hepatic RNA was isolated from C57BL/6 × 129/sv (open bar), TNFR1$^{-/-}$ (hatched bar) and IL-6$^{-/-}$ (closed bar) mice at the indicated times after an intraperitoneal injection of LPS (100 μg/mouse). Total RNA (2 μg) was separated by formaldehyde/agarose gel electrophoresis, blotted onto a nylon membrane and hybridized successively with each of the indicated ^{32}P-labelled cRNA probes: (**A**) MT-I probe; (**B**) SAA probe; (**C**) HO-I probe. Total RNA from 3–5 individual mice per group was analysed. Hybrids were quantified by phosphorimage analysis as described in the Materials and methods section; and each bar represents the mean ± S.E.M. of three to five individual determinations.

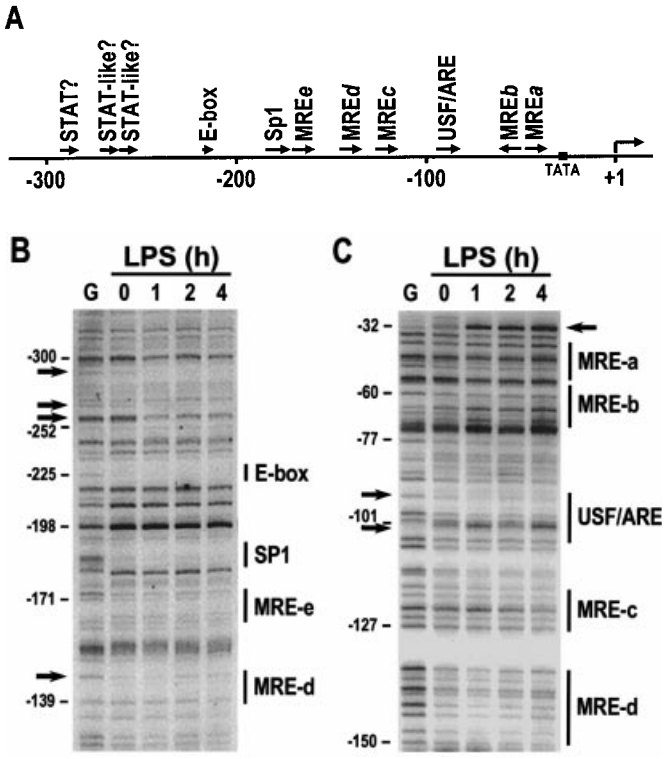

Figure 2 ***In vivo*** **genomic footprinting of the hepatic MT-I promoter after LPS injection**

Liver was collected from CD-1 mice at the indicated time (0–4 h) after an injection of LPS (100 μg/mouse). Nuclei were isolated and treated with 0.1% dimethyl sulphate to methylate guanine residues, as described in the Materials and methods section. Methylated genomic DNA was extracted, cleaved with piperidine and the proximal region of the MT-I promoter was specifically amplified using LM-PCR. The G-ladder (G) was generated by LM-PCR of naked genomic DNA methylated *in vitro*. LM-PCR products were separated on a 6% sequencing gel and detected by autoradiography. Guanine residues are numbered relative to the transcription start point in the mouse MT-I promoter. (**A**) Schematic representation of the mouse MT-I proximal promoter (+1 to −300 bp relative to the transcription start point). The locations of known regulatory elements in the MT-I promoter are as indicated: MRE, metal regulatory elements (a–e); Sp1, Sp1 binding site; USF/ARE, composite upstream stimulating factor/antioxidant response element; E-box, high affinity USF binding site; STAT, signal transducer and activator of transcription binding site consensus sequence or STAT-like sequence with lower identity. (**B**) Sense-strand amplification. (**C**) Antisense-strand amplification. Arrows demarcate guanine residues which changed (protection or hypersensitivity) in response to dimethyl sulphate methylation after LPS injection.

transgenic) was greater than that of TNFR1 deficiency (51% of non-transgenic) at 8 h after injection (Figure 1B). HO-1 mRNA, a gene induced by oxidative stress [39], was also monitored as a control (Figure 1C). LPS induction of HO-I mRNA was significant (7–9-fold) and variable among individual animals, but not dependent on IL-6 or TNFR1.

LPS induces changes in *in vivo* protein–DNA interactions within the proximal 300 bp of the MT-I promoter

Studies of the mouse MT-I promoter in transgenic mice indicate that the LPS-responsive region is located between −185 bp and −350 bp [19]. We examined protein–DNA interactions within the region from −350 to −30 bp by *in vivo* genomic footprinting using LM-PCR [34] (Figure 2A). Hepatic nuclei were isolated from mice at the indicated times after LPS injection and treated *in vitro* with dimethyl sulphate [33]. Guanine residues involved in protein–DNA interactions were visualized as either less intense (protected) or more intense (hypersensitive) relative to DNA from control mice and relative to surrounding bases within the same region of the gel. Genomic footprinting was repeated at least three times using DNA prepared from separate experiments and the results were reproducible (Figures 2B and 2C).

