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Involvement of Activator Protein-1 and Nuclear Factor-κB Transcription Factors in the Control of the DT-Diaphorase Expression Induced by Mitomycin C Treatment

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SUMMARY

The antitumor antibiotic mitomycin C is activated by several bioreductive enzymes, including DT-diaphorase. In HT29 cells, mitomycin C treatment results in the induction of DT-diaphorase as reflected in elevated steady state DT-diaphorase mRNA levels. An increase in the transcriptional rate was demonstrated by nuclear run-on assay. To investigate the molecular basis of the change in transcriptional activity caused by mitomycin C treatment, electrophoretic mobility shift assays were used to demonstrate the induction of nuclear factor binding to elements in the 5′ flanking region of the DT-diaphorase gene. Treatment of HT29 cells with mitomycin C resulted in the dose-dependent induction of binding activity directed to the activator protein-1 (AP-1) binding element with a time course similar to that of mRNA elevation. Supershift assays using specific antibodies to Jun and Fos demonstrated the participation of both proteins in the binding activities generated. A binding activity for the nuclear factor-κB (NF-κB) site was induced with a similar time course. Both competitor and supershift experiments indicated that a heterodimer of the NF-κB proteins p50 and p65 was contained in the bound complex. To further investigate the functional consequences of such binding, we transfected HT29 cells with a plasmid containing 3 kb of the DT-diaphorase 5′ region upstream of a reporter gene, chloramphenicol acetyltransferase. Treatment with mitomycin C resulted in a 5.5-fold increase in the expression of a chloramphenicol acetyltransferase construct containing 3 kb of DT-diaphorase promoter sequence. Using a series of deletion mutations based on this full-length construct, we found that two regions of the DT-diaphorase promoter region, positions −346 to −588 (containing the AP-1 element) and positions −785 to −890 (containing the NF-κB element) are required for the full expression of the mitomycin C response. The specific involvement of these binding elements was confirmed using mutational analysis. The results demonstrate that mutation of either element alone or of both diminishes the response, indicating an additive interaction between the elements at a minimum. However, inducibility characterizes a promoter fragment as small as 78 base-pairs from the transcription start site. Treatment of cells with mitomycin C induced binding to a 38-base-pair region (−40 to −78) devoid of known transcription factor binding elements. These data suggest that mitomycin C induces the overexpression of DT-diaphorase through a mechanism involving both the AP-1 and NF-κB response elements and that inducibility depends on a novel factor binding element.

The antitumor antibiotic MMC is used widely in the treatment of a variety of solid tumors, including gastric, breast, lung, pancreas, and cancers of the head and neck (1). Its use is limited by severe and cumulative bone marrow toxicity and/or unpredictable pulmonary toxicity. The activity of the drug in resistant tumors supports the broader investigation of its effects and the molecular basis for activity. The cellular pharmacology of MMC has recently been reviewed (2). The major mechanism by which MMC kills cells is by alkylating DNA at either the N2 or N7 position of guanine (i.e., in either the minor or major grooves) (3, 4). The nature of the monoadduct formed and its ability to convert into a cross-link seem to depend on whether the activated species has been formed through one- or two-electron reduction (2, 4). Cross-links are formed with substantial target sequence specificity (4). The generation of these cytotoxic lesions has been associated with the pH- and O_2-dependent metabolism of MMC by a variety of one- and two-electron reducing enzymes (2). These metabolic steps may also contribute to the detoxication of MMC, and controversy exists concerning the conditions that may promote bioreductive alkylation versus detoxication (5–11).

Among the enzymes implicated in the metabolism of MMC is the two-electron reducing enzyme DT-diaphorase (7, 9, 12,

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ABBREVIATIONS: MMC, mitomycin C; EMSA, electrophoretic mobility shift assay; bp, base-pair(s); CAT, chloramphenicol acetyltransferase; AP-1, activator protein-1; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

13). Careful analyses have elucidated the conditions that optimize the interaction of MMC and DT-diaphorase, and strategies to enhance the activity of MMC by modulation of DT-diaphorase activity have been proposed (2, 14). Colorectal tumors in particular have DT-diaphorase content that is elevated relative to that of normal colon mucosa and have been proposed as a target tumor for DT-diaphorase-based strategies (15).

The response of cells in culture to adverse conditions, including DNA damage, is to induce the expression of various proteins that may have a protective function (16). Among them are the transcription factors that make up AP-1, binding elements for which are found in the promoter regions of a wide variety of genes (17). The DT-diaphorase 5′ promoter contains several transcription factor-binding elements that may determine the level of gene expression (18). We previously identified the involvement of AP-1 and NF-κB elements in the response to hypoxia (19, 20). In the current study, we demonstrate the involvement of both of these sites in the cellular response to MMC treatment by both promoter analysis and gel mobility shift assay. We show through functional studies that treatment with MMC results in the increased expression of DT-diaphorase through a mechanism that is both AP-1 and NF-κB dependent. These data provide evidence for the involvement of transcription factor activation in determining the response of detoxicating enzymes to cytotoxic drug exposure.

Materials and Methods

Cell culture and MMC treatment. Human colon adenocarcinoma cell lines HT29 was grown in Dulbecco's modified Eagle's medium containing 10% FBS in T75 flasks (Corning Glassworks, Corning, NY), in a humidified atmosphere of 95% air/5% CO_2.

For MMC treatment, 10^6 cells were replated and allowed to adhere for 24–48 hr. MMC powder (Bristol-Myers) was diluted in sterile water and added to Dulbecco's modified Eagle's medium (GIBCO/BRL, Gaithersburg, MD) (no FBS) at the appropriate concentrations. HT29 cells were exposed to 0.15, 0.45, and 1.25 μM concentrations of MMC. These concentrations represented the IC_{10}, IC_{50}, and IC_{90} values for MMC in the HT29 cell line. At the end of the 4-hr exposure, the drug was removed, the flasks were washed twice with PBS, and fresh Dulbecco's modified Eagle's medium was added. The control cells were subjected to identical media changes and harvested at the same time as treated cells.

