NF-κB-mediated Induction of *mdr1b* Expression by Insulin in Rat Hepatoma Cells*

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The expression of P-glycoproteins encoded by the *mdr* gene family is associated with the emergence of multidrug resistance phenotype in animal cells. However, the mechanisms controlling the expression of these genes have not been well elucidated. Here, we report that the expression of rat *mdr1b* gene in cultured H-4-II-E hepatoma cells can be induced by insulin. Transient transfection assays using reporter gene constructs containing various 5' *mdr1b* sequences showed that the sequence located between base pairs −243 and −163 is important for insulin's induction of *mdr1b* promoter activity. Further analyses revealed that a NF-κB-binding site (located between base pairs −167 and −158) is required for insulin-induced promoter activity. Gel mobility shift assay demonstrated that insulin stimulates the binding of nuclear p50/p65 subunits to the *mdr1b* NF-κB sequence. Cotransfection of plasmids expressing either the p50/p65 NF-κB subunits or Raf-1 kinase or both resulted in increased expression of the gene containing wild-type but not NF-κB site-mutated *mdr1b* promoter. Finally, expression of either the antisense p65 subunit of NF-κB or dominant negative Raf-1 kinase blocked insulin's induction of the *mdr1b* promoter activity. Taken together, our results suggest that the insulin-induced *mdr1b* expression is mediated by transcription factor NF-κB via the Raf-1 kinase signaling pathway.

Multidrug resistance (MDR), a major obstacle to the effective chemotherapy of many human malignancies, is commonly associated with overexpression of a family of membrane glycoproteins called P-glycoprotein. These proteins are believed to function as energy-dependent efflux pumps that prevent intracellular cytotoxic drug accumulation (for a review, see Ref. 1). P-glycoprotein is encoded by the *mdr* gene family, which contains two members (*MDR1* and *MDR2*) in humans (2, 3) and three (*mdr1a, mdr1b, and mdr2*) in rodents (4, 5). However, only *MDR1, mdr1a, and mdr1b* are able to confer the MDR phenotype (6, 7), *MDR2* and *mdr2* only function as phospho-

*mdr* genes are tissue-specifically expressed in normal tissue and highly expressed in many tumors (1, 10, 11). Many extracellular stimuli including antitumor drugs (12), differentiating agents (13), hormones (14, 15), UV irradiation (16), heat shock (17), serum (18, 19), growth factors (18, 20), 12-O-tetradecanoylphorbol-13-acetate (21, 22), and carcinogens (23, 24) have been shown to regulate *mdr* expression. In the rat, *mdr1b* is highly expressed in lung but not in liver (25, 26). However, the expression of *mdr1b* is activated during hepatocarcinogenesis and liver regeneration (25, 27–29). In addition, expression of rat *mdr1b* mRNA can be induced by a number of cytotoxic agents including xenobiotics and protein synthesis inhibitors (23, 24, 30).

Previous studies with primary hepatocytes have shown the mRNA level of the rat *mdr1b* gene to be spontaneously up-regulated due to the increased transcription (30). Further studies have indicated that the expression of the rat *mdr1b* gene in primary hepatocyte cultures can be modulated by dexamethasone (14), extracellular matrix (31), epidermal growth factor, and insulin-like growth factor I (20), suggesting that factors or stress in culture may contribute to the induction of rat *mdr1b* expression in primary hepatocytes. However, the underlying regulatory mechanisms involved in the induced expression of *mdr1b* by these factors are still largely unclear. Although the 5'-flanking sequences of rat *mdr1b* have been isolated (26, 32) and a cis-acting element important for its promoter function has been identified (26), until now little has been known about the cis- and trans-acting factors responsible for the inducible expression of the rat *mdr1b* gene by extracellular stimuli.

In the present report, we show that expression of the rat *mdr1b* gene in rat H-4-II-E hepatoma cells can be induced by insulin. Using transient transfection assays, we confirmed that both an NF-κB-binding site located at bp −167 to −158 (5'GGGGAATTCC-3') and a previously identified palindromic sequence (5'-AGACATGTC-3') located at bp −189 to −180 from the transcription start site (26) are involved in basal transcriptional regulation as well as insulin induction of the promoter function of the rat *mdr1b* gene. In addition, we demonstrated that Raf-1 kinase is also implicated in the increased NF-κB-mediated expression of the rat *mdr1b* gene by insulin. Because a number of agents known to induce *mdr* expression are also known to modulate NF-κB activities, our present findings have a broad implication in the regulation of *mdr* expression under various conditions.

**MATERIALS AND METHODS**

Reagents—Reagents were purchased from the following companies: [α-32P]dNTPs, [α-32P]UTP, and [32]Chloramphenicol from ICN Biochemicals (Costa Mesa, CA); poly(dI-dC)poly(dI-dC) and acetyl coenzyme A from Pharmacia/LKB (Upsala, Sweden); rabbit polyclonal antibodies against p50 and p65 subunits of NF-κB from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and oligonucleotides from Genosys Biotechnologies Inc. (Houston, TX). All other reagents were pur-
chased from Sigma.

