Induction of *Mdr1b* Expression by Tumor Necrosis Factor-α in Rat Liver Cells Is Independent of p53 but Requires NF-κB Signaling

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The multidrug resistance protein *Mdr1b* in rats is up-regulated during liver regeneration after partial hepatectomy or after endotoxin treatment. We hypothesize that up-regulation of *Mdr1b* in these models is TNF-α–dependent. The mechanism of *Mdr1b* activation by TNF-α is unknown as TNF-α can signal through various pathways, including NF-κB and p53, transcription factors for which binding sites in the *Mdr1b* promoter have been identified. We aimed to elucidate the mechanism of up-regulation of *Mdr1b* by TNF-α. We selectively used constructs expressing dominant negative Fas-associated death domain protein (FADD), TNF receptor associated factor-2 (TRAF2) or IκB to inhibit pathways downstream of the TNF receptor. Further, the proteasome inhibitor MG-132 was used, which prevents the breakdown of IκB. We show a critical role for NF-κB in activation of *Mdr1b* gene expression both in primary rat hepatocytes and in rat hepatoma H-4-II-E cells. Because p53 is up-regulated by TNF-α in an NF-κB–dependent manner and the *Mdr1b* promoter contains a p53 binding site, we used liver cells expressing a dominant negative p53 to show that TNF-α up-regulation of *Mdr1b* is independent of functional p53. Using transient transfection assays, we show that *Mdr1b* up-regulation correlates with activation of the promoter. Mutation of the NF-κB site in the *Mdr1b* promoter prevents its induction by TNF-α. In conclusion our results show that activation of the rat *Mdr1b* gene by TNF-α is a result of NF-κB signaling and independent of p53. (HEPATOLOGY 2001;33:1425-1431.)

The human MDR1 (ABCB1) and its rodent homologues Mdr1a and Mdr1b belong to subcluster B of the ATP-binding cassette transporter superfamily. These transporters can confer multidrug resistance (MDR) to cells by functioning as efflux pumps for cytotoxic drugs (for review see Klein et al[1]). MDR1 and Mdr1a/1b are expressed in the blood-brain barrier, small intestine, kidney, and, to a lesser extent, in the liver where they presumably have an important role in the elimination of endogenous and exogenous compounds.

Whereas the expression of hepatic *Mdr1a* is largely unchanged, rat *Mdr1b* is up-regulated under various conditions and appears to be a "stress-responsive" gene. In cell culture, *Mdr1b* expression is influenced by the extracellular matrix.[2,3] In addition, *Mdr1b* expression increases in response to, for example, cytotoxic drugs,[4-6] carcinogens,[7-9] insulin,[10] or hydrogen peroxide.[11] *In vivo*, *Mdr1b* expression is increased in hepatocarcinogenesis, during liver regeneration, and endotoxin-induced cholestasis.[12-14] The cytokine tumor necrosis factor-α (TNF-α) plays an essential role both in liver regeneration after partial hepatectomy[15,16] and in lipopolysaccharide-induced endotoxemia.[17] We therefore speculate that TNF-α is, at least in part, responsible for the up-regulation of *Mdr1b* observed in these models. It has been reported that *Mdr1b* expression can be induced by TNF-α in cultured rat hepatocytes,[18] but the underlying mechanisms have not been elucidated.

TNF-α induces a well-described signaling cascade leading to caspase activation, but also leads to activation of NF-κB and MAP kinases. Activation of caspases involves binding of TNF-receptor-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD) to the TNF-R1 receptor. Caspase activation results in apoptosis when anti-apoptotic pathways are not effective. Activation of MAP kinases and NF-κB by TNF-α involves a cascade of factors, starting with TRADD and TNF-receptor associated factor-2 (TRAF2)/receptor-interacting protein (RIP). NF-κB activation subsequently requires activation of NF-κB-inducing kinase (NIK), and IκB kinases (IKK). Alternative pathways for the activation of IκB kinases have been shown. Reactive oxygen species, that are induced by TNF-α,[19,20] can directly act on the IκB kinases.[9,21] NF-κB is retained in the cytoplasm by IκB. For activation of NF-κB, phosphorylation of IκB by IκB kinases is essential, followed by its ubiquitination and degradation by the 26S proteasome. This releases the NF-κB subunits, which then translocate to the nucleus (for review see Wallach et al,[22] and Ashkenazi and Dixit,[23]). Thus, inhibition of NF-κB signaling can occur by either blocking proteasome mediated degradation with chemical proteasome inhibitors or using mutant IκBs that cannot be phosphorylated.[24]

Oxidative stress, as induced by TNF-α, results in activation of p53.[25] Various cellular mechanisms have been found to

Abbreviations: TNF-α, tumor necrosis factor-α; FADD, Fas-associated death domain protein; TRAF2, TNF-receptor-associated factor-2; MDR, multidrug resistance; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; Luc, luciferase.