DT-diaphorase activity. Cells were washed twice with PBS and scraped using a rubber policeman into 1 ml of PBS. The cell suspension was then sonicated for 30 sec, followed by centrifugation at 10,000 × *g* for 15 min. The clear supernatant was used for the enzyme assay. DT-diaphorase activity was measured essentially according to the method of Ernster as modified by Benson *et al*. (21). The reaction mixture contained 0.025 M Tris·HCl, pH 7.4, 0.7 mg/ml bovine serum albumin, 0.2 mM NADH, and 0.04 mM 2,6-dicholorophenolindophenol with or without 20 mM dicoumarol, in a total volume of 1 ml. DT-diaphorase was measured as the rate of dicoumarol-sensitive reduction of 2,6-dicholorophenolindophenol, which was monitored at 600 nm using a Beckman DU-70 spectrophotometer (Beckman Instruments, Palo Alto, CA).

Isolation and Northern blot analysis of RNA. Total cellular RNA was isolated by a single-step acid guanidium isothiocyanate-phenol-chloroform extraction procedure (22), subjected to electrophoresis in 1% agarose-2.2 M formaldehyde gel, transferred onto nylon membranes (Magna NT; MSI, Westboro, MA), hybridized to a 1.4-kbp ^{32}P-labeled DT-diaphorase full-length human cDNA probe[1] prepared by multiprimer labeling (Amersham), washed, and subjected to autoradiography at −70° for 3–7 days. The filter was stripped and hybridized to a 2.0-kbp *Pst*I cDNA fragment of chicken β-actin (23) as internal control of the equal loading of RNA. Hybridization and washing conditions were as described previously (19).

Nuclear run-on assay. Nuclei from MMC-treated or control cells were prepared as described by Celano *et al*. (24). The cells were washed with ice-cold PBS and scraped into buffer A (20 mM Tris·HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$), following which they were made 0.1% by volume with Nonidet P-40. Cells were vortexed, and the plasma membrane was lysed in a sterile Dounce homogenizer on ice. The nuclei were pelleted at 1000 × *g* for 10 min at 4°, washed in cold buffer A, and counted. The nuclear pellet was resuspended in transcription buffer (35% glycerol, 10 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA) and stored at −70°. After thawing, the run-on assay was carried out as described by Greenberg (25). The assay was conducted using 10^8 nuclei/reaction in a total volume of 200 μl in transcription buffer with 4 mM concentrations of ATP, GTP, and CTP and 200 μl of [α-^{32}P]UTP (3000 Ci/mM; Amersham, Arlington Heights, IL) at 26° for 10 min. Nuclei were digested with 10 μl of ribonuclease-free DNase I and 10 μl of 20 mM $CaCl_2$ at 26° for 5 min. Samples were then treated with 2 μl of proteinase K (10 mg/ml), 15 μl of 10× SET buffer (10% SDS, 50 mM EDTA, 100 mM Tris·HCl, pH 7.4) and 5 μl of yeast tRNA (10 mg/ml) at 37° for 30 min. Nuclear RNA was isolated by the guanidium-phenol-chloroform procedure described above. Finally, the RNA was dissolved in sterile Tris/EDTA buffer (1× = 10 mM Tris·HCl, pH 8.0, 1 mM EDTA) with 0.1% SDS. The DNA probes DT-diaphorase, c-*jun* (26), c-*fos* (27), c-*myc* (28), and β-actin (2 μg DNA/blot) used in the run-on assay were denatured and blotted onto a pre-wet nylon slot filter membrane in 6× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate) and allowed to dry at room temperature. The membrane was baked at 80° for 2 hr in a vacuum oven. After prehybridization of the membrane at 42° for several hours, the α-^{32}P-labeled nuclear RNA in 3 ml of hybridization buffer was added to the filter and hybridized for 24 hr at 42°. The filter was washed in 2× SSC/1% SDS at 65° for 1 hr and 0.1× SSC/0.1% SDS at room temperature for 1 hr. Autoradiography was performed at −70°, and quantification of the results was achieved by densitometric scanning normalized to the signal for β-actin.

Nuclear extract preparation. The nuclear extracts were prepared according to the procedure of Dignam *et al*. (29) as modified by Benjamin *et al*. (30). Protein content was assayed using the Bradford assay (BioRad, Richmond, CA).

Oligonucleotide labeling. The DT-diaphorase AP-1 and NF-κB oligonucleotide sequences (Fig. 1) were synthesized at the Oligonucleotide Synthesis Facility, Fox Chase Cancer Center, Philadelphia, PA. Mutant sequences had the structures shown (Fig. 1). cDNA strands were purified and annealed according to standard procedures (31). The double-strand oligonucleotide was labeled with [γ-^{32}P]ATP by phosphorylation with bacteriophage T_4 polynucleotide kinase and then ethanol precipitated to remove the bulk of the unincorporated radioactivity. Oligonucleotides that contained the accepted consensus sequence for AP-1 and NF-κB were obtained from Santa Cruz Biochemicals (Santa Cruz, CA) and used in competition studies.

EMSA. The nuclear extracts were analyzed for AP-1 and NF-κB binding activity by gel mobility shift assays. The binding reaction mixture containing 10 μg of nuclear extract and 1.8 μg of $poly(d)_{I\text{-}C}$ in a final volume of 30 μl was separated by electrophoresis in a 4% polyacrylamide gel. The gel was dried under vacuum and exposed to radiographic film overnight at −70°. For the supershift assay, the nuclear extracts were preincubated with anti-c-Jun, anti-c-Fos, anti-p65, and anti-p50 (Santa Cruz Biochemicals) before analysis by EMSA as described above.

Cloning of the human DT-diaphorase gene promoter. A HT29 cell genomic library was prepared in the λ bacteriophage

[1] K.-S. Yao and P. J. O'Dwyer, unpublished observations.

