Transcriptional Activation of the MDR1 Gene by UV Irradiation

ROLE OF NF-Y AND Sp1

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The MDR1 promoter is subject to control by various internal and external stimuli. We have previously shown that the CCAAT box-binding protein, NF-Y, mediates MDR1 activation by the histone deacetylase inhibitors, trichostatin A and sodium butyrate, through the recruitment of the co-activator, P/CAF. We have now extended our investigation to the activation of MDR1 by genotoxic stress. We show that activation of the MDR1 promoter by UV irradiation is also dependent on the CCAAT box (−82 to −73) as well as on a proximal GC element (−58 to −42). Gel shift and supershift analyses with nuclear extracts prepared from human KB-3-1 cells identified NF-Y as the transcription factor interacting with the CCAAT box, while Sp1 was the predominant factor binding to the GC element. Mutations that abrogated binding of either of these factors reduced or abolished activation by ultraviolet irradiation; moreover, co-expression of a dominant-negative NF-Y protein (NF-YA29) reduced UV-activated transcription. Interestingly, YB-1, a transcription factor that also recognizes the CCAAT motif and had been reported to mediate induction of the MDR1 promoter by ultraviolet light, was incapable of interacting with the double-stranded MDR1 CCAAT box oligonucleotide in nuclear extracts, although it did interact with a single-stranded oligonucleotide. Furthermore, a mutation that abolished activation of MDR1 by UV-irradiation had no effect on YB-1 binding and co-transfection of a YB-1 expression plasmid had a repressive effect on UV-inducible transcription. Taken together, these results indicate a role for both NF-Y and Sp1 in the transcriptional activation of the MDR1 gene by genotoxic stress, and indicate that YB-1, if involved, is not sufficient to mediate this activation.

P-glycoprotein (Pgp) was first identified by virtue of its overexpression in multidrug-resistant (MDR) tumor cells, where it mediates the energy-dependent efflux of a variety of chemotherapeutic agents. Pgp is encoded by a multigene family expressed in normal tissues, both as a consequence of differentiation triggers and in response to environmental challenges, and have long been proposed to function in the protection against cellular toxins (1–6). The human class I P-glycoproteins have been shown to transport phospholipids (7), cholesterol (8), calcium channel blockers (9), immunosuppressants (10), peptides (11), steroids (12), and xenobiotics (13, 14). More recent studies suggest that P-glycoprotein plays a general anti-apoptotic role that extends beyond resistance to chemotherapeutics, since cells that overexpress P-glycoprotein are resistant to a wide range of apoptotic inducers, including serum starvation, Fas ligand, UV irradiation, and tumor necrosis factor (15–19). It is clear that Pgp has diverse functions in different cells and tissues. Therefore, it is not surprising that the expression of P-glycoprotein is complex and highly regulated.

The human class I Pgp homologue is encoded by the MDR1 gene. In cultured cells, constitutive overexpression of MDR1 can be mediated by changes in gene dosage or transcription (20), while a recent study indicates that constitutive overexpression of Pgp in some acute myelogenous leukemia patients is associated with DNA rearrangements (21). MDR1 can be also be transiently induced in cultured cells by a variety of stimuli. In light of the apparent role of Pgp in defense against xenobiotic assault, there has been a particular interest in the activation of MDR1 by stress inducers, including heat shock (22), UV irradiation (23), and chemotherapeutic agents (24, 25). In a recent study, we have shown that MDR1 expression can also be rapidly activated in patient tumors in vivo following a short term exposure to the chemotherapeutic agent doxorubicin (26). However, despite the intensive study of Pgp-mediated drug resistance, the regulatory mechanisms underlying stress-mediated activation of MDR1 transcription are not fully understood.