**Plasmids**—The rat **mdr1b**-CAT reporter constructs (−1288 RMI-CAT, −1288 RMI-CAT-Fm1, −243 RMI-CAT, −163 RMI-CAT) were constructed as described previously (26). The site-directed mutant constructs were generated by a two-step PCR strategy (33) using −243 RMI-CAT as the template; the T7 promoter sequence and 5′-TCCATTAGCTTTCCCTAG-3′ as the exterior primers; and 5′-ACCTGAACATG-TAGCTGAATGTCTGTGTTAATGTCTG-3′, 5′-TCTGTGTTAATGTCTGCTCAATTCCAGCTCCCTT-3′, and their complementary sequences as the interior primers, respectively (mutated sequences are underlined). Then, after digestion with XbaI, the PCR products were used to replace the XbaI insert of −243 RMI-CAT. This generated −243 RMI-CAT-Fm1 and −243 RMI-CAT-xm, respectively. A similar approach was used to generate −1288 RMI-CAT-xm except that two interior primers (5′-TCTGTGTTAATGTCTGCTCAATTCCAGCTCCCTT-3′ and its complementary sequence) were used for PCR amplification. The sequences of all the mutant versions were confirmed by sequencing.

**Fig. 1.** Insulin-induced expression of the rat **mdr1b** gene. A, RNase protection analyses of rat **mdr1b** transcripts in H-4-II-E cells. Cells were maintained in medium containing 0.3% FCS for 48 h followed by incubation with 10−7 M insulin (INS) or cycloheximide (CHX) (10 μg/ml) for different times as indicated. RNA was harvested at each time point and then subjected to RNase protection as described under “Materials and Methods.” An 18 S rRNA probe was used as a reference. nt, nucleotide. The autoradiograph is representative of results from three independent experiments. B, quantitative results of time course induction of rat **mdr1b** mRNA by insulin. Data points represent means ± S.D. from three independent experiments.

**Fig. 2.** Response of rat **mdr1b** promoter to insulin stimulation. H-4-II-E cells were transfected with 5′ deletion constructs of the rat **mdr1b** promoter fused to the CAT reporter gene, and CAT activity was assayed as described under “Materials and Methods.” A, one representative result of CAT assays of extracts of cells transfected with the **mdr1b**/CAT reporter gene fusions (2 μg/well) in the absence (−) or presence (+) of insulin (INS) (10−7 M). B, average relative CAT activity in three independent experiments. C, positions of various putative transcription factor-binding sites relative to the deletion end points and the transcription start site (arrow).
Identification of an NF-κB-binding site in the rat mdr1b promoter. A, the mdr1b promoter sequence from bp -189 to -149 and oligonucleotides used. The palindromic sequence (P) (bp -189 to -180) and the putative NF-κB-binding site are in boldface type. In the mdr-κB sequence, sequences created for probe F were contained in the lower-case sequence. B, GMSA using nuclear extracts prepared from BprC1 cells cultured with 10% FCS. Extracts were assayed for binding to the labeled double-strand mdr-κB oligonucleotide in the presence or absence of a 100-fold molar excess of the indicated competitors. C, GMSA using 5-μg nuclear extracts from BprC1 cells incubated in 10% FCS with or without 12-O-tetradecanoylphorbol-13-acetate (50 ng/ml) for the indicated times. Preimmune or anti-p65 serum was also used in binding reactions as indicated. The autoradiographs are representative of results from three independent experiments. Nuclear extracts used in panels B and C were prepared from two different experiments. The exposure time of panel B was 5 days, whereas that of panel C was 1 day.

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COTGCAC-3′ (forward primer, with BamHI site created underlined) and 5′-TCGGTACGACGCTTCTCTCGA-3′ (backward primer, with the AspAsp site created underlined). The PCR products of pair 1 and 2 primers were then cleaved with BamHI and ligated. After being purified from 3% agarose gel, the ligated products were cleaved at their Asp718 site and inserted into the Asp718 site of −243 RMICAT−243 RMICAT-Fm1, and −243 RMICAT-em, respectively, to generate the 3′-243 RMICAT, 3′-243 RMICAT-Fm1, 3′-243 RMICAT-em. The orientation and sequence of each insert was then confirmed by sequencing.

The expression vectors RSV-p65 and RSV-p50 were provided by Gary J. Nabel (University of Michigan Medical Center, Ann Arbor, MD) (34). Plasmid pSVK3 was purchased from Pharmacia. Recombinant antisense pd65 (ap65) (35) was a gift of Tom Maniatis (Harvard University, Boston, MA). Plasmid pdDNA1 was purchased from Invitrogen. Plasmids expressing an activated Ras-1 kinase domain (Raf BXB), its dominant-negative mutant (Raf BXB-301), and the control expression vector pKRSPA (36) were generously provided by U. R. Rapp (Institute of Medical Radiobiology and Cell Biology, University of Würzburg, Würzburg, Germany).