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activate p53, including NF-κB.[26] The *Mdr1b* promoter contains a p53-binding site, and therefore *Mdr1b* induction by TNF-α can involve p53.[4] Moreover, NF-κB and p53 have been shown to functionally interfere with each others' transcriptional activation.[27,28] It is unknown how these 2 pathways interact in the regulation of *Mdr1b* by TNF-α because the *Mdr1b* promoter contains an NF-κB binding site in proximity to a p53 binding site.[4,10]

The aim of the present study was to show the intracellular mechanisms of TNF-α–induced *Mdr1b* expression in liver cells. We used *in vitro* cultures of either primary rat hepatocytes or rat hepatoma cells and modulated the TNF-α–induced pathways with specific inhibitors. The role of p53 in the up-regulation of *Mdr1b* by TNF-α was evaluated using rat hepatoma cells expressing dominant negative p53.

MATERIALS AND METHODS

Materials. Cell culture media, fetal calf serum (FCS), and oligonucleotides were from Life Technologies Ltd. (Paisley, UK). Antibiotics came from BioWhittaker (Walkersville, MD). Recombinant mouse TNF-α was purchased from R&D Systems (Abingdon, UK).

Cells and Culture Conditions. Rat hepatoma H-4-II-E cells (European Collection of Cell Culture, Salisbury, UK) were maintained in Earle's modified Eagle's medium supplemented with 10% FCS, 2 mmol/L glutamine, nonessential amino acids, and penicillin/streptomycin/fungizone. The rat hepatoma cell lines H35 TDN7 and NEO17 were maintained as described previously.[29] For experiments, cells were seeded on rat-tail collagen gels at a density of 2×10^5 cells/cm^2.

Primary rat hepatocytes were isolated from male Wistar rats using a 2-step collagenase perfusion as described elsewhere.[30] Cells were seeded in William's medium E supplemented with 10% FCS, 50 nmol/L dexamethasone (Sigma, St Louis, MO), 20 mU/mL insulin (Novo Nordisk, Bagsvaerd, Denmark), and penicillin/streptomycin/fungizone at a density of 1.5×10^5 cells/cm^2.

The complete medium was replaced by serum-free medium (cell lines) or serum- and dexamethasone-free medium (primary rat hepatocytes) 4 hours after seeding. Cells receiving adenovirus were then infected for 12 hours, followed by a 12-hour recovery period. TNF-α incubations were started 28 hours after seeding. The proteasome inhibitor MG-132 (Calbiochem, La Jolla, CA) was added at a final concentration of 84 nmol/L 30 minutes before addition of TNF-α where indicated. All cells were maintained in a humidified incubator at 37°C/5% CO_2.

Adenovirus Constructs. The adenovirus constructs used have been described before.[31-33] The IκB super-repressor adenovirus Ad5IκBAA contains an IκB construct in which serines 35 and 36 have been replaced by alanines. This mutated IκB cannot be phosphorylated and binds NF-κB irreversibly, preventing translocation of NF-κB to the nucleus.[31] Ad5dnFADD expresses a FADD mutant lacking the death effector domain, that is therefore unable to bind caspase 8.[32] Ad5dnTRAF2 contains a TRAF2 in which the N-terminal region of the ring finger domain has been deleted, preventing interaction with downstream signaling molecules.[33] Ad5β-Gal, expressing the *Escherichia Coli* β-galactosidase gene, was used as a control virus throughout the experiments.[31] Cells were infected at an moi of 10 (primary hepatocytes) and 50 (H-4-II-E cells) as determined by plaque assay. For co-infections, cells were infected with Ad5IκBAA and Ad5dnFADD at an moi of 10 (primary hepatocytes) and 50 (H-4-II-E).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Before RNA isolation, cells were incubated with 300 μg/mL collagenase (Sigma) for 15 minutes. RNA was isolated using the SV Total RNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions. Reverse transcription was performed on 5 μg of total RNA using random primers in a final volume of 75 μL (Reverse Transcription System, Promega). Three microliters of cDNA was used in each PCR reaction in a final volume of 50 μL, containing 50 pmol/L of sense and of antisense primers, 0.2 mmol/L dNTPs (Pharmacia, Uppsala, Sweden) and 0.5 U EuroTaq Polymerase (Eurogentech, Seraing, Belgium). Primers used were 5′-CTA TTG CGC CGC TAG AGG TG-3′ (sense) and 5′-CTG AAC GCC ACT TGT CCC TC-3′ (antisense) for *18S* (product size 525 base pairs), 5′-ATG TTC CGA GAG CTG AAT GAG G-3′ (sense) and 5′-GGA CTA GCA TTG TCT TGT CAG C-3′ (antisense) for *p53* (product size 270 base pairs) and 5′-GAA ATA ATG CTT ATG AAT CCC AAA G-3′ (sense) and 5′-GGT TTC ATG GTC GTC GTC TCT TGA-3′ (antisense) for *Mdr1b* (product size 325 base pairs). For each primer set an increasing number of PCR cycles was performed to ensure that all data were obtained from samples in the exponential phase. The number of cycles used was 15-17 for *18S*, 25 for *p53*, and 28 for *Mdr1b*. PCR reactions were performed as described before.[13] *18S* and *Mdr1b* RNA levels were related to internal-competitor fragments made according to Celi et al.[34] These fragments were generated using the sense primers as mentioned earlier and the antisense primers 5′-CTG AAC GCC ACT TGT CCC TCA GAC AAA TCG CTG CAC CAA C-3′ and 5′-GGT TTC ATG GTC GTC GTC TCT TGA GCA CCC ATT TAT AAC AGC ACA AA-3′ for *18S* and *Mdr1b*, respectively. Ten microliters of each PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide. Band intensities of digitized images were quantified using Imagemaster 1D Elite software version 3.00 (Pharmacia).

