0026-895X/95/040696-11$3.00/0

MOLECULAR PHARMACOLOGY, 47:696–706 (1995).

Topoisomerase IIα Promoter *Trans*-Activation Early in Monocytic Differentiation of HL-60 Human Leukemia Cells

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Received August 8, 1994; Accepted January 23, 1995

SUMMARY

The cytotoxic efficacy of antitumor drugs targeted at DNA topoisomerase II (topo II) in many cases varies in direct proportion to cellular topo II content. To investigate the transcriptional control of the predominant α form of topo II, the 5′ flanking region of the human topo IIα gene (positions −562 to +90) was subcloned into a firefly luciferase reporter plasmid and transiently transfected into HL-60 human leukemia cells, a line capable of monocytic differentiation after treatment with various agents. Early in phorbol-12-myristate-13-acetate (30 nM)-induced differentiation (18–24 hr after treatment), an unexpected 3–5-fold activation of topo IIα gene promoter activity was observed. Activation was observed in HL-60 cells and U-937 cells, but not in HeLa human cervical carcinoma cells. Sodium butyrate (NaB) (0.4 mM) also led to activation (4–17-fold) of the topo IIα promoter in HL-60 and U-937 cells. Promoter sequences between position −90 and position +90 mediated the inducing effects of NaB. This NaB-dependent promoter-reporter induction was partly mirrored by a transient ~2-fold increase in endogenous topo IIα enzyme. The stimulus for promoter activation could be partly attributed to a 2-fold increase in DNA synthesis at 16 hr for NaB, but not phorbol-12-myristate-13-acetate. Regardless of the primary stimulus for topo IIα promoter *trans*-activation, it could be bypassed by treatment of HL-60 cells with NaB for 48 hr before transfection, revealing the expected 60–70% suppression of topo IIα promoter activity. Further study of topo IIα promoter down-regulation later in monocytic differentiation may serve as a model for elucidating the transcriptional mechanisms that may also be exploited by tumor cells expressing intrinsic or acquired resistance to topo II-directed drugs.

Topo II is a nuclear enzyme target for the pharmacological action of a chemically diverse class of anticancer drugs. From a basic cell biology standpoint, evidence points to at least a partial role for topo II activity in gene transcription, DNA replication, and chromosomal segregation at mitosis (1, 2). The latter action of topo II is essential for cellular proliferation; in the absence of topo II activity in yeast, proliferating cells undergo mitotic catastrophes characterized by cytokinesis in the absence of chromosomal segregation (3).

Topo II catalyzes its DNA relaxation, knotting/unknotting, and catenation/decatenation activities via a concerted DNA double-strand breakage-reunion reaction. During the DNA cleavage reaction, each subunit of a topo II homodimer becomes transiently bound, in a covalent linkage, to the 5′-phosphate of each DNA phosphodiester backbone via a highly conserved tyrosyl residue in the enzyme (1, 2). Passage of a second DNA duplex through the break site results in DNA relaxation if the passage helix is part of the same molecule; unknotting or decatenation occurs if the passage helix is from another DNA molecule.

A growing series of agents from several chemical families are now known to be useful in cancer chemotherapy, due to their ability to stabilize topo II in its normally transient covalent complex with DNA. These agents include the epipodophyllotoxins, anthracyclines, acridine orange derviatives, and anthracenediones, among others (2). Drug-induced stabilization of these "cleavable complexes" is believed to cause cell death either directly via replication fork collisions (4) or indirectly via pathways that involve ATP hydrolysis and protein synthesis (5, 6).

Because most topo II-directed drugs act as poisons rather than classical competitive inhibitors of the enzyme, the extent of the cytotoxic action of these drugs is often directly proportional to cellular topo II content. Rapidly proliferating cells, which maintain relatively high topo II levels (7, 8), are exquisitely sensitive to topo II poisons (9). Differentiated

This work was conducted with the support of a National Institutes of Health National Research Service Award (DK08854), a grant from the Cancer League of Colorado, an American Cancer Society/University of Colorado Cancer Center Institutional Grant, and a New Investigator Grant from the American Association of Colleges of Pharmacy, supported by the Burroughs-Wellcome Foundation (to D.J.K.). This work was presented in part at the 33rd Annual Meeting of the Society of Toxicology, March 13–17, 1994 (Dallas, TX).