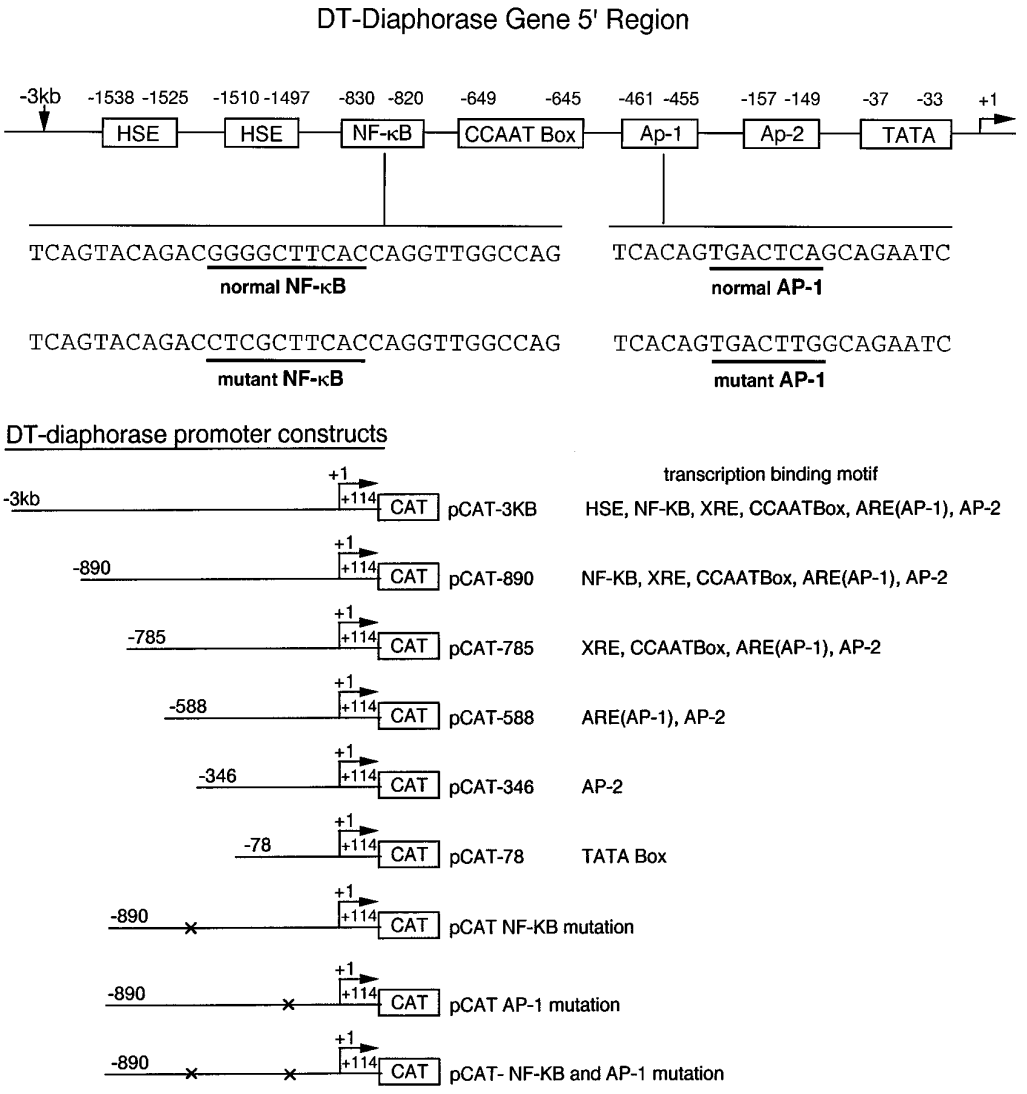

Fig. 1. Structure of the DT-diaphorase promoter region and promoter-CAT constructs (18) show the positions and sites of transcription factor binding elements and the sequences used as double-stranded probes for EMSAs. *HSE*, heat shock element.

Fig. 2. Construction of various DT-diaphorase gene 5′-flank regions in CAT vector. *HSE*, heat shock element; *XRE*, xenobiotic response element; *ARE*, antioxidant response element.

EMBL3 using standard procedures.[2] The library was screened with a probe containing human DT-diaphorase cDNA. Seven positive plaques were purified by four rounds of plaque purification. Genomic inserts were mapped by restriction digestion. A 3-kb *Bam*HI fragment, designated pDT3kb, was subcloned into the plasmid pUC18 for further characterization. The promoter region was sequenced by the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing procedure based on the manufacturer's protocol (Applied Biosystems, Norwalk, CT) with a Perkin-Elmer Cetus model 380 thermal cycler (Norwalk, CT). The sequence data were verified through use of the GenBank data base of the DT-diaphorase promoter region (32).

Construction of DT-diaphorase/CAT constructs. The 3-kb DNA segment containing DT-diaphorase promoter region was synthesized by PCR amplification and cloned into the pCAT-Basic vector (Promega, Madison, WI) *Sal*I and *Xba*I sites. The resulting recombinant plasmid was called pCAT-3KB. The deletion mutants pCAT-890, pCAT-785, pCAT-588, pCAT-346, and pCAT-78 were made through PCR amplification from pCAT-3KB through the use of specific primers. The details of promoter constructs was shown on Fig. 2. The mutant constructs were synthesized by the Ex-site PCR-based site-directed mutagenesis method following the manufacturer's procedure (Stratagene, La Jolla, CA). The mutation site was the same site as used in the gel mobility shift assay (Fig. 1).

[2] K.-S. Yao and P. J. O'Dwyer, unpublished observations.

Transfections and CAT assay. HT29 cells were plated at 8×10^5 cells/100-mm dish at 24 hr before transfection. Transfections were performed with 20 μg of plasmid DNA using the calcium phosphate precipitation Kit (5′ prime-3′ prime, Inc., Boulder, CO). After an additional 24-hr incubation, cells were harvested, washed, and pelleted. Finally, the pellet was resuspended in 200 μl of 0.25 M Tris, pH 7.8, buffer containing 1.0 mM phenylmethylsulfonyl fluoride/100-mm dish and frozen at −80°. The lysates were obtained by three freeze/thaw cycles. The protein concentration was determined by the Bradford assay (BioRad). A total of 50 μg of total cellular protein was assayed by an enzyme-linked immunosorbent assay-CAT method following the manufacturer's procedure (5′ prime-3′ prime, Inc.). Transfection efficiency, as determined by cotransfection with a β-gal plasmid (Promega), was highly similar within HT29 cell line. The relative CAT activities were expressed as the ratio (in percentage) of pCAT-78 activity that contains TATA box basal transcription apparatus. All reported values were from at least three different transfections performed with different plasmid preparations as well as different cell stocks.

Results

The effect of MMC in inducing DT-diaphorase was studied in HT29 cells. The base-line activity of DT-diaphorase in HT29 ranges from 300 to 700 nmol/min/mg of protein, de-

pending on a number of factors, including, in particular, the density of the cells in culture. When the cells were exposed to MMC and recultured in fresh media, a dose-dependent increase in the catalytic activity of DT-diaphorase was observed at 48 hr after treatment. The maximal effect was observed for the IC_{50} value: further increases in drug concentration had no additional effect (Fig. 3A). Examination of the time course in HT29 cells showed that although the effect was detectable at 24 hr, it reached a peak at 48 hr after treatment (Fig. 3B). Values had returned to base-line by 72 hr.