Nuclear Extracts. Nuclear extracts were prepared from H-4-II-E cells as described previously.[35] Cells were washed twice with PBS, harvested in 500 μL PBS, spun down, and resuspended in 400 μL of buffer containing 10 mmol/L Hepes, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, and Complete (Roche Diagnostics, Almere, The Netherlands). After a 15-minute incubation on ice, Nonidet-P-40 was added to a final concentration of 0.25%. After centrifugation, the pellet was resuspended in 50 μL of buffer containing 20 mmol/L Hepes, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and Complete. After constant agitation for 30 minutes at 4°C, nuclear debris was pelleted by centrifugation. The supernatant was stored at −80°C until analysis.

Electrophoretic Mobility Shift Assays (EMSAs). EMSAs were performed with approximately 5 μg of nuclear proteins in a total volume of 15 μL in a buffer containing 20 mmol/L Hepes pH7.9, 60 mmol/L KCl, 0.06 mmol/L EDTA, 0.6 mmol/L DTT, 2 mmol/L spermidine, 10% glycerol, 2 μg poly [d(I-C)] (Roche Diagnostics) and radiolabeled probe at 26°C for 25 minutes.[35] DNA probe was radiolabeled with [α-^{32}P]dATP (Amersham, Buckinghamshire, UK) using Klenow Polymerase (Promega). The mdr-κB probe used, 5′- gat cCT GGG GAA TTC CAG CTC-3′ with the NF-κB site underlined, has been published before.[10] The consensus sequence for AP1 was used in competition experiments. For supershifts, samples were incubated with a p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 30 minutes before the addition of the radiolabeled probe. The final reaction mixture was analyzed on a 4% polyacrylamide gel with 0.5 × Tris borate-EDTA electrophoresis buffer.

Plasmids. The rat *Mdr1b*-luciferase reporter construct −736WT-Luc has been described.[7] The mdr-κB mutant −736κM-Luc was generated by direct site directed mutagenesis (QuikChange, Stratagene, LaJolla, CA) using −736WT-Luc as a template and 5′-TCT GTG TTA ATG TCT GCT CAA TTC CAG CTC CCT T-3′ and its complementary sequence as primers (mutated base pairs are underlined), analogous to Zhou and Kuo.[10]

Transient Transfections and Luciferase Assays. 10^5 H-4-II-E cells, cultured on plastic, were transfected using Effectene (Qiagen, Hilden, Germany) with 0.6 μg of Qiagen Endofree purified plasmid DNA according to manufacturer's instructions. After 12 hours, medium was replaced by serum-free medium. Cells were lysed 30 hours later by incubation with passive lysis buffer (Promega). Luciferase activity was assayed on 20 μL of lysate using the luciferase assay system (Promega), utilizing the Anthos LUCY1 luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) with a 10-second

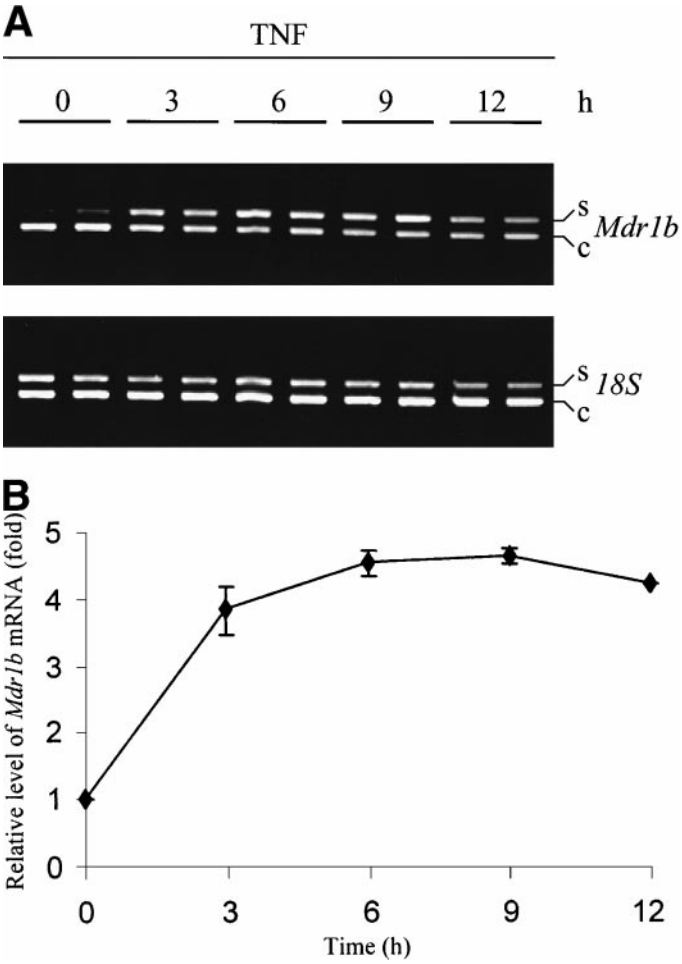

FIG. 1. TNF-α-induced expression of the rat *Mdr1b* gene. (A) Competitive RT-PCR analyses for *Mdr1b* and *18S* in H-4-II-E cells. s, signal from sample; c, signal from the competitor fragment. Cells were cultured on collagen and serum-starved for 24 hours before incubation with TNF-α (20 ng/mL) for different times as indicated. RNA was isolated and RT-PCR performed as described in the Materials and Methods. Data are representative of results from 2 independent experiments performed in duplicate. (B) Quantitative results of time-dependent induction of rat *Mdr1b* mRNA by TNF-α. Data points represent means ± S.D. from 2 independent experiments performed in duplicate.