ABBREVIATIONS: topo II, DNA topoisomerase II; PMA, phorbol-12-myristate-13-acetate; NaB, sodium butyrate; PCR, polymerase chain reaction; DTT, dithiothreitol; LU, relative luciferase light units; TCA, trichloroacetic acid; CMV, cytomegalovirus; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

cells maintain relatively low topo II levels and are intrinsically resistant to topo II poisons (10); this observation at least partly accounts for the selective toxicity of these agents. Similarly, tumor cells selected in culture for drug resistance to this class of cytotoxic agents often display topo II levels lower than those of their drug-sensitive parental counterparts (11). Conversely, cells selected for overexpression of topo II (12) or engineered to overexpress topo II (13) exhibit hypersensitivity to topo II poisons. Hence, altered topo II regulation clearly influences tumor cell susceptibility to this important class of anticancer agents.

Two human cDNAs for topo II have been identified and classfied as α and β. The 170-kDa α form of the enzyme has been studied in greatest detail, because it is the predominant form of the enzyme in most cells. The 180-kDa β form of the enzyme has recently been cloned and is present only in certain cells (14), usually but not always at levels below that of α (15, 16). The relative contribution of this newly described isoform of the enzyme is only beginning to be appreciated (16).

Although much is known regarding the implications of altered topo II regulation (specifically with regard to the α form), very little is known about the molecular mechanisms governing topo II transcription. Recently though, Hochhauser *et al.* (17) cloned the 5′ flanking region of the human topo IIα gene and demonstrated that this region had gene promoter activity. With this tool now available, we sought to investigate what genetic elements might be involved in normal topo IIα gene regulation. The rationale for addressing this question was that an understanding of normal topo IIα regulation, such as during the transition from proliferation to cellular differentiation, would form a rational basis for elucidation of the mechanisms exploited by topo II-directed drug-resistant cells to suppress topo II levels. The HL-60 human promyelocytic cell line represented an excellent model for these studies because it could be induced by a variety of chemical agents to differentiate down either the granulocyte or monocyte/macrophage lineage (18). Terminal differentiation down either pathway has been well described previously to result in a diminution of topo II mRNA and protein levels (10, 19).

In the present study, we generated a variety of topo IIα gene promoter fragments by PCR, to enable their subcloning into a firefly luciferase reporter vector (20). These topo IIα promoter-luciferase plasmids were then introduced into HL-60 cells by electroporation, as described recently (21). Luciferase enzyme activity produced under the control of these various topo II gene promoter fragments was measured in both control HL-60 cells and cells induced to differentiate down the monocytic pathway with either the phorbol ester PMA or NaB. Surprisingly, topo IIα promoter activity was stimulated 2–17-fold, rather than suppressed, at early time points during differentiation. In the case of NaB, promoter stimulation was accompanied temporally by a ~2-fold increase in cellular topo IIα protein detected by immunoblotting. Although this stimulation could be attributed only partially to a transient increase in DNA synthesis (and only with NaB), the stimulatory event governing topo IIα promoter *trans*-activation could ultimately be bypassed by introducing promoter-reporter plasmids into cells at later times during differentiation. These results demonstrate the first known investigation of topo IIα gene promoter regulation in human cellular differentiation. In addition, the studies described herein have potential implications for differentiation therapy of leukemias, as well as the involvement of proto-oncogene product transcriptional activators in the regulation of this important anticancer drug target.