Northern blot analysis revealed that the increase in DT-diaphorase catalytic activity was associated with a dose-dependent increase in steady state mRNA content in HT29 cells (Fig. 4). The increase showed a similar dose-dependence to that of enzyme activity. The time course showed that induction of DT-diaphorase mRNA was evident by 4 hr after the end of the period of drug exposure, and elevated content was maintained for as long as 48 hr.

To investigate further the basis for elevated steady state DT-diaphorase mRNA content, we performed nuclear run-on assays in HT29 cells treated at the IC_{50} for 4 hr (Fig. 5). At the end of drug exposure, the rate of DT-diaphorase mRNA synthesis was only slightly increased, suggesting that

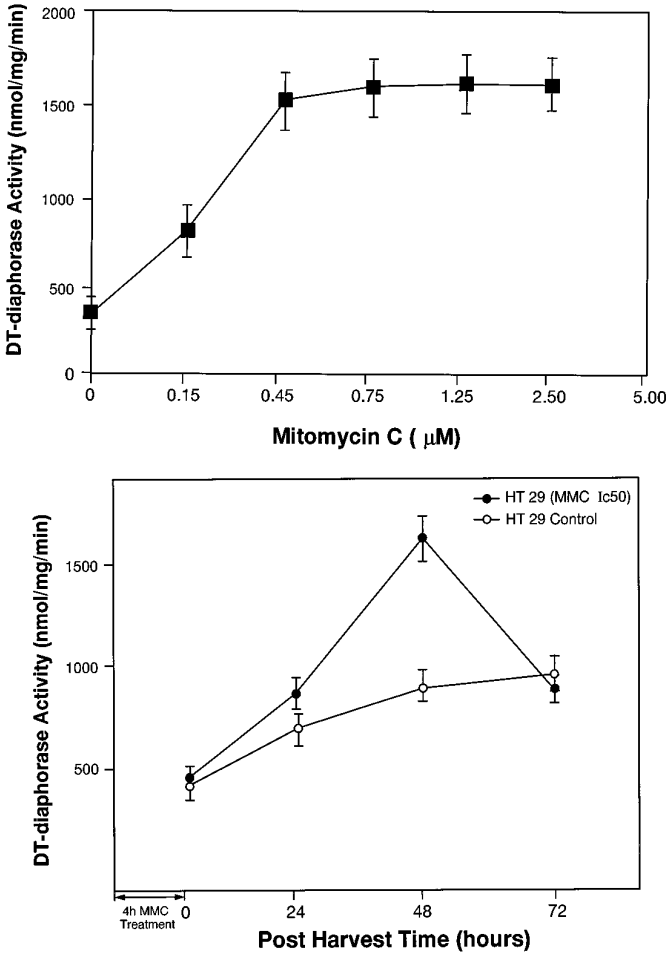

Fig. 3. Induction of DT-diaphorase catalytic activity after a 4-hr exposure to MMC in HT29 cells. *Top*, dose response for induction measured at 48 hr after exposure to MMC. *Bottom*, time course of response after a 4-hr exposure to MMC at the IC_{50} values for HT29 cells (0.45 μM).

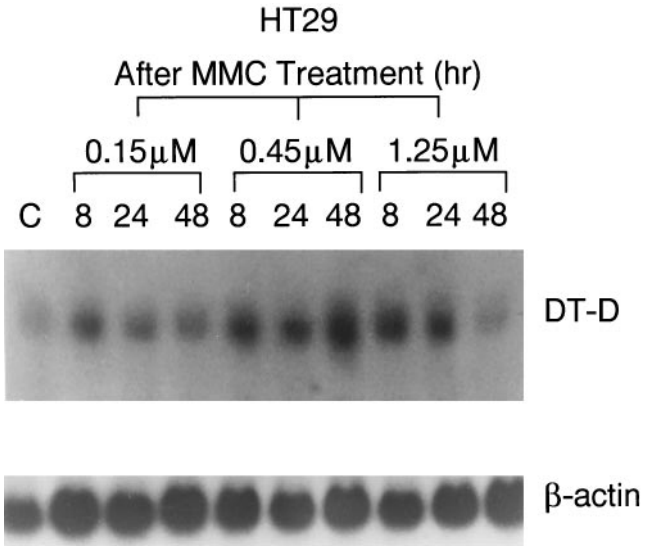

Fig. 4. Northern analysis of RNA (10 μg/lane) isolated from HT29 colon cancer cells at intervals (8, 24, and 48 hr) after harvesting after a 4-hr exposure to MMC at the concentrations shown. After hybridization to a DT-diaphorase probe, the blot was stripped and rehybridized to a β-actin probe to demonstrate equal loading.

changes in steady state mRNA levels at this early time point were a consequence of enhanced message stability. The rate of DT-diaphorase mRNA synthesis increased by 6.9-fold at 12 hr. An early increase in c-*fos* and c-*jun* transcription by 3.1- and 15.6-fold, respectively, was declining (c-*jun*) or had vanished (c-*fos*) by 24 hr. The nature of the AP-1 transcription factor response is quite different than that occurring after hypoxia (19). However, the response of c-*myc* transcription is similar, which is consistent with the view that in normal cells, the early induction of c-*myc* expression is a protective mechanism. Therefore, these data indicate that transcriptional induction plays a role in the DT-diaphorase response to MMC treatment.

We investigated the participation of the AP-1 binding element in mediating responses to MMC by EMSA. Nuclear extracts from untreated HT29 cells contained AP-1 binding activities (Fig. 6). The composition of the complexes was investigated by supershift analysis using specific antibodies directed to Fos and Jun: the participation of members of both protein families was indicated (Fig. 6). These data suggest that induction of Fos and Jun binding to AP-1 participates in the observed DT-diaphorase response to MMC.

Another element in the DT-diaphorase promoter shared by a number of genes responsive to environmental stimuli is the NF-κB element. The induction of transcription factor binding to this element occurs as a response to redox changes and requires the dissociation and translocation to the nucleus of a preformed inactive cytoplasmic complex (33). EMSA analysis showed that factors binding to this element also were present in control cells and that a dose- and time-dependent increase in binding followed MMC treatment (Fig. 7). Competitor experiments confirmed the specificity of binding for the NF-κB sequence (Fig. 8).