In control mouse liver, constitutive footprints were evident over the composite upstream stimulating factor/antioxidant response element (USF/ARE), Sp1 and E-box sites (Figures 2A and 2B). Similar results have been reported previously in cultured mouse cell lines [34,40]. *In vivo* footprinting was not noted over the metal regulatory elements (MRE) core sequences in control liver, nor in the putative LPS-responsive region upstream of the E-box (−225 bp) (Figure 2A). However, by 1 h after LPS injection several new footprints were noted. Guanine −292 was protected. Similarly, guanines in a four guanine cluster at −256 to −259, and guanine −267 were protected. These guanines remained protected for at least 4 h after LPS injection. In contrast, a weak, transient footprint was noted over MRE-d. Guanine −146, in the MRE core sequence, was protected up to 1 h after LPS, but not thereafter (Figure 2B). No footprints were detected over other MRE sequences (Figures 2B and 2C).

Within the more proximal MT-I promoter, after LPS injection, guanine −32 became strikingly hypersensitive (Figure 2C) and remained so for up to 4 h. This base is immediately upstream of the TATA box (Figure 2A). In addition, a change in the constitutive footprint over the USF/ARE was noted by an apparent increase in the hypersensitivity of guanine −101 and by increased protection of guanine −94. Metals and oxidative stresses also cause changes in the *in vivo* footprint around the USF/ARE [34].

Identification of a STAT-binding site at −297 bp in the MT-I promoter and of LPS-dependent *in vitro* binding of STAT1 and STAT3

Analysis of the MT-I promoter region from −250 bp to −300 bp, around those bases *in vivo* footprinted after LPS injection (Figure 2), revealed three STAT-like binding sites. The consensus STAT sequence (TTN_5AA) is present beginning at −297 bp and this sequence (TTCTCGTAA) is similar to the IL-6-responsive STAT-binding site (TTCTGGGAA) in the rat α_2MG promoter [10]. Two tandem STAT-like binding sites were located at −272 bp and −262 bp. Although neither conforms to the STAT-binding consensus sequence, the last five bases of the −272 bp site are identical with those of the −297 bp site (CGTAA), and seven bases of the −262 bp site (**ATCGGGGAA**, indicated in bold) are identical with those in the α_2MG site.

EMSA was used to detect protein interactions with oligonucleotides containing the −297 bp site (MT-I/-297) or an oligonucleotide spanning the −272 bp and −262 bp sites (MT-I/-277) (see below). Hepatic nuclear protein was extracted from liver taken after LPS injection. Two MT-I/-297 binding complexes (I and II; Figure 3A) were detected by 1 h and remained until 4 h after LPS injection. The amount of complex I increased between 1 h and 4 h, whereas the amount of complex II was apparently maximal by 1 h and declined by 4 h. Formation of both binding complexes was effectively inhibited by a 100-fold molar excess of unlabelled MT-I/-297 oligonucleotide, as well as by a 100-fold molar excess of unlabelled rat α_2MG oligonucleotide (Figure 3B). Supershift analysis using antiserum specific for STAT1 and STAT3 showed that complex I contained STAT1 and complex II contained STAT3 (Figure 3B). Neither complex was supershifted using antisera against STAT5, Sp1 or glucocorticoid receptor.

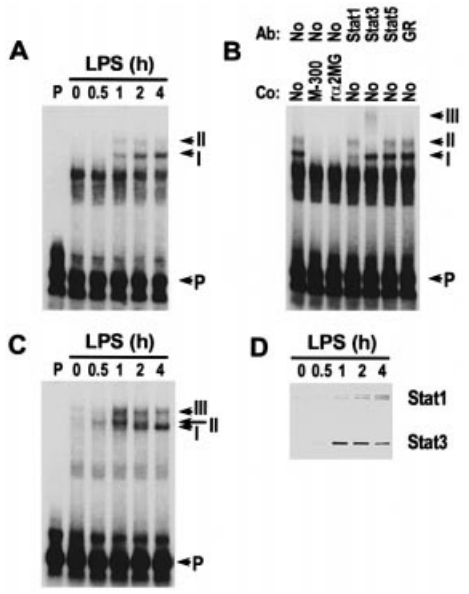

Figure 3 EMSA detection of LPS-induced STAT1 and STAT3 binding to the −297 bp region of the mouse MT-I promoter and Western blot detection of nuclear STAT1 and STAT 3 proteins in the liver