counting window. Luciferase activity was normalized to the amount of living cells as determined by a standard microculture tetrazolium test (MTT). We were unable to normalize to β-galactosidase or *Renilla* luciferase as TNF-α had a strong effect on the promoters driving the expression of these reporters.

RESULTS

TNF-α Induces Mdr1b Expression in Rat Hepatoma Cells and in Primary Rat Hepatocytes. Rat hepatoma H-4-II-E cells cultured on collagen gels and serum-starved for 24 hours were treated with TNF-α (20 ng/mL) for different time intervals. As shown in Fig. 1A, *Mdr1b* mRNA levels increased within 3 hours of TNF-α addition and reached a maximal level of approximately 4.5-fold after 6 to 9 hours (Fig. 1B). Likewise, when primary rat hepatocytes cultured under comparable conditions were treated with TNF-α, an approximately 3-fold increase in *Mdr1b* mRNA levels was observed after 12 hours (data not shown).

IκBAA Inhibits Induction of Nuclear NF-κB Binding Activity by TNF-α. We selectively inhibited the various pathways activated by TNF-α using adenoviral dominant expression constructs for mutated TRAF2, FADD, or IκB with an adenovirus expressing β-galactosidase as a control. Functionality of the different viral constructs was shown in multiple ways. Ad5IκBAA inhibits NF-κB activation as shown by EMSA (Fig. 2). Ad5dnFADD inhibits apoptosis. Whereas cells exposed to TNF-α following treatment with IκBAA die within 4 to 5 hours, combined treatment with dnFADD prevents this. Moreover, caspase-3 activation as observed after treatment of cells with TNF-α in combination with actinomycin-D is inhibited by pretreatment with dnFADD (data not shown). Expression of exogenous TRAF-2 was determined by RT-PCR. Functionality was shown by Western blot analysis using an antibody raised against phospho-c-Jun. Exposure of control cells to TNF-α resulted in an increase in phospho-c-Jun in nuclear extracts, which was absent in cells pretreated with dnTRAF2 (data not shown).

To study the effect of TNF-α and the various adenoviral constructs on the nuclear NF-κB binding activity, we performed EMSAs using the NF-κB binding site of the rat *Mdr1b* promoter (mdr-κB) as a probe. Nuclear extracts were pre-

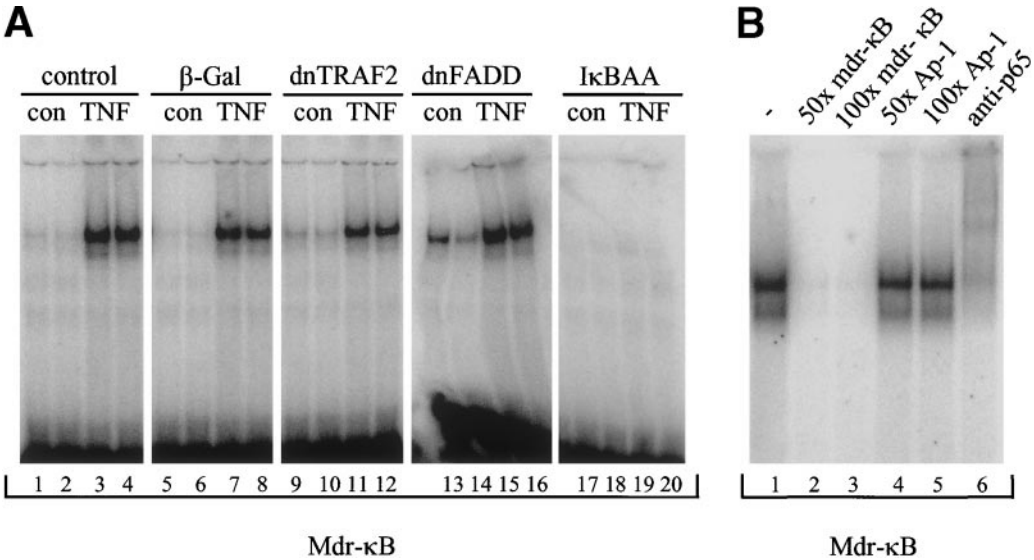

FIG. 2. Activation of nuclear NF-κB activity in H-4-II-E cells by TNF-α. (A) H-4-II-E cells were cultured on collagen and infected with the various adenovirus constructs as indicated. Twenty four hours after serum removal and 12 hours after virus removal, cells were treated with TNF-α (20 ng/mL) for 2 hours. Nuclear extracts were prepared and assayed for NF-κB binding activity as described in Materials and Methods. con, control. (B) Nuclear extracts from uninfected TNF-α-treated cells were assayed for NF-κB binding activity in the absence or presence of excess unlabeled competitors (mdr-κB, AP-1) or NF-κB p65 antibody as indicated.