The active DNA-binding NF-κB protein complex consists of a heterodimer that includes a 50-kDa and a 65-kDa protein (p50, p65) (33). The participation of both of these proteins in the complexes induced in HT29 cells was demonstrated by supershift analysis (Fig. 9), implicating the NF-κB site in the

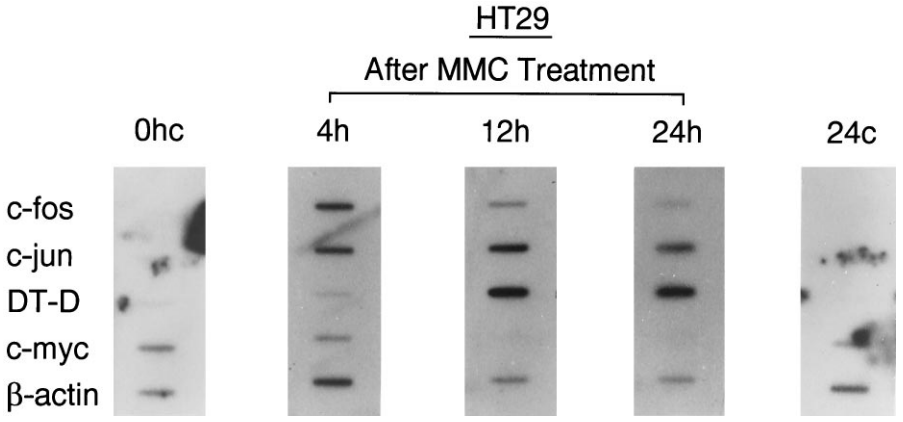

Fig. 5. Nuclear run-on assay showing the time course of variation in the rate of expression of several genes involved in the response of DT-diaphorase expression to MMC treatment in HT29 cells. *Ohc*, untreated control. Cells were treated with 0.45 μM MMC for 4 hr and harvested immediately (4 hr) or after an additional 8 or 20 hr in drug-free media.

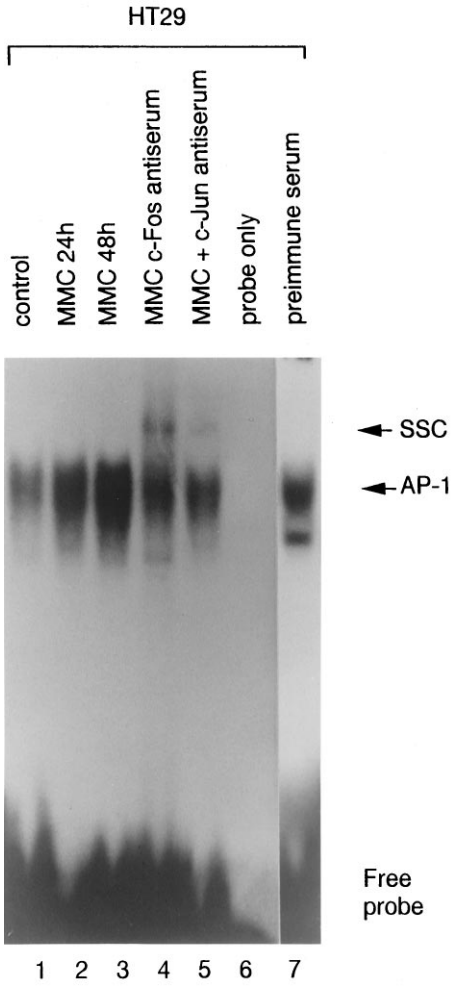

Fig. 6. EMSA demonstrating the induction of nuclear binding activity to an AP-1 double-strand probe at 24 and 48 hr after a 4-hr exposure to MMC in HT29 colon cancer cell (*lanes 1–3*). Nuclear extracts were incubated with preimmune serum (*lane 7*) and with specific antibodies to c-Fos (*lane 4*) and c-Jun (*lane 5*). *Lane 6*, probe only. Positions of the bound complex (AP-1) and the supershifted complex (*SSC*) are shown.

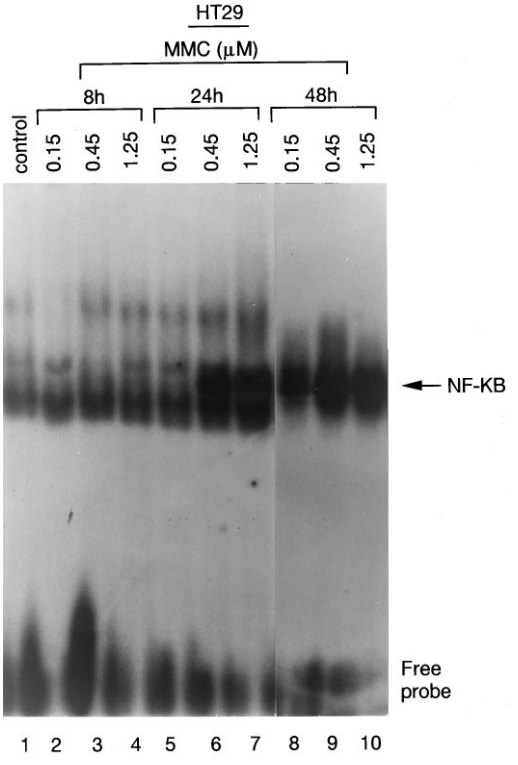

Fig. 7. EMSA demonstrating dose-dependent binding of nuclear factors to a double-strand probe with the sequence of the DT-diaphorase NF-κB element at various times after a 4-hr treatment of HT29 cells with the indicated concentrations of MMC. Positions of the bound complex (NF-κB) and free probe are indicated.

response to MMC. It should be noted that additional rel family members may form DNA binding heterodimers with p50 and p65 and could be responsible for the observed effects.