EMSA was performed using hepatic nuclear extracts prepared at the indicated times after LPS injection (100 μg/mouse). Nuclear protein (5 μg) was incubated with the ^{32}P-labelled double-stranded MT-I/-297 oligonucleotide (**A** and **B**) or the labelled rat α_2MG oligonucleotide (**C**) for 20 min at room temperature, as described in the Materials and methods section. Protein–DNA complexes were analysed by 4% polyacrylamide-Tris/glycine gel electrophoresis and detected by autoradiography. P, free probe. The arrows indicate specific binding complexes (I and II). (**B**) Nuclear extract was prepared 2 h after LPS injection. Co refers to EMSA binding reactions which also contained a 100-fold molar excess of the indicated unlabelled double-stranded oligonucleotide as a competitor. Ab refers to EMSA supershift reactions which contained the indicated STAT antiserum. The arrows indicate specific MT-I/−297 binding complexes: I, STAT1; II, STAT3. Complex III is the supershifted STAT3 complex. (**D**) Hepatic nuclear proteins (5 μg) were analysed by Western blotting using rabbit anti-STAT1 or anti-STAT3 antisera. Antigen–antibody complexes were detected using goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase and enhanced chemiluminescence, as described in the Materials and methods section.

EMSA using the α_2MG oligonucleotide was compared with that using the MT-I/-297 oligonucleotide. Three LPS-dependent α_2MG oligonucleotide-binding complexes (I, II and III) were detected using liver nuclear extracts (Figure 3C). The relative mobility and time-course changes in amounts of complexes I and III were similar to those of complexes I and II formed with the MT-I/-297 oligonucleotide respectively (Figures 3A and 3C). Supershift EMSA demonstrated that complex I with the α_2MG oligonucleotide contains STAT1 and complex III contains STAT3 (results not shown). The STAT1 antisera inhibited formation of complex I and the STAT3 antisera supershifted complex III. Complex II was not supershifted or inhibited with STAT1, STAT3 or Sp1 antisera (results not shown).

Western blotting was used to monitor the time course of LPS-induced appearance of STAT1 and STAT3 proteins in the nuclei from liver cells (Figure 3D). STAT1α and STAT1β levels were detectably increased at 1 h after LPS injection and continued to increase until 4 h. In contrast, STAT3 levels were detectably increased by 0.5 h after LPS injection and were high at 1 h, after which they declined. Therefore, the changes in total nuclear STAT1 and STAT3 proteins in the liver after LPS injection paralleled the amount of STAT complex formation *in vitro* with both the MT-I and the α_2MG oligonucleotides.

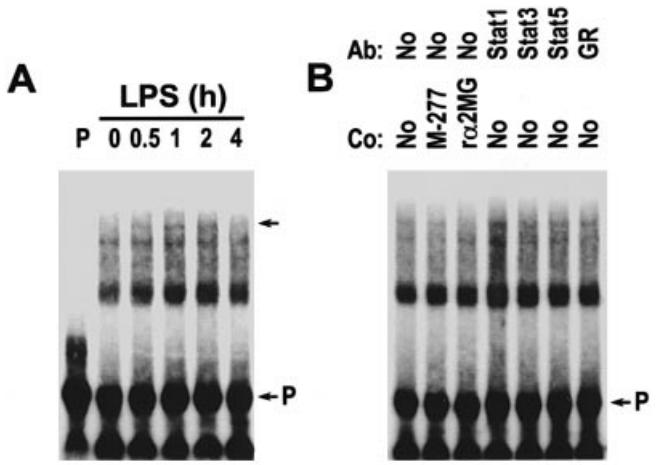

Figure 4 EMSA detection of LPS-induced binding of hepatic nuclear protein to the −277 bp region of the mouse MT-I promoter

EMSA was performed using hepatic nuclear extracts prepared at the indicated times after LPS injection. Nuclear protein (5 μg) was incubated with the ^{32}P-labelled double-stranded MT-I/−277 oligonucleotide for 20 min at room temperature, as described in the Materials and methods section. Protein–DNA complexes were analysed by 4% polyacrylamide-Tris/glycine gel electrophoresis and detected by autoradiography. P, free probe. The arrow indicates a binding complex that increases after LPS injection. (**B**) Nuclear extract was prepared 2 h after LPS injection. Co refers to EMSA binding reactions which also contained a 100-fold molar excess of the indicated unlabelled double-stranded oligonucleotide as a competitor. Ab refers to EMSA supershift reactions which contained the indicated STAT antiserum. No STAT supershift was detected and competition with excess unlabelled oligonucleotide was incomplete.