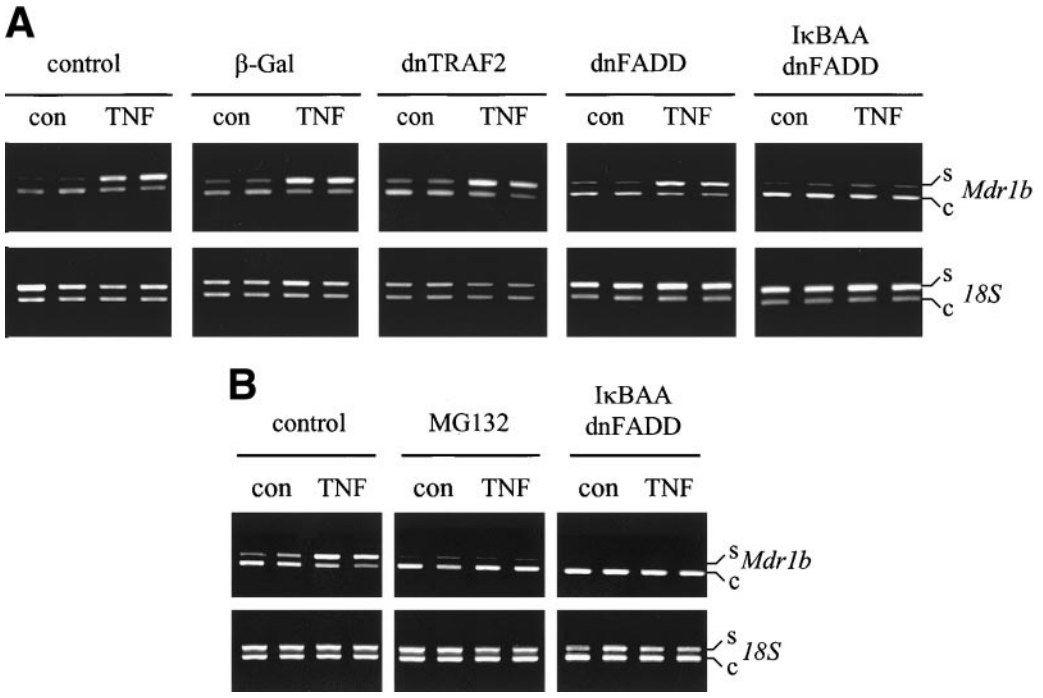

FIG. 3. TNF-α-induced expression of the rat *Mdr1b* gene is NF-κB dependent. Competitive RT-PCR analyses for *Mdr1b* and *18S* in H-4-II-E cells (A) and primary rat hepatocytes (B). Cells were cultured on collagen and serum-starved for 24 hours before incubation with TNF-α (20 ng/mL) for 9 hours. Cells were infected with adenoviral constructs or treated with MG-132 as indicated. RNA was isolated and RT-PCR performed as described in Materials and Methods. s, signal from sample; c, signal from the competitor fragment. Data are representative of results from 2 independent experiments performed in duplicate.

pared from H-4-II-E cells in the presence or absence of TNF-α. As shown in Fig. 2A, stimulation with TNF-α resulted in a clear formation of a DNA-protein complex. This complex was absent in cells expressing the IκB super-repressor (lanes 17-20). The DNA-protein complex was competed by unlabeled mdr-κB (Fig. 2B, lanes 2 and 3) but not by an excess of unlabeled unrelated probe (AP-1, lanes 4 and 5). Supershift analysis with NF-κB p65 antibody showed protein specificity of binding to the mdr-κB oligonucleotide (lane 6).

***Mdr1b* Expression Induced by TNF-α Is Dependent on Activation of NF-κB.** To investigate the intracellular signal transduction pathway involved in the induction of rat *Mdr1b* by TNF-α, we selectively inhibited the pathways with the various adenoviral constructs. When the NF-κB pathway was inhibited with IκBAA, incubation of the cells with TNF-α for periods longer than 4 hours resulted in massive cell death. Therefore, in these experiments IκBAA was used in combination with dnFADD to simultaneously inhibit the activation of both NF-κB and