To verify the participation of both AP-1 and NF-κB binding elements in the observed response to MMC, we performed functional analysis of DT-diaphorase induction using various promoter constructs. The sequence of the first 1.8 kb of human DT-diaphorase gene 5′ flanking region was determined previously (32). As described, this region includes a TATA sequence 33 bases upstream of the start site, an AP-2 (position −157 to −149), an AP-1 (position −461 to −455), an NF-κB (position −830 to −820), and two heat shock elements (positions −1510 to −1497 and −1538 to −1525) (Fig. 1). To identify the regulatory sequences necessary for the induction of DT-diaphorase gene expression after MMC treatment in HT29 cells, transient expression studies were done using a series of deletion mutant CAT recombinants (pCAT-3KB, pCAT-890, pCAT-785, pCAT-588, pCAT-346, and pCAT-78) as shown in Fig. 2. The results of the CAT activity after normalization with nonpromoter vector pCAT-Basic was reported as an activity relative to that of the pCAT-78 (TATA

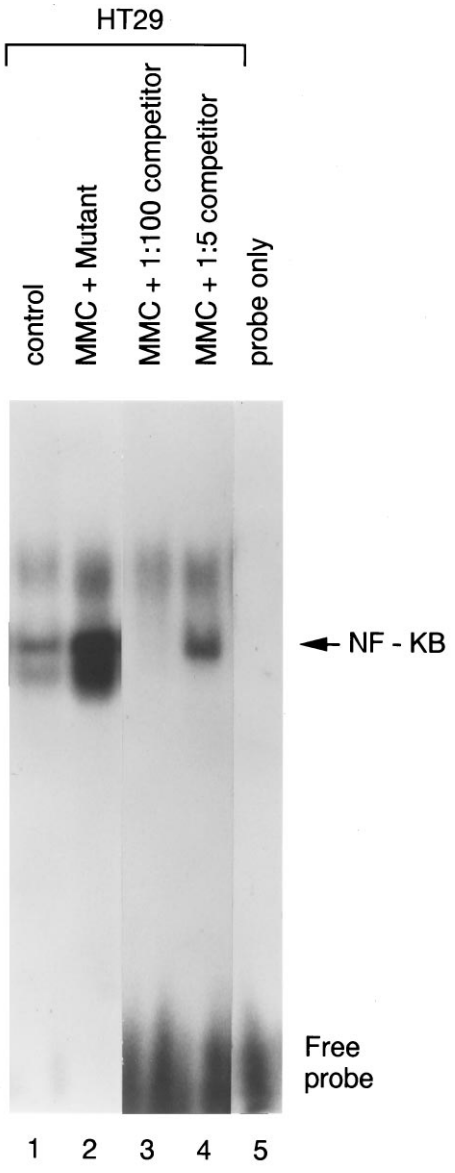

Fig. 8. EMSA demonstrating the specificity of binding to the NF-κB double-strand probe. *Control*, induction of nuclear factor binding to the probe 24 hr after the exposure of HT29 cells to 0.45 μM MMC. The addition of the mutant probe (*lane 2*), a 100-fold (*lane 3*), or 5-fold (*lane 4*) excess of unlabeled probe to the nuclear extract alters binding as shown. *Lane 5*, free probe.

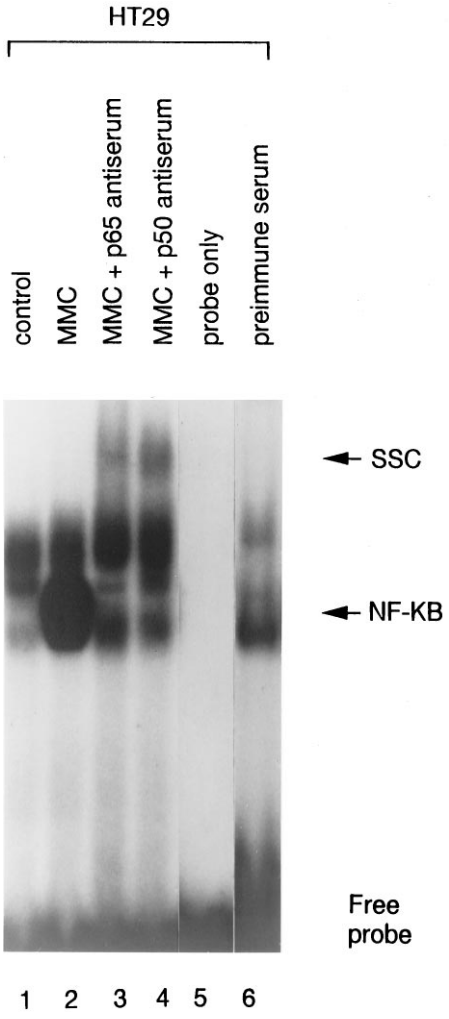

Fig. 9. Supershift assay demonstrating the participation of both p65 and p50 in the NF-κB binding activity induced by exposure of HT29 cell to MMC at IC_{50} value for 4 hr. Nuclear extracts were prepared 24 hr after drug exposure. *SSC*, supershifted complex.

basal transcription apparatus). CAT gene expression under control of different promoter constructs in the presence and absence of MMC treatment at the IC_{50} value was measured using the CAT/enzyme-linked immunosorbent assay method.

The HT29 cells transfected with plasmid pCAT-3KB expressed the CAT gene at a high level (Fig. 10). Activity that was 82.4- and 453.9-fold that of pCAT78 activity was observed, in control and MMC-treated cells, respectively. Thus, there was a 5.5-fold induction of DT-diaphorase transcription with MMC treatment at the IC_{50} value (Fig. 10). Using the plasmids containing progressive deletion mutants of the DT-diaphorase promoter, several regions were identified as containing elements that mediated basal transcription. Elimination of the 2 kb upstream of −890 had little effect on basal transcription. A large decrement in basal transcription was observed on deletion of sequences upstream of −785 (41%), implying that NF-κB has an important role in basal transcriptional control. A small decrement (18%) in basal transcriptional activity was observed between −785 and −588, a region that contains a CCAAT box. The further deletion of sequences upstream of −346 that contain the AP-1 element resulted in a very striking decrement (84.5%) in basal activity.

The plasmid lacking these sequences had lost almost all of the activity of the larger promoter construct. An additional 6.2-fold decrement was observed by deletion of the sequences upstream of the TATA box, implying a role for AP-2 among other potential elements.

The behavior of these constructs in MMC-treated cells was somewhat different. As with basal transcription, sequences upstream of −890 were relatively noncontributory (15.2%). However, a substantially larger decrement (44.3%) was observed with the elimination of the NF-κB element. Similarly, elimination of the sequence containing the AP-1 element resulted in a larger decrement (97% was observed). It is noteworthy, however, that all of the promoter constructs were capable of mediating some response after MMC treatment, even those constructs that lacked the sequences containing both NF-B and AP-1 sites (Fig. 10).