LPS-induced *in vitro* binding activity for the −277 bp region of MT-I promoter does not involve STAT proteins

The two tandem STAT-like binding sites, located at −272 bp and −262 bp, were footprinted *in vivo* after LPS treatment, although neither site conforms to the STAT-binding consensus sequence. EMSA revealed two relatively faint complexes with the MT-I/−277 oligonucleotide using nuclear extracts from liver (Figure 4A). One of these complexes was transiently induced after LPS injection, and reached a peak at 1 h, after which it decreased. Competition experiments demonstrated that formation of this binding complex was completely inhibited by a 100-fold molar excess of unlabelled rat α_2MG oligonucleotide, but not by excess unlabelled MT-I/−277 oligonucleotide (Figure 4B). Supershift EMSA using antisera specific for STAT1, STAT3, STAT5 and glucocorticoid receptor suggest that none of these proteins are a part of this binding complex (Figure 4B).

The STAT binding site at −297-bp in the MT-I promoter directs the response to IL-6 in transiently transfected HepG2 cells

Transient transfection assays were used to determine whether the −297 bp STAT-binding site (MT-I/-297) or the −300 bp to −251 bp region of the MT-I promoter represent functional IL-6-responsive promoter elements. Human HepG2 cells were transfected in these experiments. This cell line has been used extensively to study IL-6 regulation of acute-phase protein gene expression [2,10,13]. Two tandem copies of the MT-I/−297 oligonucleotide or the rat α_2MG oligonucleotide were cloned in front of the minimal MT-I (−42 bp) promoter in a Luc expression vector. These constructs were compared in transfection assays, since it is known the rat α_2MG oligonucleotide is IL-6 responsive under these conditions [2]. A single copy of the −300 bp to −251 bp oligonucleotide was also cloned into this vector and

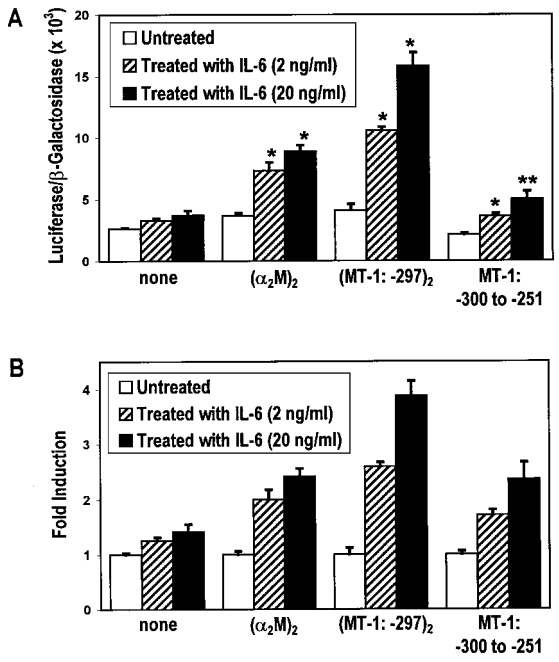

Figure 5 The STAT binding site at −297 bp in the mouse MT-I promoter directs the response to IL-6 in transiently transfected HepG2 cells

The MT-I/−297 and the rat α_2MG were each cloned as two tandem copies in the forward orientation in front of a minimal (−42 bp) MT-I promoter [29] driving Luc expression. In addition, a single copy of the MT-I promoter region from −300 bp to −251 bp was cloned in this reporter construct. HepG2 cells were transfected using LipofectAMINE reagent containing 200 ng of SV-βGal transfection control plasmid and 300 ng of the indicated Luc reporter plasmid. Transfected cells were treated for 18 h with human recombinant IL-6 at 2 ng/ml or 20 ng/ml, as indicated. The cells were treated with IL-6 for 18 h and then lysed and assayed for Luc and βGal activities. (**A**) Luc activity was normalized to βGal activity to correct for transfection efficiency and the values shown are means ± S.E.M. of six determinations. *P value was < 0.001 relative to untreated. **P value was 0.0013 relative to untreated. (**B**) Data from (**A**) are expressed as fold induction in the IL-6-treated samples relative to the untreated control for each of the Luc reporter constructs.

examined in these assays. HepG2 cells were transfected and then treated overnight with human recombinant IL-6 (Figure 5). Under these conditions, none of these promoter constructs had an increased basal level of expression relative to the −42 bp promoter (Figure 5A). However, each of the oligonucleotides examined conferred IL-6 responsiveness on the −42 bp promoter. The response was dose dependent, was detectable at an IL-6 concentration of 2 ng/ml and was near maximal at 20 ng/ml (higher concentrations are not shown). Luciferase expression driven by the MT-I/−297 and the rat α_2MG oligonucleotides was increased 3.9- and 2.4-fold respectively. In repeated experiments, the −300 to −251 promoter element was responsive to IL-6, whereas the −42 bp promoter was not. These results suggest that IL-6 responsiveness of the MT-I promoter involves the −300 bp to −251 bp region which contains a STAT binding site.