Cell Culture, DNA Transfections, and Chloramphenicol Acetyltransferase (CAT) Assay—The mouse hepatoma cell line Hepa1c1c-BprC1 (a generous gift from James Whitlock, Stanford University Medical School, Palo Alto, CA) (37) and the rat hepatoma cell line H-4-II-E (American Type Culture Collection number 1548) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Life Technologies, Inc.), 1 mM glutamine, and 50 μg of neomycin/ml in a humidified incubator containing 5% CO2. Prior to stimulation of insulin, cells were grown to 70–80% confluence in Dulbecco’s modified Eagle’s medium containing 10% FCS (low serum medium). For insulin stimulation, 10−7 m insulin was added directly to the spent low serum medium 48 h after incubation. After 12 h of insulin treatment, cells were harvested. Activities in the cell extracts were measured by a method previously described (39) using total extract protein (as measured by the Bio-Rad protein assay kit) as reference. Relative CAT activity levels were calculated from the conversion of [14C]chloramphenicol into acetylated chloramphenicol by PhosphorImager (model 400S, Molecular Dynamics).

Preparation of Nuclear Extracts and GMSA—Nuclear extracts were prepared from BprC1 and H-4-II-E cells by the method of Digman et al. (40) with modifications. Briefly, cells were washed with ice-cold PBS. After centrifugation, the cell pellet was resuspended in 5 packed cell volumes of buffer A (10 mM HEPES/KOH, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 0.74 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin) and then incubated on ice for 10 min. Supernatants were removed after centrifugation, and 2 packed cell volumes of buffer A was added. Cells were then lysed by 10–15 strokes in a tight fitting Dounce homogenizer. Then, 1/5 volume of 68% RNase-free sucrose was mixed with the cell lysates and centrifuged at 16,000 × g for 30 s at 4 °C. The resulting pellet was washed with buffer A and resuspended in 1/5 packed cell volumes of buffer B (20 mM HEPES/KOH, pH 7.9, 0.42 mM NaCl, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin). After constant agitation for 30 min at 4 °C, nuclear debris was centrifuged at 15,000 × g for 6 min, and supernatants (nuclear extracts) were stored at −80 °C until analysis by EMSA.

GMSAs were performed with approximately 5 μg of nuclear proteins in a total volume of 20 μl of binding mixture (10 μl Tri-HCl, pH 7.5, 50 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, 2% Nonidet P-40, 3 μg of poly(dI-dC)·poly(dI-dC)) and radiolabeled DNA probe at room temperature for 20 min. If antibodies were added, the mixtures were preincubated for 10 min before the addition of the radiolabeled probe. Protein concentration was determined using the Bio-Rad kit according to the manufacturer’s instruction. DNA probe was radiolabeled with [32P]dCTP using the Klenow fragment of DNA polymerase I (Boehringer Mannheim). For competition assays, a 100-fold excess of unlabeled oligonucleotide was added to the reaction. The consensus sequences of AP-1 (41), HNF-1 (41), HNF-3 (41), HNF-4 (41), C/EBP (41), IgB (42), PRDI (43), and IgC-m (44) were synthesized, annealed to double strands, and used in competition experiments. The final reaction mixture was analyzed on a 4% nondenaturing polyacrylamide gel with 0.25 × Trisborate–EDTA electrophoresis buffer.
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Fig. 4. Involvement of mdr1b NF-κB-binding site in the rat promoter function. A, CAT assays of −1288 wild-type and mutant constructs shown in panel B after transfection into H-4-II-E cells in the presence or absence of insulin treatment. Results are expressed as the average relative CAT activity (means from three independent experiments) after normalization to the protein concentration of the cellular extracts. S.D. values are represented by the bars. 2 μg of DNA was used in each transfection. B, schematic diagrams of −1288 RMICAT, RMICAT-Fm1, and RMICAT-xm constructs used in transient transfections. Mutated bases in the palindromic sequence and NF-κB site are indicated in boldface type.

RNase Protection Assay—A 162-nucleotide (−37 to +125) antisense RNA probe was synthesized using T7 RNA polymerase as described previously (26). Either 20 μg (for the mdr1b probe) or 1 μg (for the 18 S rRNA probe) of total RNA from serum-treated, insulin-treated, or untreated H-4-II-E cells was hybridized with 32P-labeled antisense RNA probes (2 × 106 cpm) and subjected to the RNase protection assays as described previously (26, 45). The protected RNA products were analyzed on a 7% denaturing polyacrylamide gel and quantitated using a Personal Densitometer SI (Molecular Dynamics).