The proximal promoter of MDR1 contains several regulatory regions, including an inverted CCAAT box at −82 to −73 and a GC element at −56 to −42, both of which have been shown to be required for constitutive promoter activity in some cell types (27–29, 31). Binding of Sp1 (28) or Sp3 (30) to the GC element activates the MDR1 promoter in Drosophila cells, while interaction of the trimeric transcription factor NF-Y with the CCAAT box has been implicated in constitutive regulation of the MDR1 promoter in several cell lines by ourselves and others (27, 29, 31). Recently, we have shown that NF-Y functions in MDR1 transcription by recruiting P/CAF, a co-activator with histone acetyltransferase activity, to the promoter (31).

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CCAT-binding protein in nuclear extracts. In light of this, we have revisited the activation of MDR1 by UV irradiation in an effort to determine whether NF-Y, YB-1, or both transcription factors are required for induction by this stressful stimulus. We now report that NF-Y is the double-stranded CCAAT box-binding protein, which, along with Sp1, mediates activation by ultraviolet light. We further propose that the single-stranded binding protein YB-1, if involved, may mediate its effect through an alternate mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmids, and Transfections—**Human KB-3-1 epidermoid carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The wild type MDR1 promoter/luciferase construct (pMDR1–1202) and two CCAAT box mutant constructs (pMDR1-mutC1 and pMDR1-mutC2) included MDR1 promoter sequence from −1202 to +118 and were generated from the luciferase vector pGL-2B as described previously (31). The MDR1 promoter/luciferase construct containing a mutant GC element (pMDR1-MutGC) was created by mutagenesis using a 4% non-denaturing oligonucleotide gel-inverted 5′-GTGGGCT-3′ to 5′-cGGGga 3′ at −51 through −45 (50). The expression plasmid NF-YA29 carries a dominant negative form of the NF-YA subunit, capable of trimerization with NF-YB and NF-YC, but incapable of activation (32). pGL-2C contains an SV-40 promoter and enhancer inserted upstream of the luciferase coding region (Promega, Madison, WI). The YB-1 expression vector (pSFFV-YB-1) and the corresponding control vector (pSFFV-neo) have been described (33).

KB-3-1 cells were transfected with 0.5 μg of pMDR1 reporter construct and varying amounts of the appropriate expression vector using Lipofectin as recommended by the vendor (Life Technologies, Inc.). The total amount of DNA was adjusted to 2.0 μg/well by the addition of sonicated salmon sperm DNA (Stratagene, La Jolla, CA). Transfected cells were incubated for 9–16 h prior to treatment with ultraviolet light, then incubated for an additional 40–48 h before harvesting. Luciferase assays were performed as recommended by the vendor (Promega, Madison WI), and activity was expressed as luminescence units normalized to protein concentration as determined by the bichoninic acid protein assay (Pierce).

**Electrophoretic Mobility Shift Assays—**Nuclear extracts were prepared from KB cells as described previously (31) with or without prior UV irradiation. Two different buffer systems were used in binding reactions on the MDR1 CCAAT box oligonucleotide: buffer I (20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM MgCl2, 1 mM DTT, 10% glycerol) and buffer II (25 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol). Proteins interacting with the GC element were identified in a buffer containing 12 mM HEPES, pH 7.5, 42 mM KCl, 3 mM MgCl2, 60 μM ZnCl2, 1 mM DTT, 0.03% Nonidet P-40, and 7.2% glycerol. Approximately 11 μg of nuclear extract were incubated for 10 min at room temperature in a 20-μl reaction containing the appropriate buffer and 0.5 μg of poly(dI-dC). Following preincubation, 60,000 cpm (−0.5 ng) of 5′-32P-end-labeled probe was added and the reaction mixture was incubated at room temperature for an additional 20 min. For supershift analyses, nuclear extracts and antibodies were preincubated on ice for 2–3 h, followed by the addition of 5′-32P-labeled probe. The reaction products were resolved on a 5% non-denaturing polyacrylamide gel. 10 μl of 0.5× Tris borate-EDTA or 1× Tris-glycine-EDTA at 4°C. The sequences of the upper and lower strands of the oligonucleotides corresponding to the MDR1 CCAAT box and two CCAAT box mutants, mutC1 and mutC2, have been described previously (31). The sequence of the upper strand of the oligonucleotides that included either the wild-type(GC) or mutant GC (mutGC) elements are shown below, along with the sequence of the upper strand of the double-stranded oligonucleotide that included both the inverted CCAAT box and the GC element (NF-YGC): GC, 5′-GAAACAGCCCGGCCTGGTGGAGCA- CACCGCTTCTCCGC-3′; mutGC, 5′-GAAACAGCCCGGCCTGGTGGAGCA- CACCGCTTCTCCGC-3′; mutC1, 5′-GGAACAGCCCGGCCTGGTGGAGCA- CACCGCTTCTCCGC-3′; and 5′-GTGGGCT-3′.