DISCUSSION

Expression of mouse MT genes is upregulated in response to a variety of stresses including inflammation [17,41–43]. The observation that hepatic expression of MT is dramatically elevated in response to bacterial infection, an effect mediated by endotoxin (LPS) [41], has led to the classification of MT as an acute-phase protein. This effect of LPS on hepatic MT gene expression has been conserved during evolution [44], which suggests a fundamental physiological role of MT in the acute-phase response. Results presented herein suggest that IL-6 is a direct mediator of hepatic MT gene expression in mice exposed to endotoxin, as it is for the expression of many APPs [1]. Induction of SAA, haptoglobin, α-1 acid glycoprotein, α_2MG and serum amyloid P is dramatically decreased in IL-$6^{-/-}$ mice during acute-phase responses [27,45,46]. Therefore, the effects of other cytokines on hepatic MT gene expression, reported previously [18,21,22,47,48], may be attributable to IL-6. Consistent with this concept, IL-6 has little effect on IL-1 or TNF-α gene expression in the mouse liver. In contrast, IL-1 or TNF-α can rapidly induce IL-6 mRNA in the liver [18]. Recent studies demonstrate that antiserum against IL-6 can block the ability of serum from LPS-treated mice to induce expression of MT in a hepatoma cell line [24], and IL-6 has been shown to induce MT gene expression in rat hepatocyte cultures [25,26]. Remarkably, IL-6 has also been suggested to play a key role in hepatic MT gene expression during inflammation caused by organic solvents [23] and by immobilization stress (J. Carrasco, J. Hernandez, H. Bluethmann, G. K. Andrews and J. Hidalgo, unpublished work).

Multiple promoter elements may be involved in LPS-induced MT-I gene expression. In the mouse liver and in mouse Hepa cells, MT-I is expressed constitutively and it appears that constitutive interactions of Sp1-, USF- (at two sites) and ARE-binding proteins occur within the proximal 250 bp of the MT-I promoter [34,49]. In addition to those protein–DNA interactions, LPS apparently induces the binding of STAT proteins (1 and 3) to a binding site at −297 bp. Furthermore, changes in protein–DNA interactions occur downstream of the STAT-binding site, near the TATA box and at the USF/ARE. Thus, efficient induction of MT-I gene transcription during inflammation is likely to involve the cooperation of multiple promoter elements.

The functionality of the MT-I promoter STAT site was demonstrated in transiently transfected cells. This site maps within a region of the MT-I promoter that is important for LPS responsiveness *in vivo* [19], and LPS induces STAT1 and STAT3 binding to this site, as suggested by increased *in vitro* binding and increased *in vivo* protein interactions at this site. Furthermore, STAT1 and STAT3 binding *in vitro* to the MT-I STAT-binding site was qualitatively similar to that with the rat α_2MG IL-6-responsive element (STAT-binding site) [10,11]. These results suggest that the Jak/STAT signal-transduction cascade potentiates transcription of the MT-I gene, as it does the APP genes [27]. The individual roles that activated STAT1 and/or STAT3 may play in regulating MT-I gene expression remain to be determined. STAT1 and STAT3 are activated by a variety of growth factors and cytokines, including IL-6 [7,8,50]. However, studies of STAT1 knockout mice demonstrate that STAT1 functions to regulate genes that provide innate immunity [51]. Thus, loss of STAT1 activity attenuates the response to IFN, but not to several other cytokines [8,51]. Whether STAT1 is important for IL-6 activation of MT-I gene expression has not been demonstrated. However, LPS induction of the mouse MT-I gene is not attenuated in IFN-γ receptor knockout mice [52]. STAT3 is expressed in many cell-types [53], and is activated by the IL-6 family of cytokines [4,7,10,54]. STAT3 is also activated in the liver in response to LPS [50]. Targeted disruption of the mouse STAT3 gene is lethal to the embryo, but over-expression of dominant negative mutants of STAT3 block IL-6 activation of murine erythroleukaemic cells [5,55]. Therefore, STAT3 plays an important role in transcriptional activation of IL-6-responsive genes.

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