RESULTS

Insulin Induces mdr1b Expression in Rat Hepatoma Cells—To investigate whether the expression of the rat mdr1b gene expression is regulated by insulin, we treated serum-starved rat hepatoma H-4-II-E cells (0.3% serum, 48 h) with insulin. At various time intervals, cells were harvested and mdr1b mRNA levels were measured by RNase protection assay. As shown in Fig. 1A, the steady-state rat mdr1b mRNA levels elevated rapidly in the first hour after the addition of insulin, reaching to about 3.5-fold by 12 h (Fig. 1B). Similarly, when serum-starved H-4-II-E cells were treated with serum (20%) for 12 h, a 3–4-fold increase of the mdr1b mRNA level was also observed (data not shown).

Consistent with previous observations (23, 30), treating these cells with the protein synthesis inhibitor cycloheximide (10 μg/ml) also induced rat mdr1b expression (Fig. 1A, lane 7). This suggests that rat mdr1b expression may be regulated by an inhibitor that can be repressed by protein synthesis inhibition (see below).

Rat mdr1b Promoter Responds to Insulin Stimulation—To investigate the possible involvement of transcriptional regulation in the induction of the rat mdr1b gene expression by insulin and to identify DNA sequences involved in the insulin induction of mdr1b expression, we generated a set of 5′ deletion mutant CAT constructs (Fig. 2C) and transfected them into H-4-II-E cells in the presence or absence of insulin stimulation (Fig. 2A). When −1288 RMICAT and −243 RMICAT containing 1288 and 243 bp of the rat mdr1b upstream sequences, respectively, plus 125 bp downstream from the transcription start site, were transfected into H-4-II-E cells followed by insulin treatment, CAT activities increased an approximately 2-fold in comparison with the untreated controls (p < 0.05) (compare Fig. 2A, lanes 2 and 1 and lanes 4 and 3). However, when −163RMICAT, which contains additional deletion to −163 bp was transfected, basal transcriptional activities were reduced more than 80% (compare lanes 5 and 3), consistent with our previous findings that sequences between −243 and −163 are important for the basal transcription of mdr1b (26). More importantly, the deletion also resulted in the loss of insulin inducibility (lane 6). Together, these results indicated that the rat mdr1b promoter can respond to insulin stimulation and that the sequence from −243 to −163 bp is essential for the promoter’s insulin responsiveness.

Identification of an NF-κB-binding Site in the Rat mdr1b Promoter—In examining the −243−163 sequence of the rat mdr1b promoter, we found that a sequence between bp −167 and −158 (5′-GGGAATTC-3′) (Fig. 3A) was strikingly similar to the transcription factor NF-κB-binding consensus sequence (5′-GGGRNNYYCC-3′) (46). A comparison of the putative mdr1b NF-κB-binding site with several known NF-κB-binding sequences, including the interferon-β gene’s PRDII site (INFβ PRDII) (43) and the immunoglobulin gene’s κB site (IgκB) (42) is shown in Fig. 3A. Since insulin can reportedly activate NF-κB activity in CHO-R cells that overexpress insulin receptor (47), we speculated that this putative NF-κB-binding site in the rat mdr1b promoter may be involved in the observed insulin’s induction of the promoter function. We therefore designed experiments to test this possibility.
First, we determined whether the sequence located between bp −167 and −158 is indeed an NF-κB-binding site. To this end, we carried out GMSA using a double-stranded oligonucleotide spanning bp −169 to −153 as the probe (designated the mdr-κB fragment in Fig. 3A) and various unlabeled oligonucleotides as competitors. As shown in Fig. 3B, a DNA-protein complex was formed in nuclear extracts prepared from mouse hepatoma BprC1 cells (lane 1). More importantly, formation of this complex was efficiently competed only by the unlabeled probe (lane 2) and NF-κB sequences derived from IgκB (lane 8) and PRDII (lane 9), and not by the mutated sequence of IgκB (IgκBm, lane 10) or other unrelated consensus sequences, i.e. HNF-1, HNF-3, HNF-4, AP-1, or C/EBP (lanes 3–7).

NF-κB is an inducible transcription factor, and its nuclear DNA binding activity could be induced by mitogens such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (48). To further substantiate the aforementioned results, we treated BprC1 cells with 12-O-tetradecanoylphorbol-13-acetate (50 ng/ml) and prepared nuclear extracts from the treated cells. GMSA using the mdr-κB probe showed that the protein binding activity was rapidly induced in nuclear extracts from cells treated for 30 min and that the increase lasted at least 1 h (Fig. 3C, lanes 1–3). Furthermore, the formation of protein-DNA complex could be blocked by antiseraum raised against the p65 subunit of NF-κB (Fig. 3C, lanes 5, 7, and 9), whereas the preimmune serum did not inhibit the formation of DNA-protein complexes (lanes 4, 6, and 8). Thus, taken together, these results strongly suggest that the rat mdr1b promoter sequence located between bp −167 and −158 (5′-GGGGAGTTCC-3′) is an authentic NF-κB-binding site.