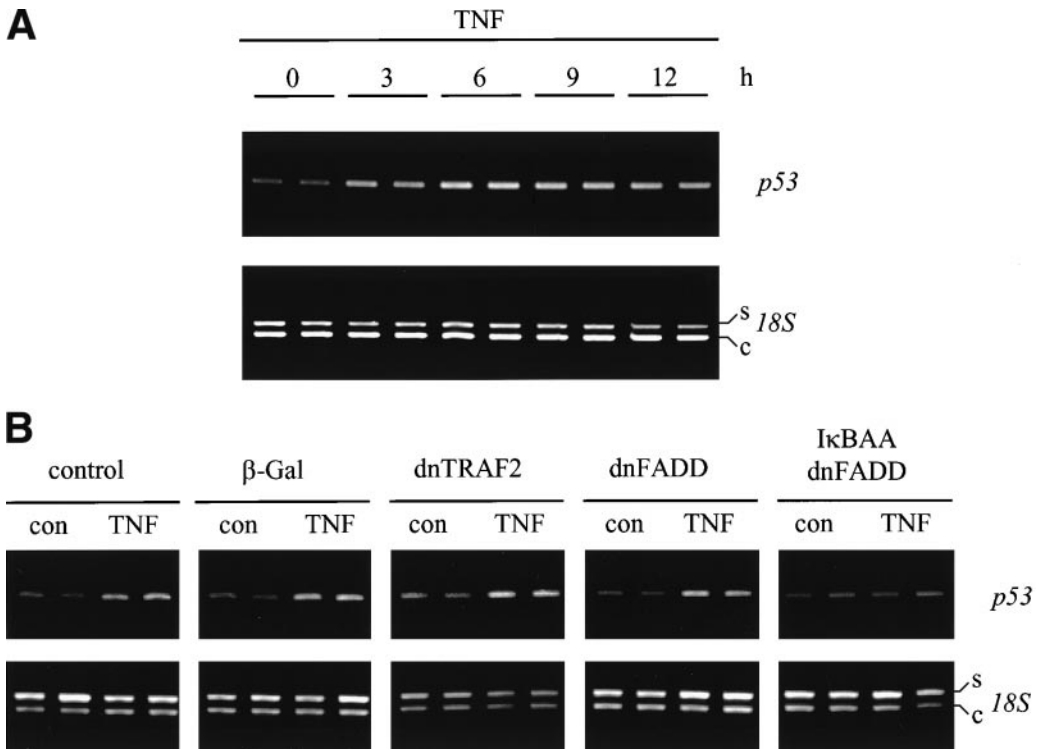

FIG. 4. Increase in *p53* mRNA level in H-4-II-E cells by TNF-α is NF-κB dependent. RT-PCR analyses for *p53* and *18S* in H-4-II-E cells. Cells were cultured on collagen and serum-starved for 24 hours before incubation with TNF-α (20 ng/mL) for different times as indicated. RNA was isolated and RT-PCR performed as described in Materials and Methods. For *18S* PCR: s, signal from sample; c, signal from the competitor fragment. Data are representative of results from 2 independent experiments performed in duplicate. (A) Time course of induction of rat *p53* mRNA by TNF-α. (B) Rat *p53* mRNA levels after incubation with TNF-α for 9 hours. Cells were infected with adenovirus constructs as indicated.

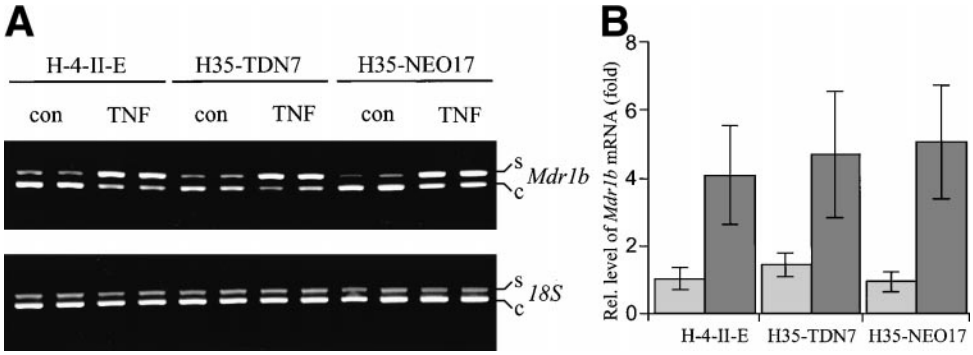

FIG. 5. Trans-dominant negative p53 expression in H35 hepatoma cells does not affect the induction of *Mdr1b* gene expression by TNF-α. (A) Competitive RT-PCR analyses for *Mdr1b* and *18S* in H-4-II-E, H35 TDN7, and H35 Neo17 cells. Cells were cultured on collagen and serum-starved for 24 hours before treatment with TNF-α for 12 hours. RNA was isolated and RT-PCR performed as described in Materials and Methods. s, signal from sample; c, signal from the competitor fragment. (B) Quantitative results of TNF-α-induced expression of the rat *Mdr1b* gene in H-4-II-E, H35 TDN7, and H35 Neo17 cells. Values are normalized to control values for H-4-II-E cells. Data points represent means ± S.D.; n = 2; □ = −TNF-α, ■ = +TNF-α.

caspases. Twelve hours after the removal of the virus-containing media, the cells were incubated with TNF-α for 9 hours. Figure 3A shows that in H-4-II-E cells induction of *Mdr1b* expression by TNF-α is observed under all conditions except when the cells were treated with IκBAA/dnFADD, indicating that NF-κB plays a crucial role in the induction of *Mdr1b* by TNF-α. When primary rat hepatocytes were treated either with IκBAA/dnFADD or with the proteasome inhibitor MG-132, the expression of *Mdr1b* was decreased both in cells stimulated with TNF-α and in untreated cells (Fig. 3B). This indicates that in cultured primary rat hepatocytes, NF-κB is important not only for the induction of *Mdr1b* by TNF-α but also for the basal expression of *Mdr1b*.