Mouse monoclonal anti-NF-Y antibodies and rabbit polyclonal anti-YB-1 antibodies have been described previously (29, 34). Rabbit polyclonal anti-Sp-1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

**RESULTS**

**Activation of the MDR1 Promoter by UV Irradiation Depends on Both the CCAAT Box and the GC Element—**It had previously been demonstrated that activation of the MDR1 promoter in KB-3-1 cells by UV irradiation required sequences between −136 and −76 within the promoter region (23). We have shown that an inverted CCAAT box located between −82 and −73 is required for transcriptional activation by the histone deacetylase inhibitors trichostatin A and sodium butyrate (31). To determine whether the CCAAT box played a role in activation of transcription by UV light, luciferase reporter constructs containing either the wild-type MDR1 promoter (pMDR1–1202) or two different constructs in which the CCAAT box was mutated (pMDR1-mutC1 and pMDR1-mutC2) were transiently transfected into human epidermoid KB-3-1 cells, which were then exposed to UV light (16 J/m2, 254 nm). As shown in Fig. 1A, MDR1 promoter-driven transcription was activated ∼20-fold by UV irradiation, and this activation was abrogated by both mutations in the CCAAT element; the low level of activation of the mutant constructs was comparable to nonspecific activation of the promoterless pGL-2B vector (data not shown). Using the same approach, we then determined whether the proximal GC element (−56 to −42) contributed to the UV activation observed. Mutation of this element within the context of the full-length promoter also abolished transcriptional activation (Fig. 1A), indicating that both the inverted CCAAT box and the GC element were necessary for this response, but that neither alone was sufficient.

**NF-Y Interacts with the Double-stranded MDR1 Inverted CCAAT Element in KB Nuclear Extracts—**Although several transcription factors, including C/EBP, NF-Y, and YB-1, have been shown to interact with CCAAT sequences in different promoters (35), only NF-Y requires all five base pairs for binding. We and others had previously determined that NF-Y binds to the CCAAT box of the MDR1 promoter in the colon cell lines SW620 (31) and HCT116 (29) as well as in HepG2 liver cells (29). To identify the MDR1 CCAAT box-binding protein in KB-3-1 cells, gel and supershift assays were performed using a double-stranded oligonucleotide corresponding to the MDR1 promoter sequence from −89 to −64 (dsMDR-CCAAT). Two major bands were detected with nuclear extracts from KB-3-1 cells (Fig. 1B, lane 1), the pattern of which was indistinguishable from that seen with SW620 cells (31). Formation of both complexes was prevented by the presence of excess, unlabeled dsMDR-CCAAT (lane 2), but not with two oligonucleotides containing mutations in the CCAAT box (dsMDR-mutC1 and dsMDR-mutC2, lanes 3 and 4). Mammalian NF-Y comprises three subunits, NF-YA, NF-YB, and NF-YC (24), which are highly conserved throughout evolution. An antibody against the NF-YA subunit was therefore used to investigate the presence of NF-Y in the gel-shift complexes. Precipitation with anti-NF-YA supershifted the upper, but not the lower complex (lane 5). Neither the control mouse IgG antibody (lane 6) nor antibodies to C/EBPβ or YB-1 (data not shown) affected the formation of either complex. Moreover, no qualitative or quantitative differences in complex formation were observed when extracts from UV-irradiated cells were used (lane 7). Taken together, these results indicate that NF-Y, not YB-1 or C/EBP, is a component of the higher molecular weight complex that forms on the MDR1 CCAAT box in both untreated and UV-irradiated KB-3-1 cells; the identity of the proteins involved in lower complex formation is under investigation. The failure of the two CCAAT box mutants, Mut C1 and Mut C2, to compete for formation of the NF-Y-containing complex is consistent with their inability to mediate MDR1 activation (Fig. 1A).