Both NF-κB-binding Site and Palindromic Sequence Are Involved in Basal and Insulin-inducible Promoter Function of Rat mdr1b—To determine whether the mdr-κB site is functionally involved in mdr1b promoter activity, we first generated site-directed mutations in the NF-κB sequence of the wild-type −1288 RMICAT recombinant (designated −1288 RMICAT-κm, Fig. 4B) and then transiently transfected them into rat H-4-II-E cells. In the absence of insulin, basal expression of CAT activity was reduced about 65% in cells transfected with mutant −1288 RMICAT-κm compared with that in cells transfected with the wild-type construct (−1288 RMICAT) (Fig. 4A). More importantly, the induction of CAT expression by insulin was only seen in cells transfected with the wild-type construct (−1288 RMICAT) and not with the mutated version (−1288 RMICAT-κm). Together, these results suggest that the NF-κB binding site is involved in both basal and insulin-inducible mdr1b promoter function.

We previously identified in the rat mdr1b promoter region a palindromic sequence (5′-AGACATGCTT-3′) located between bp −189 and −180 (designated the F site in Fig. 4B). This site is important for basal promoter function in many different cell lines (26). Since the F site also falls within the bp −243 to −163 region, which is essential for the induction of the promoter activity as described above, we tested whether the palindromic sequence is also involved in the insulin-inducible promoter activity. Thus, a mutant designated −1288 RMICAT-Fm1 was generated (Fig. 4B) and subjected to transient transfection assays. The assays demonstrated that, like the NF-κB site, the palindromic sequence is also required for insulin induction. The disruption of this palindromic sequence concomitantly abolished basal and insulin-induced mdr1b promoter activity in rat H-4-II-E cells (Fig. 4A).

To further substantiate these findings, we generated two additional sets of constructs. The first set contained mutations in either the NF-κB site or F site of −243 RMICAT. The constructs were designated −243 RMICAT-κm and −243 RMICAT-Fm1, respectively (Fig. 5). These mutants were then transfected into rat H-4-II-E cells and subjected to insulin inducibility analysis. Consistent with the results shown in Fig. 4A, mutations in either site not only reduced basal expression...
levels of CAT activity but also abolished the insulin inducibility of the reporter gene’s expression (Fig. 5). The second set of plasmids consisted of three constructs, each containing three copies of a −243 to −127 mdr1b sequence linked in tandem to the 5’ end of a promoter sequence beginning at bp −127. Mutations in either the F site or NF-κB site were introduced in wild-type constructs (3x-243 RMICAT), and the resulting mutant plasmids were designated 3x-243 RMICAT-Fm1 and 3x-243 RMICAT-km (Fig. 5). These constructs then transiently transfected into H-4-II-E cells. In the absence of insulin, wild-type 3x-243 RMICAT showed an approximately 3-fold higher CAT activity than −243 RMICAT, which contains only one copy of the −243/−127 sequence (Fig. 5). CAT expression was also induced by insulin in 3x-243 RMICAT, although no additive induction was evident. However, mutations in either the NF-κB site or F site impaired not only basal promoter activity but also insulin inducibility. Collectively, these results suggest that both NF-κB and the palindromic sequence are involved in insulin-induced up-regulation of the rat mdr1b expression. Since the transcription factor(s) recognizing the F site (palindromic sequence) are currently unknown, the involvement of this palindromic sequence in insulin induction was not characterized further.

Insulin Induces Nuclear NF-κB Binding Activity in Rat Hepatoma Cells—To test whether treatment of rat H-4-II-E cells with insulin could induce nuclear NF-κB activity, we prepared nuclear extracts in the presence or absence of insulin stimulation and performed GMSAs using mdr-κB (Fig. 3A) as the probe. As shown in Fig. 6A, a major DNA-protein complex (arrow) was rapidly induced by insulin treatment within the first 0.5 h (lanes 1 and 2). Binding activity levels remained elevated throughout the 12-h induction period (lanes 3–6).

Cycloheximide, already shown to be capable of inducing rat mdr1b expression (Fig. 1A, lane 7) (23, 30), also induced DNA binding activity (Fig. 6A, lane 7). Furthermore, the induced DNA-protein complex was efficiently competed only by unlabeled mdr-κB and IκB consensus sequences (Fig. 6B, lanes 2 and 4), and not by a nonspecific competitor AP-1 consensus sequence (lane 3).