Materials and Methods

Cell culture. HL-60 human promyelocytic leukemia cells (CCL 240) and U-937 monoblastic leukemia cells (CRL 1593) were obtained from the American Type Culture Collection (Rockville, MD). HeLa human cervical carcinoma cells were provided by Dr. James P. Hoeffler (University of Colorado School of Medicine). All cells were maintained at 37° in a humidified atmosphere containing 5% CO_2. Exponentially growing suspension cultures of leukemia cells were propagated by reseeding at 5×10^5 cells/ml every 3–5 days, in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. HeLa cells were maintained in Dulbecco's modified Eagle's medium with the same serum and antibiotic supplements and were propagated at 5×10^5 cells/100-mm dish every 2–3 days. For gene transfer experiments, all cultures were freshly seeded at the same cell densities 48 hr before transfection.

Promoter-reporter plasmid constructions. The 5′ flanking region of the human topo IIα gene promoter from position −562 to position +90, relative to the transcriptional start site, was obtained by PCR as follows. Two oligonucleotide primers (TOP-562–5′, 5′-GGGCCCGGGTACCGGGGCGGGGTTGAGGCAGATGCC-3′; TOP+90–3′, 5′-GTGCAGATCTGGTACCGGTGACGGTCGTGAAGGGGC-3′), which anneal to the antisense strand of the topo IIα promoter at position −562 and to the sense strand at position +90, respectively, were synthesized based on the sequence of the human placental topo IIα promoter (17). Both oligonucleotides also included a *Kpn*I site (underlined) for subcloning into the reporter vector pA_3LUC (20). PCRs contained 10 mM Tris·HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM $MgCl_2$, 1 μM concentrations of each primer, 1 μg of HeLa cell genomic DNA, and 2.5 units of *Thermus aquaticus* DNA polymerase (Promega, Madison, WI). Template DNA was denatured by boiling for 10 min before the addition of *T. aquaticus* DNA polymerase. The cycling conditions were 93° for 2 min, 63° for 1 min, and 72° for 1 min for 37 cycles, with a final elongation of 7 min at 72°. The presence of the appropriately sized PCR product was confirmed by 1% agarose gel electrophoresis in 0.5× TBE (45 mM Tris-Borate, 1 mM EDTA) containing 0.05 μg/ml ethidium bromide. For DNA sequencing purposes, the PCR product was directly subcloned into the vector pCRII (which has the T7 and SP6 promoters flanking the cloning site) provided with the TA cloning kit (Invitrogen, La Jolla, CA). The entire PCR product was sequenced by the Sanger dideoxy chain termination method, using Sequenase enzyme (United States Biochemicals), and was found to differ from the published placental sequence by only two base pair substitutions, at positions −287 (cytosine to thymine) and −114 (guanine to cytosine). At present it is not clear whether these substitutions reflect PCR artifacts or actual differences between the HeLa and placental topo IIα promoter sequences.

The position −562 to +90 PCR product was digested with *Kpn*I, purified on a low-melting point agarose gel using standard hot phenol extraction, and then subcloned, using T4 DNA ligase, into *Kpn*I-digested, phosphatase-treated, pA_3LUC vector immediately 5′ to the coding sequence for firefly luciferase. Ligation mixtures were used to transform *Escherichia coli* strain DH5α, and DNA was prepared by an alkaline-SDS miniprep method (22). A subclone containing the resulting vector (−562TOP2LUC) with the promoter in the correct orientation was propagated for large-scale plasmid preparation. All DNAs used for transient transfection assays were purified twice by isopycnic ultracentrifugation on CsCl gradients.

Deletions from the 5′ end of this promoter in the context of

pA_3LUC were also constructed. Two *Hind*III sites, at position −295 of the promoter and in pA_3LUC, facilitated the subcloning of −295TOP2LUC. Other deletions (yielding −219-, −148-, −90-, and −51TOP2LUC) were constructed by PCR addition of *Sal*I and *Pst*I sites into individual 5′ primers and a common 3′ primer (at position +90), respectively, using linearized −562TOP2LUC as the template. Each of these PCR products was digested and purified as described earlier and was subcloned into a modified pA_3LUC vector (pA_3LUC/SP) that contains the *Sma*I-*Hind*III polylinker (containing *Sma*I, *SalI*/*Acc*I, *Pst*I, and *Hind*III sites) from pSP65 (Promega). Hence, this modification enabled the directional subcloning of each PCR product into *SalI*/*Pst*I-digested pA_3LUC/SP.