**Sp1 Interacts with the GC Element in Untreated and UV-
induced KB-3-1 Cells—Several factors have been shown to bind to the MDR1 GC element in different cells under different conditions, including Sp1 (28), Sp3 (30), Egr1 (36), and WT-1 (37). To identify protein(s) interacting with the GC element in treated and untreated KB-3-1 cells, we next performed gel shift analyses using an oligonucleotide spanning both the inverted CCAAT box and the GC element (dsNF/Y/GC). Three specific complexes were detected in extracts from untreated and UV-irradiated cells (Fig. 1C, lanes 1 and 2, respectively), all of which were eliminated by the addition of the unlabeled probe (lane 3). Addition of dsMDR-CCAAT competed for the formation of complexes I and III (lane 4), while dsMDR-mutC1 had no effect on complex formation (lane 5); preincubation with the mouse anti-NF-YA antibody supershifted these complexes (lane 8), indicating that they included NF-Y. An oligonucleotide corresponding to the GC element of the MDR1 promoter (dsMDR-GC) also competed for complex I as well as for complex II (lane 6), while an oligonucleotide containing a mutation in the GC element (MDR-mutGC) did not (lane 7); preincubation with rabbit anti-Sp1 antibodies resulted in a supershift of these complexes (lane 10), indicating the presence of Sp1. Incubation of dsNFY/GC with recombinant Sp1 protein and in vitro synthesized NF-Y resulted in formation of the same size complexes with the same supershift pattern (data not shown). Taken together, these results identified Sp1 as a binding component of complex II and NF-Y as a component of complex III; both NF-Y and Sp1 are present in complex I, demonstrating that their binding is not mutually exclusive.

**YB-1 Interacts with Single-stranded, but Not Double-stranded, MDR1 Promoter Sequences**—In light of our findings, prior studies by two laboratories using gel shift assays have suggested that the transcription factor interacting with the double-stranded MDR1 inverted CCAAT element is YB-1. However, this interaction was not tested directly. In initial studies, it was shown that multiple complexes were formed on the MDR1 CCAAT element in the presence of KB-3-1 nuclear extracts; it was assumed, but not confirmed, that the complexes formed included YB-1 (23, 38). In a subsequent study by another laboratory, the presence of YB-1 in nuclear extracts was assayed using a consensus YB-1 oligonucleotide derived from the MHC DRA promoter rather than one derived from the MDR1 promoter sequence (39). Since we and others have identified NF-Y rather than YB-1 as the transcription factor interacting with dsMDR1-CCAAT using nuclear extracts prepared from several human cell lines (29, 31), we were prompted to readdress the binding of YB-1 to the MDR1 CCAAT box as well as the role of YB-1 in UV-mediated activation of the MDR1 promoter. Since we had used a different assay buffer for the detection of NF-Y binding than had been used in the earlier YB-1 studies (buffers I and II, respectively; see “Experimental Procedures”), we considered the possibility that the interaction of YB-1 with the MDR1 promoter required different binding conditions than NF-Y. However, as shown in Fig. 2A, the complexes formed in buffer II were indistinguishable from those identified in buffer I (Fig. 1B). Since YB-1 has been shown by several laboratories to prefer single-stranded DNA as a binding substrate (40), we next determined whether YB-1 could bind to a single-stranded oligonucleotide containing the MDR1 CCAAT box. Gel shift analyses were performed using both buffer systems indicated above. As shown in Fig. 2B, incubation of the upper strand of the MDR1 CCAAT box oligonucleotide (up-MDR-CCAAT) with nuclear extract from KB-3-1 cells resulted in the formation of a complex (complex A, lane 2) that was competed for by an excess of unlabeled upMDR-CCAAT oligonucleotide (lane 3). Addition of excess unlabeled lower-strand oligonucleotide (loMDR-CCAAT) resulted in the generation of the double-stranded free probe, which migrated somewhat slower than the single-stranded probe (compare lane 4 to lane 1), as well as two slower migrating complexes (lane 4) that were indistinguishable from those complexes binding to the double-stranded probe in initial experiments (Fig. 1B); the presence of NF-Y complexed with the double-stranded probe was confirmed by supershift assays (data not shown). A single complex also formed on loMDR-CCAAT (lane 5); however, addition of a 200-fold molar excess of unlabeled loMDR-CCAAT had no effect on its formation, indicating that it was nonspecific (lane 7). Addition of excess unlabeled upMDR-CCAAT again yielded the double-stranded oligonucleotide and the specific NF-Y complex (lane 6). Essentially the same results were obtained using buffer II (lanes 8–14) except that an additional slower-migrating, specific complex (complex B) was observed when using upMDR-CCAAT as probe.