To further characterize the identity of insulin-induced DNA-protein complex, antibodies directed against the p50 and p65 NF-κB subunits were used. Both antibodies, especially the p65 antibody, markedly reduced the formation of DNA-protein complex, whereas preimmune serum had no effect (Fig. 6B, lanes 5–7). These results indicate that the induced complex is composed of both p50 and p65 NF-κB subunits and most likely a p50/p65 heterodimer, a major effective and inducible form of NF-κB (49).

Transfection of p50/p65 NF-κB Subunit Expression Vectors Activates Wild-type but Not NF-κB Site-mutated Rat mdr1b Promoter Activity—To verify whether p50 and p65 subunits of the NF-κB family directly trans-activate the expression of the rat mdr1b promoter, we cotransfected p50/p65 expression vectors with reporter constructs containing either wild-type NF-κB-binding site (−243 RMICAT or 3x-243 RMICAT) or mutated κB site (−243 RMICAT-km or 3x-243 RMICAT-km). As shown in Fig. 7, a p50/p65 plasmid DNA dose-dependent increase in CAT activities was observed in cells cotransfected with wild-type constructs but not in cells cotransfected with constructs containing mutated NF-κB site. These results further demonstrated that p50/p65 subunits of the NF-κB family are able to trans-activate the rat mdr1b promoter activity specifically via the NF-κB-binding site.

Raf-1 Kinase Is Involved in the NF-κB-regulated Promoter Function of the Rat mdr1b Gene—The biological function of insulin is believed to be mediated by several signal transduction pathways, including the Ras/Raf mitogen-activated protein kinase pathway (for a review, see Ref. 50). Coincidentally, it has been shown that NF-κB site-dependent induction of gene expression by diverse inducers of the nuclear factor NF-κB requires Raf-1 kinase (51). Thus, we deemed it important to investigate whether Raf-1 kinase also regulates the rat mdr1b
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**Fig. 7.** Activation of wild-type but not NF-κB site-mutated mdr1b promoter by p50/p65 subunits of NF-κB. A and B, 2 μg of wild-type (−243, and 3x-243 RMICAT) or NF-κB site-mutated (−243, and 3x-243 RMICAT-xm) mdr1b promoter reporter construct was transfected alone or in combination with increasing amounts of an equimolar mixture of p50 and p65 expression vectors as indicated. Empty control vector (pSVK3) was used to normalize the amounts of the transfected DNA to a total 4 μg of DNA in each transfection reaction. Each column represents the mean of relative CAT activities from three independent experiments after normalization to the protein concentration of the cellular extracts. S.D. values are represented by the bars. Levels of CAT activities in each panel were calculated in reference to control, i.e. first column from the left of each panel.

Promoter through the NF-κB-binding site. Plasmid expressing Raf-1 kinase (Raf-BXB) (36) was cotransfected with −243 RMICAT, −243 RMICAT-xm, or −243 RMICAT-Fm1 into H-4-II-E cells. As shown in Fig. 8A, a Raf-1 DNA concentration-dependent increase in CAT activity was seen in cells cotransfected with the wild-type construct but not in those cells transfected with constructs containing a mutant NF-κB-binding or in the F site. These results indicate that the integrity of both the NF-κB site and the palindromic sequences is required for the promoter to be activated by Raf-1 kinase. Similar results were obtained using 3x-243 RMICAT and the corresponding mutants (Fig. 8B). Furthermore, cotransfection of expression recombinants of p50/p65 NF-κB subunits and Raf-1 resulted in a synergistic effect on rat mdr1b promoter activity (Fig. 8C).

Both Antisense p65 and Dominant Negative Raf-1 Mutant Inhibit Insulin-Induced Promoter Function of the Rat mdr1b Gene—To obtain more direct evidence of the involvement of NF-κB and Raf-1 signaling in insulin-induced rat mdr1b expression, we cotransfected antisense p65 expression vector (αp65) (35) or a dominant negative Raf-1 mutant (Raf-BXB301) (36) into H-4-II-E cells with the −243 RMICAT vector. Approximately 48 h after transfection, cells were either mock-stimulated or insulin-stimulated for 12 h, and CAT activity was determined. As shown in Fig. 9, insulin inducibility was abolished when cells were transfected with either αp65 or Raf-BXB301 plasmids. These results, taken together, lend additional credence to the notion that both NF-κB and Raf-1 kinase are involved in the insulin-induced expression of rat mdr1b.

**DISCUSSION**

We have previously identified a palindromic sequence located at bp −189 to −180 that is essential for rat mdr1b promoter function in many types of cells (26). Here, we report the identification of a NF-κB-binding site located at bp −167 to −158 that is important for the basal as well as insulin-inducible promoter activity of the rat mdr1b gene. To the best of our knowledge, this is the first evidence that NF-κB is involved in the transcriptional regulation of genes in the mdr family.