To confirm promoter *trans*-activation with a more extensive topo IIα promoter region, −1200TOP2CAT was generously provided by Dr. Ian Hickson (University of Oxford, Oxford, UK). The position −1200 to +90 promoter was excised by digestion of −1200TOP2CAT with *Xba*I, blunting with Klenow fragment and deoxynucleoside triphosphates, and then digestion with *Pst*I to yield a *Pst*I-blunt fragment. This promoter fragment was then directionally subcloned into pA_3LUC/SP that had been digested with *Hind*III, blunted, and then digested with *Pst*I.

Transient gene transfection protocol. Exponentially growing HL-60 or U-937 cells were seeded as described above and then collected by centrifugation at 1000 × *g* for 5 min. The resulting cell pellet was resuspended in fresh RPMI 1640 medium, with all supplements, at a cell density of at least 5×10^7 cells/ml. Two hundred microliters of this cell suspension (~1×10^7 cells) were combined with 20–50 μg of reporter plasmid and then transferred to a 0.4-cm gap width, metal-lined, electroporation cuvette (Invitrogen). DNA was introduced into the HL-60 cells by delivery of a charge to the cell suspension using an IBI geneZAPPER electroporator set at 250 V and 950 μF. The observed time constant τ was usually within the range of 48–56 msec. The cell suspension was then removed to a 60-mm culture dish containing 3 ml of supplemented RPMI 1640 medium. Cells were then treated with the indicated differentiating agent, i.e., PMA (30–300 nM; Sigma, St. Louis, MO) or NaB (0.4 mM; Sigma). Each agent remained in the culture medium continuously until cell harvest, unless noted otherwise. Harvest times varied from 4 to 48 hr, as indicated in each figure. In general, each group contained three samples and experiments were performed three to five times.

For the HeLa cell experiments, the procedure was essentially the same as described above, except that Dulbecco's modified Eagle's medium was used for resuspension and plating, 1×10^6 cells were used in each transfection, and the electroporation conditions were 180 V and 500 μF. Time constants generally ranged from 21 to 26 msec.

Cell harvest and luciferase assay. Cell pellets collected by centrifugation at 1000 × *g* for 5 min were washed once with PBS and then resuspended in 100 μl of 100 mM potassium phosphate, pH 7.8, containing 1 mM DTT. Cells were lysed by three cycles of freezing in dry ice, thawing at 37° for 30–45 sec, and vortex-mixing for 20–25 sec to liberate luciferase enzyme. After the third freeze-thaw cycle, lysates were centrifuged at 10,000 × *g* for 10 min, and then the supernatants were removed, quantitated, and immediately assayed for luciferase activity. Luciferase activity was assayed by combining 25–40 μl of cell supernatant with 160–175 μl of luciferase assay buffer (100 mM potassium phosphate, pH 7.8, 1 mM DTT, 15 mM $MgSO_4$, 5 mM ATP). The luciferin substrate (Analytical Bioluminescence, San Diego, CA) was prepared at a final concentration of 1 mM in 100 mM potassium phosphate, pH 7.8, 1 mM DTT, and 100 μl were used to initiate each reaction. Luminescence of each reaction was measured in arbitrary LU, as quantitated over 15 sec with a Los Alamos Diagnostics 535 luminometer (Turner Designs, Mountain View, CA). Each sample was assayed twice, background values were subtracted, and individual data were expressed as total LU back-calculated for the entire cell sample. Group data were expressed as mean ± standard error.