Identification of YB-1 as the protein involved in complex formation on the upMDR-CCAAT oligonucleotide was accomplished by supershift analysis using an anti-YB-1 antibody (Fig. 2C). Preincubation with anti-YB-1 resulted in a reduction in formation of complex A and the appearance of a supershifted complex in both buffers (compare lane 3 to lane 2, and lane 6 to lane 5). Anti-YB-1 also reduced the formation of complex B in buffer II. Taken together, these results indicate that YB-1 specifically interacted with the upper-strand of the inverted CCAAT box of the MDR1 promoter. Complex B may represent a multimer form of YB-1, which is consistent with previous results obtained with purified recombinant proteins (41). No difference in YB-1 binding was seen between untreated and UV-treated nuclear extracts (compare lanes 2 and 4).

In light of our findings, we next considered the possibility that the binding of YB-1 to one strand of the CCAAT box may regulate UV-induced activation of the MDR1 promoter. Since we had identified two CCAAT box mutations (MDR-mutC1 and MDR-mutC2) that abrogated the UV response, we tested these mutants for their ability to compete for YB-1 complex formation (Fig. 2D). Addition of the unlabeled upper strand of the MDR-mutC1 oligonucleotide (lane 4) reduced formation of the YB-1 complex more effectively than an upper strand oligonucleotide corresponding to the MDR1-GC element (lane 6), although not as effectively as the wild-type oligonucleotide (lane 2). However, the upper strand of MDR-mutC2 eliminated YB-1 complex formation as effectively as did the wild type (lane 5). Therefore, both of the CCAAT box mutations that eliminated NF-Y binding and UV-induced activation were still able to support YB-1 complex formation, suggesting that YB-1 binding was not dependent on an intact CCAAT box and that YB-1, if involved, was not sufficient to mediate activation of the MDR1 promoter by UV-irradiation.

**Evidence for Involvement of NF-Y, but Not YB-1, in Activation of the MDR1 Promoter by UV Irradiation**—To determine whether activation of the MDR1 promoter by UV irradiation was dependent upon NF-Y, a dominant negative NF-YA expression vector (NF-YA29) was co-transfected with pMDR1–1202 into KB-3-1 cells. This mutant NF-YA, which sequesters NF-YB and NF-YC into a functionally inactive heterotrimer (32), significantly reduced activation of the MDR1 promoter by UV irradiation (Fig. 3A), indicating a role for NF-Y in the UV response.