The transcription factor NF-κB/Rel family has been shown to be involved in immune, inflammatory, and acute phase response, cellular proliferation; oncogenic transformation; and programmed cell death (for reviews, see Refs. 46 and 52–54). This family is made up of five members (p50, p52, p65, c-Rel, and RelB) that are able to bind DNA as homo- or heterodimers and are implicated in the expression of many inducible genes. Indeed, NF-κB is functionally active in many tissues, and its activity can be induced by a wide variety of stimuli, including serum, insulin, growth factors, UV irradiation, cytotoxic compounds, and protein synthesis inhibitors (for a review, see Ref. 49). Strikingly, many of these stimulants are also known to induce mdr gene expression (Fig. 1A) (18, 19, 23), suggesting that the induction of mdr gene expression by these stimulants may be mediated through the NF-κB signaling pathway.

The pleiotropic function of insulin is mediated by several signal transduction pathways, including the mitogen-activated protein kinase-dependent pathway (for a review, see Ref. 50). The Raf-1 kinase works downstream of the Ras signaling pathway and upstream of the mitogen-activated protein kinase kinase and mitogen-activated protein kinase pathway (for a review, see Ref. 55). Consistent with the previous observation that Raf-1 is involved in the signal transduction pathway of κB site-dependent induction of gene expression by many NF-κB inducers (51), our present results demonstrate that NF-κB-dependent induction of rat mdr1b expression by insulin also requires Raf-1. As we noted in the Introduction, epidermal growth factor and insulin-like growth factor I have been shown to induce expression of the rat mdr1b gene in primary rat hepatocyte culture (20). Given that a similar signal transduction pathway is elicited by these growth factors in activating gene expression (56, 57), it is likely that regulation of the rat mdr1b gene by these growth factors may follow a similar signal transduction pathway as described in this paper.

It has been known that in unstimulated cells, NF-κB is sequestered in the cytoplasm by virtue of its association with members of a set of inhibitor proteins belonging to the IκB family (for a review, see Ref. 53). Through noncovalent association, the IκB proteins mask the nuclear localization signal of NF-κB. Extracellular inducers of NF-κB usually activate signal transduction pathways that result in the phosphorylation and subsequent degradation or dissociation of IκB. As a result, the released NF-κB translocates into the nucleus where it regulates gene expression by binding directly to cognate DNA sequences (for reviews, see Refs. 46, 52–54, and 58). The mechanism by which Raf-1 activates NF-κB activity is not clear. Although there was a controversial report suggesting that IκB-α can be phosphorylated by Raf-1 (59), it is now clear that Raf-1 cannot directly phosphorylate IκB-α (60). A recent study showed that phosphorylation of IκB-α appears to be mediated by a large, multisubunit IκB-α kinase complex (61). However, it remains unclear that whether Raf-1, like other MEKs such as MEKK1, can activate NF-κB by phosphorylating IκB-α kinase directly (62) or by an intermediate activator. It also remains possible that Raf-1 may induce synthesis of a factor, possibly a cytokine, which in turn could have been the true activator of one or more mitogen-activated protein kinase pathways lead-
FIG. 8. Raf-1 kinase activation of wild-type but not NF-κB site-mutated mdr1b promoter. A and B, H-4-II-E cells were transfected with 2 μg of wild-type (−243, and 3×-243 RMICAT), NF-κB site-mutated (−243, and 3×-243 RMICAT-κm), or palindrome-mutated (−243, and 3×-243 RMICAT-Fm1) mdr1b promoter reporter alone or in combination with increasing amounts of Raf-1 kinase expression vector (Raf-BXB) as indicated. Empty control vector (pKRSPA) was used to normalize the amounts of the transfected DNA to a total of 4 μg of DNA in each transfection reaction. C, synergy of Raf-1 kinase with p50/p65 in activating the rat mdr1b promoter activity. 2 μg of wild-type −243 RMICAT construct was transfected into H-4-II-E cells together with p50/p65 expression plasmids, Raf-BXB, or both. Lane 1, 0.5 μg each of pKRSPA and pSVK3 (empty vectors); lane 2, 0.5 μg each of an equimolar mixture of p50/p65 expression vectors and pKRSPA; lane 3, 0.5 μg each of Raf-BXB and pSVK3 vectors; lane 4, 0.5 μg each of Raf-BXB and an equimolar mixture of p50/p65 expression vectors. Each column represents the mean of relative CAT activities from three independent experiments after normalization to the protein concentration of the cellular extracts. S.D. values are represented by the bars.

there is increasing evidence to suggest that NF-κB plays an important role in the proliferation and malignant transformation of tumor cells (for reviews, see Refs. 52 and 67), implying that NF-κB may be involved in the regulation of mdr gene expression in these tumors. Furthermore, mdr gene expression in cultured rodent cells can be acutely induced by various cytotoxic compounds (68). Many of these compounds exert cytotoxic stress on the cells, resulting in the concomitant induction of stress-response NF-κB transcription factors (for a review, see Ref. 69). Thus, it is likely that the induction of mdr expression by many of these cytotoxic compounds may be due to the elevated NF-κB activities.