TNF-α Induces p53 Via the NF-κB Pathway. It has been reported that NF-κB activates the p53 promoter.[26] We therefore tested whether TNF-α increased endogenous p53 mRNA levels in H-4-II-E cells. Figure 4A shows that p53 mRNA expression was induced time-dependently by TNF-α and reached a maximum after 6 to 9 hours. We compared the expression of p53 in H-4-II-E cells with that in H-4-II-E cells transduced with dnTRAF2, dnFADD, or IκBAA in combination with dnFADD to see which pathway activated by TNF-α is involved in this induction. Figure 4B shows that induction of p53 mRNA expression by TNF-α is abolished in cells where the NF-κB pathway is inhibited by IκBAA/dnFADD transduction.

Mdr1b *Expression Induced by TNF-α Is Not Dependent on p53 Activation.* As the *Mdr1b* promoter contains a p53 binding site and TNF-α induces p53 via NF-κB, we next investigated the possible involvement of p53 in the induction of rat *Mdr1b* gene expression by TNF-α. We compared the effect of TNF-α treatment on *Mdr1b* mRNA levels in 3 different cell lines: H-4-II-E, H35 TDN7, and H35 Neo17. H35 TDN7 cells are stably transfected with trans-dominant negative (TDN) p53. Presence of the dominant negative p53 construct was confirmed by determination of p53 levels by Western blot analysis and *Mdr1a* mRNA levels by RT-PCR. The results (data not shown) conformed with those from Thottassery et al.,[29] who showed that loss of functional p53 results in increased expression of Mdr1a. H35 Neo17 cells contain the backbone vector CMV-Neo-Bam and were used as a control.[29] These cells, cultured on collagen and serum-starved for 24 hours, were treated with TNF-α (20 ng/mL) for 12 hours before RNA isolation. As shown in Fig. 5A, *Mdr1b* mRNA levels increased similarly in the 3 different cell lines. Increases of 4 to 5-fold were observed in this experiment (Fig. 5B). These results show that the induction of rat *Mdr1b* expression by TNF-α is independent of p53 because cells lacking functional p53 upregulate *Mdr1b* just as readily as those that retain functional wild-type p53.

TNF-α Induces the Rat* Mdr1b *Promoter Through its NF-κB Binding Site. To investigate whether binding of NF-κB to the rat *Mdr1b* promoter was sufficient for the induction of *Mdr1b* by TNF-α, we performed transient transfection assays using a rat *Mdr1b* promoter construct coupled to the luciferase gene. When –736WT-Luc, containing 736 base pairs of the rat *Mdr1b* promoter plus 608 base pairs downstream from the transcription start site, was transfected into H-4-II-E cells followed by TNF-α treatment, luciferase activity increased approximately 2.8-fold compared with controls (Fig. 6). This induction is lower than found for endogenous *Mdr1b* (Fig. 1).

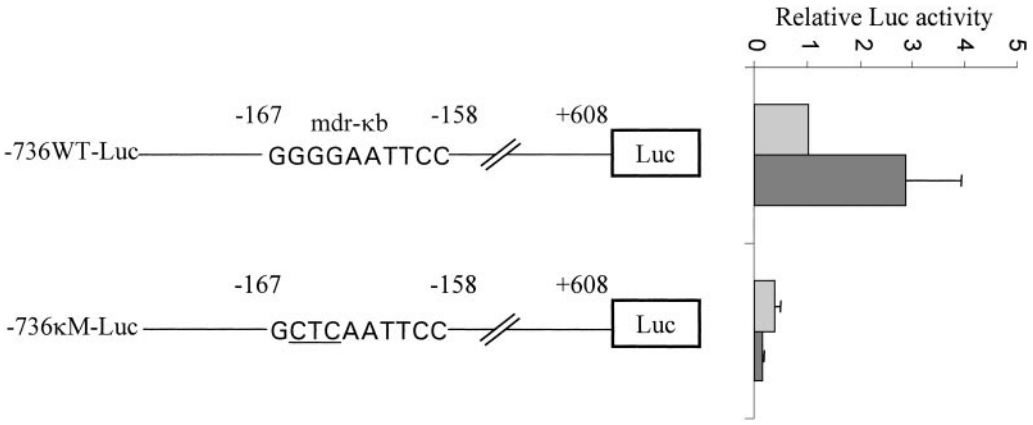

FIG. 6. A functional mdr-κB site is required for TNF-α-induced *Mdr1b* promoter activity. H-4-II-E cells were transfected with the rat *Mdr1b* promoter constructs as described in Materials and Methods. Cells were stimulated with TNF-α (20 ng/mL) for 18 hours before lysis. Values are expressed relative to control values for the wild-type construct. Data points represent means from 3 independent experiments. □ = −TNF-α, ■ = +TNF-α. S.D. values are represented by bars.

However, in transfection experiments, cells could not be cultured on collagen because this would interact with the DNA-liposome particles. Moreover, the transfection itself can induce stress. This could result in increased basal expression levels of the *Mdr1b*-reporter construct, and consequently a lower fold of induction. Mutation of the mdr-κB site abolished this activation completely and instead caused a repression of the *Mdr1b* promoter. These results show that a functional mdr-κB site is required in the rat *Mdr1b* promoter to allow induction of *Mdr1b* by TNF-α.