By co-expression assays, YB-1 has been shown to be a transcriptional activator of some promoters and a transcriptional repressor of others (33, 42–48). To evaluate the effect of the single-stranded interaction of YB-1 with the MDR1 promoter on either basal or UV-induced expression, the YB-1 expression vector, pSFFV-YB-1, or the control vector, pSFFV-neo, were co-transfected with pMDR1–1202 into KB-3-1 cells and lucif-
untreated cells. Activity from treated cells are expressed as fold activation relative to measured and normalized to protein concentration. Levels of luciferase were transiently transfected with 0.5 \( \mu \)g of plasmid for 48 h prior to assaying. Luciferase activity was measured and normalized to protein concentration. Levels of luciferase activity from treated cells were expressed as fold activation relative to untreated cells. A double-stranded oligonucleotide corresponding to the MDR1 promoter sequence be-comes activated by ultraviolet light. A plethora of studies have shown that the MDR1 promoter is inducible by a variety of stimuli (20). One critical question in the field is how these seemingly disparate inducers converge on the promoter, and what cis elements and protein factors are involved in translating these signals into transcriptional activation. One factor that has been shown by our laboratory (31) and others (29) to be involved in constitutive expression of the MDR1 promoter is the trimeric transcription factor NF-Y. We have shown that NF-Y activates transcription by recruiting the histone acetyl transferase P/CAF to the MDR1 promoter (31) and that this activation is accompanied by the hyperacetylation of promoter-associated histones, which has been proposed to promote transcription factor access to nucleosomal DNA and relieve inhibitory effects on transcriptional initiation and elongation. More recently, we have shown that the ubiquitous transcription factor Sp1 cooperates with NF-Y to mediate the effects of histone modifying enzymes. Previous studies have shown that Sp1 binds to a GC element within the MDR1 promoter and is required for basal expression in a number of cell lines (28, 29, 49). In the present study, we demonstrate that both NF-Y and the Sp1 site are also required for MDR1 activation by UV irradiation.

The mechanism by which NF-Y and Sp1 transduce the signal from UV light to the MDR1 promoter has not yet been elucidated. We were unable to detect a quantitative change in complex formation using extracts from untreated or UV-irradiated cells. While these in vitro assays do not allow us to rule out the possibility that in vivo binding patterns are altered, it is intriguing to speculate that UV light may somehow alter the NF-Y/Sp1/P/CAF complex, possibly through a post-translational modification. In support of this, a recent study by Nakatani and co-workers (50) indicates that the histone acetylase activity of P/CAF is augmented by DNA damage. This suggests that histone hyperacetylation may be enhanced under these conditions; alternatively, acetylation of NF-Y itself may be affected, since it has recently been shown that the Xenopus laevis NF-YB subunit can be acetylated by the P/CAF partner, p300 (51). A recent study has also suggested that the activity of Sp1 may be regulated by UV irradiation, since activation of the p21 promoter by UV light requires two Sp1 sites within the proximal promoter (52). It is interesting to note that NF-Y and Sp1 have been shown to cooperate in the regulation of other

**DISCUSSION**

A plethora of studies have shown that the MDR1 promoter is inducible by a variety of stimuli (20). One critical question in the field is how these seemingly disparate inducers converge on the promoter, and what cis elements and protein factors are involved in translating these signals into transcriptional activation. One factor that has been shown by our laboratory (31) and others (29) to be involved in constitutive expression of the MDR1 promoter is the trimeric transcription factor NF-Y. We have shown that NF-Y activates transcription by recruiting the histone acetyl transferase P/CAF to the MDR1 promoter (31) and that this activation is accompanied by the hyperacetylation of promoter-associated histones, which has been proposed to promote transcription factor access to nucleosomal DNA and relieve inhibitory effects on transcriptional initiation and elongation. More recently, we have shown that the ubiquitous transcription factor Sp1 cooperates with NF-Y to mediate the effects of histone modifying enzymes. Previous studies have shown that Sp1 binds to a GC element within the MDR1 promoter and is required for basal expression in a number of cell lines (28, 29, 49). In the present study, we demonstrate that both NF-Y and the Sp1 site are also required for MDR1 activation by UV irradiation.