Cycloheximide has been shown to induce rat mdr1b expression in primary rat hepatocyte cultures and in other different cell lines (23, 30). A previous study even suggested that such induction may be due to the release of an unknown trans-acting transcriptional repressor (23). In the present study, we have confirmed that cycloheximide can induce rat mdr1b expression (Fig. 1A, lane 7). More importantly, we have shown that cycloheximide can also induce NF-κB activity in rat H-4-II-E cells (Fig. 6A, lane 7), as previously reported in other types of cells (47, 70). The mechanism underlying the induction of nuclear NF-κB activity by cycloheximide is still unclear. However, because IκB has a shorter half-life than NF-κB (71), the levels of NF-κB relative to IκB may be increased in the presence of

ing to the activation of NF-κB. A similar example was observed in HeLa cells in which Raf-1 activates stress-activated protein kinase via an induced cytokine (63). We also cannot rule out the possibility that Raf-1 may modulate NF-κB activity by directly targeting p50/p65 rather than IκB. It has recently been demonstrated that a splice variant of adenovirus E1A product can activate NF-κB activity not only by the IκB phosphorylation pathway but also through direct interaction with the C-terminal 80 amino acids of the p65 component (64). Furthermore, Perkins et al. (65) reported that regulation of NF-κB may be achieved by cyclin-dependent kinases through association with the p300 coactivator. Additional studies are needed to determine the exact roles of Raf-1 in NF-κB regulation.

Overexpression of rat mdr1b is associated with liver regeneration (25, 27). NF-κB activity in normal liver is very low but rapidly induced in regenerating liver (for a review, see Ref. 66). Many of these compounds exert cytotoxic stress on the cells, resulting in the concomitant induction of stress-response NF-κB transcription factors (for a review, see Ref. 69). Thus, it is likely that the induction of mdr expression by many of these cytotoxic compounds may be due to the elevated NF-κB activities.
protein synthesis inhibitor, thereby resulting in the elevated expression of rat mdr1b. This would be consistent with the IκB/NF-κB scenario discussed above, implying that IκB may be the previously unidentified trans-acting repressor. All in all, our present findings suggest that NF-κB signaling may play an important role in the regulation of the rat mdr1b expression by many environmental influences that have been previously described in the literature.

Our present study also demonstrates that the induction of rat mdr1b promoter function by insulin requires the integrity of both the NF-κB-binding site and the palindromic sequence, suggesting that these two cis-acting elements somehow cooperate in regulating rat mdr1b expression. But because there is no clear information on the trans-activating factors that recognize the palindromic sequence, the activation mechanism remains elusive. We still cannot rule out the possibility that the palindromic-binding protein is also directly induced or regulated by insulin and Raf-1 kinase. Therefore, the identification, through molecular cloning, of transcription factors that recognize the palindromic sequence will be very helpful in elucidating the activation mechanism. It has been known that most inducible cis-acting elements contain multiple, distinct transcription factor-binding sites that are part of a combinatorial mechanism that relies on cooperative binding, interaction of transcriptional activator proteins, and transcriptional synergy (for reviews, see Refs. 72 and 73). Moreover, NF-κB transcription factor-binding sites that are part of a combinatorial mechanism will be very helpful in elucidating the activation mechanism. It has been known that most inducible cis-acting elements contain multiple, distinct transcription factor-binding sites that are part of a combinatorial mechanism that relies on cooperative binding, interaction of transcriptional activator proteins, and transcriptional synergy (for reviews, see Refs. 72 and 73). Moreover, NF-κB transcription factor-binding sites that are part of a combinatorial mechanism will be very helpful in elucidating the activation mechanism. It has been known that most inducible cis-acting elements contain multiple, distinct transcription factor-binding sites that are part of a combinatorial mechanism that relies on cooperative binding, interaction of transcriptional activator proteins, and transcriptional synergy (for reviews, see Refs. 72 and 73). Moreover, NF-κB transcription factor-binding sites that are part of a combinatorial mechanism will be very helpful in elucidating the activation mechanism.

Finally, the observation that insulin can induce mdr1b gene expression in rat liver cancer cells in culture may have important clinical implications. For example, breast cancer MCF-7 cells treated with insulin or insulin-like growth factor I have reportedly high levels of resistance to many antitumor drugs, including actinomycin D, adriamycin, and puromycin (80). Although the levels of mdr expression in the treated cells were not reported for that study, the drugs used are known substrates for the mdr-encoded P-glycoproteins (for a review, see Ref. 1). It is not known, however, if insulin would modulate such drug resistance to chemotherapeutic agents in vivo. Nevertheless, our present communication provides an important basis for future investigations on the potential roles of NF-κB, insulin, and other growth factors in the regulation of drug resistance during cancer chemotherapy.

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