DISCUSSION

In this study we show a time-dependent stimulation of the rat *Mdr1b* expression by TNF-α in H-4-II-E rat hepatoma cells as well as in primary rat hepatocyte cultures. We show that NF-κB activation is essential for TNF-α-induced *Mdr1b* expression. Moreover, EMSA experiments show that up-regulation of the *Mdr1b* gene correlates with nuclear translocation and binding of NF-κB to its target site in the *Mdr1b* promoter. Although the promoter is up-regulated by TNF-α and this is lost when the mdr-κB site is mutated, we cannot rule out other factors, regulated by NF-κB, playing a role in *Mdr1b* up-regulation. Nevertheless, despite NF-κB increasing p53 (Fig. 4 and Wu and Lozano[26]), p53 does not play a role in TNF-α up-regulation of *Mdr1b* because a liver cell line expressing a dominant negative p53[29] is still capable of up-regulating *Mdr1b* after TNF-α treatment (Fig. 5).

On stimulation with TNF-α, *Mdr1b* mRNA levels reached a maximum after 6 to 9 hours. A similar time-frame of induction has been observed *in vivo* after endotoxin treatment or partial hepatectomy in rats.[13,14] Induction of *Mdr1b* mRNA by TNF-α has been shown previously by Hirsch-Ernst et al., in primary rat hepatocyte cultures, yet, in their system, maximal *Mdr1b* mRNA levels were reached only after 3 days of incubation with TNF-α.[18] In their experiments, TNF-α was added 4 hours after seeding the cells, whereas in our system TNF-α was added to the cells 28 hours after plating. This and other differences in culture conditions might explain the divergent time kinetics observed.

Besides NF-κB and p53 binding sites, other cis-elements have been identified in the rat *Mdr1b* promoter, including Sp1 and AP1 binding sites.[7,36] The Sp1 binding site is essential for optimal basal activity.[7] Our findings with both MG-132 and IκBAA leading to decreased levels of *Mdr1b* in primary rat hepatocyte cultures support the idea that NF-κB also plays a role in regulating basal *Mdr1b* expression.[10] It is unlikely that Sp1 plays a role in NF-κB-mediated regulation of the *Mdr1b* promoter because the *Mdr1b* promoter with the mutated NF-κB binding site still retains these elements and is not activated by TNF-α. Further, as deletion of the AP1 site has no effect on the promoter activity, AP1 does not appear to play a role in its regulation.[7]

TNF-α induces both anti-apoptotic and pro-apoptotic pathways. Activation of the NF-κB pathway results in activation of genes that mediate protective functions because after inhibition of NF-κB activation, the apoptotic pathways prevail.[37] This also occurred in our system when cells treated with the IκB super-repressor were exposed to TNF-α for periods longer than 4 hours. It is possible that up-regulation of *Mdr1b* may help to protect the cell against apoptosis. A direct relation between Mdr1 over-expression and apoptosis was established by Johnstone et al.,[38] who observed that a T-cell leukemia cell line transduced with a retroviral construct containing human MDR1 was more resistant to TNF-α-induced apoptosis than control cells. Also drug-selected cell lines have been shown to be less sensitive to apoptosis, but the cytotoxic compounds used to select resistant cells may induce other changes besides up-regulation of Mdr1 proteins.[39-41] In these studies PSC833 is often used as a Mdr1 inhibitor, but may itself increase apoptosis.[42]

Regarding the mechanism involved, it has been observed that increased expression of MDR protein lowers the sensitivity of the cell to caspase-dependent apoptosis by decreasing the caspase-3 activity.[40] Because MDR proteins can transport small peptides,[43] it is possible that MDR proteins modulate the levels of proteins essential for effective activation of caspase-3. Alternatively, it has been proposed that MDR proteins affect the intracellular pH.[38] Thirdly, MDR proteins may influence intracellular ceramide levels.[39] TNF-α treatment increases intracellular ceramide levels.[44] Primary rat hepatocytes are, however, resistant to ceramide treatment unless RNA synthesis is inhibited,[45] implying that transcriptional up-regulation of protective factors is required. Up-regulation of MDR1 has been reported to increase the translocation of sphingomyelin from the inner to the outer leaflet of the cell membrane, thereby keeping intracellular concentrations of sphingomyelin low and preventing its conversion to ceramide by neutral sphingomyelinases.[39]

An alternative explanation for the profound up-regulation of *Mdr1b* expression during acute phase response is that its expression is intimately coupled to the expression of cytochrome P450 genes. During liver regeneration after partial hepatectomy, endotoxin treatment, or bile duct ligation the levels of various cytochrome P450 proteins are decreased whereas *Mdr1b* is up-regulated.[46-48] TNF-α can also down-regulate cytochrome P450 proteins, including P450 2C11.[49] Schuetz et al. have recently shown the interrelationship between the expression of cytochrome P450 and Mdr1a/1b levels. Members of the cytochrome P450 family were up-regulated in *Mdr1a*, *Mdr1a/b*, and to a lesser extent in *Mdr1b* nullizygous mice.[50] Up-regulation of *Mdr1b* by the cell could be a compensatory pathway to enable transport of compounds no longer metabolized via cytochrome P450 directly into the bile.

The rapid up-regulation of *Mdr1b in vivo* after partial hepatecomy and during endotoxin-induced cholestasis implies that *Mdr1b* is part of the hepatic acute phase response.[13,14] This article shows that expression of *Mdr1b* by TNF-α is under NF-κB control. Clearly future studies will elucidate how Mdr1b affects apoptotic signaling and impacts cell survival.

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