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**FIG. 1.** The NF-Y and Sp1 binding sites are required for activation of the MDR1 promoter by ultraviolet light. A, KB-3-1 cells were transiently transfected with 0.5 \( \mu \)g each of the MDR1 promoter wild type construct (pMDR1–1202), two CCAAT box mutant constructs (pMDR1-mutC1, pMDR1-mutC2), or an Sp1 site mutant construct (pMDR1-mutGC). Cells were irradiated with UV light (16 J/m\(^2\), 254 nm) and then incubated for 48 h prior to assaying. Luciferase activity was measured and normalized to protein concentration. Levels of luciferase activity from treated cells were expressed as fold activation relative to untreated cells. B, 0.5 ng (60,000 cpm) \( ^{32} \)P-labeled double-stranded oligonucleotides corresponding to the MDR1 promoter sequence between −89 and 64 were incubated in buffer I with 11 \( \mu \)g of nuclear extracts prepared from untreated KB cells (lanes 1–6) or UV-irradiated cells (lane 7). 100-fold molar excess of wild type (lane 2) and two mutant oligonucleotides (mutC1 and mutC2, lanes 3 and 4) were used in competition assays. For supershift analysis, 250 ng of anti-NF-YA antibody (lane 5) or mouse IgG (lane 6) were preincubated with nuclear extracts on ice for 3 h prior to the addition of the probe. Lane 8, free probe. C, simultaneous binding of NF-Y and Sp1 to the MDR1 promoter. A double-stranded probe including −91 to −29 of the MDR1 promoter (NFY/GC) was incubated with untreated nuclear extract (lane 1) or UV-treated nuclear extracts (lanes 2–7) in gel mobility shift assays. 50-fold molar excess of various cold oligonucleotides were used in competition assays (lanes 3–7). Lane 3, wild type oligonucleotide NFY/GC. Lane 4, wild type CCAAT box oligonucleotide. Lane 5, mutant CCAAT box oligonucleotide (mutC2). Lane 6, wild type MDR1GC box oligonucleotide (−67 to −26). Lane 7, mutant GC oligonucleotide (mutGC). In supershift analysis, 250 ng of anti-NF-YA or mouse IgG and 750 ng of anti-Sp1 or rabbit IgG were preincubated with nuclear extracts for 3 h at 4 °C (lanes 8–11). Lane 12, free probe. Protein-DNA complexes were resolved on a polyacrylamide gel in 1× TBE.

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promoters, including the rat fatty acid synthase promoter (53), the mouse polysialic acid synthase promoter (54), the human MHC class II promoter (55), and the human cdc25C promoter (56). Furthermore, computer analysis has identified several other promoters in which the orientation and relative proximity of NF-Y and Sp1 binding sites is similar to that identified in
investigating qualitative changes in these proteins in response to UV irradiation and other genotoxic stress.

Previous studies have shown that NF-Y is involved in constitutive activation of the MDR1 promoter (29, 31), while the transcription factor YB-1 had been suggested to be responsible for activation of the MDR1 promoter by UV irradiation (23, 38, 59). YB-1 was originally cloned as a CCAAT box-binding protein (60) and can function as either a transcriptional activator or transcriptional repressor (40). It shows a strong preference for single-stranded DNA substrates and, unlike NF-Y, does not show an absolute requirement for the 5-base pair CCAAT motif. In fact, many YB-1-binding sites contain either an imperfect CCAAT box or no CCAAT box at all, suggesting that flanking sequences play an important role in sequence-specific recognition by YB-1 (42–47). The predicted involvement of YB-1 in genotoxic stress-induced activation of MDR1 originated from the isolation of a YB-1 cDNA in a library screen using a double-stranded probe corresponding to −136 to +4 of the MDR1 promoter (38, 59). Subsequent studies identified a binding protein in KB nuclear extracts that interacted with an oligonucleotide containing the MDR1 CCAAT element; however, the identity of this binding protein as YB-1 was postulated, but not directly evaluated (i.e. by supershift assays) (23, 38, 61). In light of this, it is interesting to note that the pattern of DNA-protein complexes observed in these previous studies is very reminiscent of the complexes that we have shown to include NF-Y. Moreover, we and others (this report; Refs. 29 and 31) have been unable to detect binding of YB-1 to the double-stranded MDR1 CCAAT box element in nuclear extracts from either KB-3-1 or SW620 cells. In fact, we can only detect YB-1 binding to a single-stranded oligonucleotide representing the upper strand of this element. We have also shown that a mutation within the CCAAT box that abrogates induction by UV light has no effect on YB-1 binding to the single-stranded MDR1 oligonucleotide, in contrast to its dramatic effect on NF-Y binding. We believe that our repeated failure to detect YB-1 binding in gel shift assays, despite its presence in the nuclear extracts, is due to the very low affinity of this protein for the double-stranded MDR1 CCAAT element. This is supported by our finding that conversion of the single-stranded oligonucleotide to a double-stranded probe eliminates YB-1 binding in favor of NF-Y binding. It is also in agreement with the studies of Sundseth and co-workers (29), who showed that, although high concentrations of purified recombinant YB-1 protein were able to interact with the MDR1 CCAAT box, NF-Y was the interacting species in nuclear extracts from the several cell line studied.