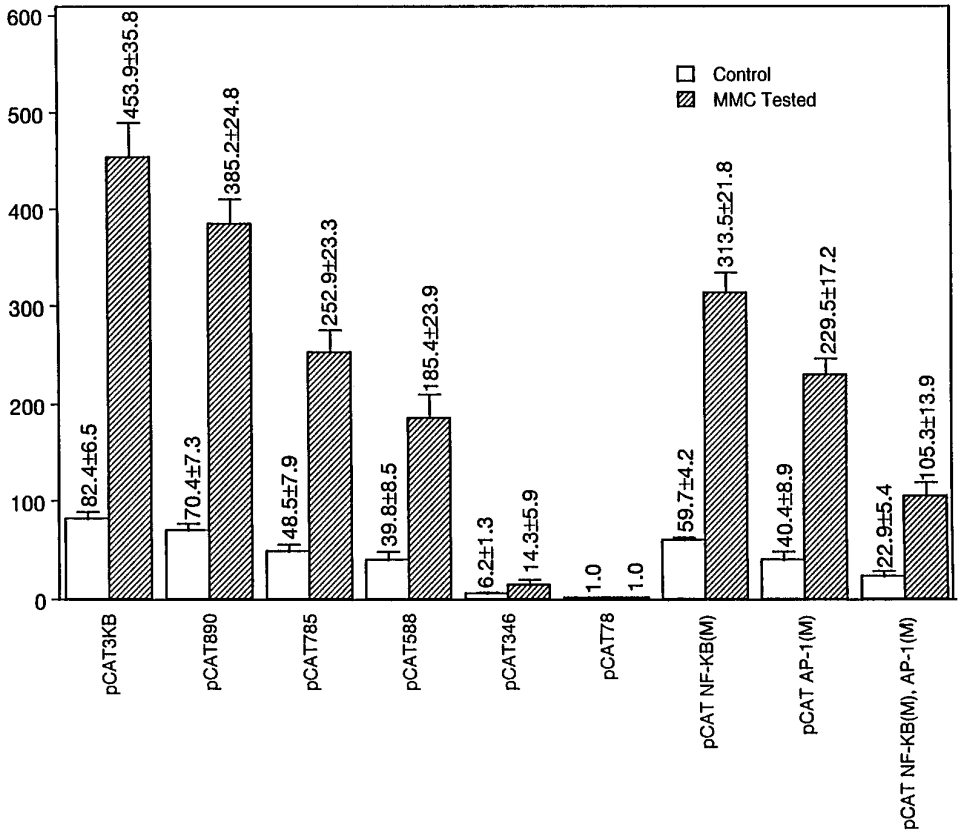

Fig. 10. Effects of MMC on the activities of CAT constructs containing various lengths of DT-diaphorase gene 5′-flank regions in HT29 cells. CAT activity of each construct was calculated as ng of CAT protein/mg of total protein after normalizations to the nonpromoter containing plasmid pCAT-Basic.

To determine the specificity of these deletions for the NF-B and AP-1 binding elements, we performed site-specific mutagenesis of the PCAT-890 construct as described (Fig. 2). Transfection of the mutant constructs into HT29 cells showed results similar to those with the deletion mutants. After transfection of the plasmid (pCAT-890) containing AP-1 and NF-κB elements, CAT gene expression levels elevating above the pCAT78 level were 70.4- and 385.2-fold in control and MMC-treated cells, respectively. Deletion of NF-κB causes loss of CAT gene activity of 15.2% and 18.6% in control and MMC-treated cells, respectively, compared with pCAT-890 activity (that plasmid contains intact AP-1 and NF-κB elements). When the plasmid with deletion of the AP-1 element was used, 42.7% and 40.5% of transcription activity was lost in the control and MMC-treated cells, respectively. When both NF-κB and AP-1 elements were mutated, the CAT activity was lost 67.5% and 72.7% in both control and MMC-treated cells, respectively. These data clearly indicate that the AP-1 and NF-κB elements are required to determine both basal and induced DT-diaphorase expression.

The inducible response that characterized even the smallest fragment was studied further by EMSA. A 38-bp fragment (-40 to -78) was found to bind nuclear extracts from MMC-treated cells in a dose- and time-dependent manner (Fig. 11). This region is devoid of known transcription factor binding elements. Additional studies to characterize this region are in progress.

Discussion

Controversy exists concerning the role of DT-diaphorase in MMC cytotoxicity (5, 10). Early work from Keyes *et al*. suggested that metabolism by DT-diaphorase was a determinant of the cytotoxicity of MMC to EMT6 cells under oxic but not hypoxic conditions (34). Some research with lung cancer cell lines that vary in DT-diaphorase activity has suggested that the IC_{50} value for MMC was inversely proportional to DT-diaphorase activity, implying a role for this enzyme in the activation of MMC to cytotoxic species (35). More detailed studies have demonstrated that MMC is a substrate for DT-diaphorase (12) and that the absence of DT-diaphorase can diminish cytotoxicity (32, 36). However, the absence of a clear linear relationship between DT-diaphorase activity and sensitivity to quinones in multiple cell lines casts doubt on the existence of a simple relationship between MMC toxicity and DT-diaphorase activity (35–38). Studies with expression constructs for DT-diaphorase are needed to define its contribution to cytotoxicity. Gustafson and Pritsos have adduced evidence that the bioreduction of MMC to toxic species is accomplished mainly by xanthine oxidase/xanthine dehydrogenase (39, 40); coregulation of DT-diaphorase and xanthine oxidase or other unidentified reductases could therefore result in misleading associations between DT-diaphorase and toxicity. However, because DT-diaphorase is ordinarily a protective enzyme that facilitates the detoxication of quinones (38), changes in its expression may be interpreted as reflecting a general induction of protective responses on cytotoxic drug challenge.