A number of studies have investigated the effect of altering YB-1 levels on MDR1 gene expression. An initial study by Kohno and co-workers (59) showed that stable transfection of YB-1 antisense expression vectors into KB-3-1 cells led to a decrease in YB-1 levels; while these transfectants exhibited resistance to UV irradiation and cisplatin, they showed no change in their sensitivity to MDR drugs, including vincristine and doxorubicin, implying no change in functional Pgp levels. However, in a recent study by the same group (61), the −3-fold decrease in YB-1 levels in the stably transfected KB cells correlated with a 1.5–2-fold decrease in MDR1 gene expression, although it was not determined whether this effect was mediated by an interaction of YB-1 with the MDR1 CCAAT box. It was also reported that levels of YB-1 in the nucleus of KB-3-1 cells increased in response to UV irradiation (62), although effects on MDR1 gene expression were not evaluated in that study. A correlation between nuclear localization of YB-1 and MDR1 expression in osteosarcomas (63) and breast tumors (39) has also been reported. Although these observations suggest a
role for YB-1 in the regulation of MDR1 gene expression, they do not establish a cause-effect link, nor do they allow for a distinction between direct and indirect effects, since neither the binding of YB-1 to the MDR1 CCAAT box nor a direct effect of YB-1 on MDR1 promoter activity was evaluated. Indeed, YB-1 has been shown to indirectly modulate transcription of some promoters by affecting the activity of other transcription factors, including Sp1 (64).

In contrast to the studies showing a positive, albeit potentially indirect, effect on MDR1 gene expression, we have shown that overexpression of YB-1 in KB cells had a weak repressive effect on both basal and UV-activated transcription of the MDR1 promoter. Moreover, co-expression of a dominant-negative NF-Y construct decreased UV-activated transcription, indicating a role for this transcription factor in UV-mediated activation. While the basis for this difference is not clear, one possibility is that YB-1 exerts a post-transcriptional effect on MDR1 expression, since the YB-1 family of proteins have been shown to interact with both DNA and RNA and have been proposed to have multiple functions in addition to transcriptional regulation (65).

Taken together, our data indicate that NF-Y, not YB-1, is the MDR1 CCAAT-binding protein in nuclear extracts. Activation of the MDR1 promoter by UV-irradiation, much like activation by histone hyperacetylation (31), requires both the NF-Y and Sp1 binding sites. It is possible that YB-1 interacts at a site outside of the CCAAT box, or that it modulates the activity of Sp1 binding sites. It is possible that YB-1 interacts at a site outside of the CCAAT box, or that it modulates the activity of other transcriptional activators on the MDR1 promoter, such as Sp1 or NF-Y; alternatively, YB-1 may act at a post-transcriptional level to effect the UV response. Nevertheless, it is clear that the regulation of this response is complex, involving multiple transcription factors whose levels and/or activities may be altered by post-translational modifications in response to genotoxic stress. This predicts that the relative levels and activities of these factors in different cell types will play a role in determining cellular response to stress inducers. Therefore, one should be cautious about assigning functional significance to a correlation between levels of any one factor and changes in MDR1 expression.

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