We have shown that MMC treatment of HT29 cell lines results in the transient induction of steady state DT-diaphorase mRNA levels in a dose-dependent manner. A substantial proportion of the increase is accounted for by transcriptional induction, although a change in message stability may also contribute, especially given the early rise in DT-diaph-

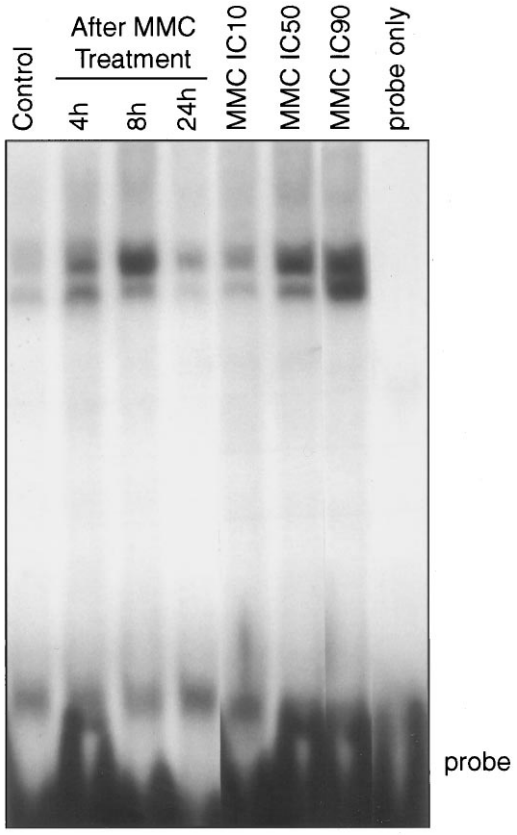

Fig. 11. EMSA demonstrating binding to a 38-bp double-strand probe representing the positions −40 to −78 bp. Induction of nuclear factor binding is demonstrated after a 4-hr treatment with MMC at IC_{50} value (with cells harvested at 4, 8, and 24 hr) and a 4-hr treatment with MMC at IC_{10}, IC_{50}, and IC_{90} values (cells harvested at 8 hr).

orase steady state mRNA content (Fig. 2), before the transcription rate had increased materially (Fig. 3). The determinants of DT-diaphorase message half-life have not been studied; exposure of HT29 cells to hypoxia results in prolongation of the message half-life in addition to stimulation of transcription (19).

Previous evidence has associated cellular responses to DNA damage with the induction of transcription factors, including AP-1 (16, 41). Because the 5′ promoter region of the DT-diaphorase gene has an active AP-1 site (18), we hypothesized that MMC could alter the regulation of DT-diaphorase gene expression. Nuclear run-on analysis revealed that the rate of DT-diaphorase mRNA transcription was increased after exposure of colon cancer cells to MMC. Therefore, we first examined the participation of the AP-1 binding element in the observed transcriptional effects.

Induction of nuclear factor binding to the AP-1 response element was demonstrated, and both Jun and Fos were shown to be components of the DNA-binding complex at an early stage. In this respect, the activation through AP-1 differs from that induced by exposure of the cells to hypoxia, which seems to activate AP-1 selectively through *jun* family dimers (19). Also, in a series of ovarian cancer cell lines expressing stable cisplatin resistance, we have shown elevated expression of DT-diaphorase and c-Jun and proportional increases in AP-binding activity, implying that altered regulation of c-*jun* was associated with resistance (42). Jun regulation is achieved by phosphorylation of various residues that may either activate or repress its dimerization (43). The responsible kinases and phosphatases are subject to growth factor and cell cycle regulation. It will be important to compare the phosphorylation status of Jun in each context to elucidate further the pathways responsible for the conversion of transient to stable transcription factor overexpression. Activation of factor binding to the NF-κB site was also shown to follow exposure to MMC. This may result from DNA damage or from redox changes induced by the drug. The quinone function of MMC is subject to cyclical oxidation/reduction reactions resulting in the generation of oxygen radicals, which may themselves damage macromolecules or be reduced in a process that results in hydrogen peroxide production (44). In the stably resistant ovarian cancer cell lines, overexpression of rel family proteins that make up NF-κB was not found (42). The induction of NF-κB in the context of a brief cytotoxic exposure will therefore provide a useful control for further studies, and studies incorporating antioxidants will help in dissociating the effects of DNA damage from those of the production of oxygen radicals.

The functional consequences of factor binding to elements in the DT-diaphorase promoter were studied using deletional and site-specific mutation analysis. These results indicate that elements upstream of NF-κB (which include the heat shock elements) are involved neither in basal nor induced DT-diaphorase transcription. The NF-κB element has a more striking role in MMC-induced than in basal transcription, and accounts for about 40% of the latter. Of course, "basal" is a relative term in the context of these experiments. Tumor cells are maintained in log phase, supplemented by growth-factor-containing fetal bovine serum. Thus under the resting conditions of most human tissues, the role of NF-κB may be less prominent. On the other hand, many human tissues are characterized by loss of proliferative control, and a role for NF-κB activation in resistance to therapy merits consideration.

The effect of deleting the AP-1 element on both basal and induced transcription was even more dramatic. Elimination or mutation of AP-1 resulted in a decrease in transcriptional activity to 23% and 30% of maximum in untreated and treated cells respectively. These data do not imply any specificity of AP-1 for induced transcription however. This observation may be somewhat surprising in that pronounced differences in cellular levels of the transcription factors that may bind in various considerations to the AP-1 site follow various types of stimulus. Following hypoxia, c-jun overexpression is an early event, while c-fos occurs later and to a lesser extent. In contrast, MMC treatment results in an immediate and short-lived induction of c-fos, with a more sustained induction of c-jun (Fig. 5). These differences in the presumed composition of the dimers binding AP-1 do not appear to have detectable consequences in DT-diaphorase expression.

Our data also suggest that inducibility *per se* may depend upon a promoter fragment close to the transcriptional start site and devoid of known factor-binding elements. A response element in this region would be ideally placed to mediate cooperative interactions between DNA-bound transcription factors and the basal transcription apparatus. Further characterization of this element is in progress.

Our findings with MMC in colon cancer cells will be extended to additional models. As noted above, co-regulation of

many of the enzymes involved in detoxication pathways is established: such commonality has clear advantages to a cell responding to adverse environmental conditions. Models of tumor cell selection for resistance are often associated with overexpression of groups of detoxicating enzymes. The biology of this phenomenon has not clearly been elucidated: an explanation based upon altered transcription factor action is an attractive hypothesis for the induction of these and other resistance mechanisms. The data presented in this paper may provide a model for understanding the transition between the transient and stable induction of such changes.

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