In some cases a plasmid containing the CMV immediate early promoter/enhancer upstream of the *E. coli lacZ* gene (encoding β-galactosidase), denoted pCMV-β-gal, was co-transfected (0.5–1 μg) as an internal control. After the luciferase assay, an aliquot of cell supernatant was used for assay of β-galactosidase activity, using 2-nitrophenyl-β-D-galactopyranoside as the substrate, according to the method detailed by Sambrook *et al.* (22). *E. coli* β-galactosidase (Boehringer-Mannheim, Indianapolis, IN) was used as the standard. Where valid, luciferase values were corrected for β-galactosidase internal control activity by dividing total LU by total milliunits of β-galactosidase activity. It should be noted that, under conditions where topo II promoter activity was decreased (due to truncation or in the NaB pretreatment experiment) (see Fig. 7), β-galactosidase activity was still robust, due to this gene being driven by the strong CMV promoter. In general, the assay was conducted under conditions where 0.3125 milliunit was the limit of detection; the normal assay volume of test samples was always adjusted to yield values 10–20-fold greater than this lower limit of detection.

[^{3}H]Thymidine incorporation. Incorporation of [*methyl*-^{3}H]thymidine (73 Ci/mmol; ICN) into TCA-precipitable material was performed as follows. HL-60 cells were established in six-well culture dishes at 5×10^5/ml, in 2 ml of supplemented RPMI 1640 medium (four samples/treatment time point), and permitted to grow for 48 hr. At the indicated time points, medium was supplemented with 1 μCi of [^{3}H]thymidine and cells were incubated for an additional 2 hr at 37°. Cells were harvested by centrifugation and lysed with 0.5 ml of 1% SDS containing 200 μg of BSA as carrier. After the addition of 0.5 ml of ice-cold 20% (w/v) TCA and a 5-min incubation on ice, pellets were obtained by centrifugation at 10,000 × *g* for 10 min. Pellets were washed twice with ice-cold 10% TCA and once with 100% ethanol. Pellets were solubilized with 100 μl of 1 M NaOH and transferred to scintillation vials containing 5 ml of ScintiVerse BOA (Fisher, Pittsburgh, PA). Quantitation of ^{3}H in each pellet was performed by liquid scintillation counting, and the data are expressed as percentages of untreated control incorporation at each time point (mean ± standard deviation). Treatment means were compared with the respective control means and significant differences were assessed by using Dunnett's multiple-comparison test, with $p < 0.05$ as the criterion for significance.

Topo IIα immunoblotting. Cellular topo IIα content after NaB treatment was assessed by Western immunoblotting, as follows. HL-60 cells were seeded exactly as for the transfections and treated for 2–48 hr with either 0.4 mM NaB or an equivalent amount of sterile water. Cells were harvested by centrifugation at 1000 × *g* for 5 min and were washed once with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride. Each cell pellet (~10^7 cells) was resuspended in 950 μl of hypotonic lysis buffer (30 mM Tris·HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM KCl, plus the protease inhibitors aprotinin, antipain, leupeptin, and pepstatin A at 20 μg/ml and phenylmethylsulfonyl fluoride and benzamidine at 1 mM each) and lysed by the addition of 4.75 μl of 10% (v/v) Nonidet P-40 (final concentration, 0.05%). These concentrations of $MgCl_2$ (23) and Nonidet P-40 (24) are absolutely essential for the maintenance of HL-60 nuclear integrity. Nuclei were pelleted at 1400 × *g* for 5 min and the Nonidet P-40 supernatant was discarded. Each nuclear pellet was then lysed by resuspension in 180 μl of DNase nuclear lysis buffer (50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 100 μg/ml grade II DNase I, 50 μg/ml RNase A, 4.5% glycerol, plus protease inhibitors as described above) and 20 μl of 10% SDS (final concentration, 1%). Most insoluble nuclear proteins were completely solubilized at this point by the addition of 200 mg of crystalline urea, yielding a lysate with roughly 8 M urea. Preliminary immunoblotting studies indicated that >99% of detectable topo IIα partitioned into the nuclear lysate under these conditions.

After quantitation of protein in each nuclear lysate (bicinchoninic acid protein assay; Pierce, Rockford, IL), using BSA as the standard, 30 μg of nuclear protein were loaded onto each lane of a 7.5% polyacrylamide-SDS gel and samples were run at 7 mA for 16 hr.

Duplicate gels were also stained with Coomassie blue, to confirm equivalent protein loading in each lane. Proteins were electrophoretically transferred to nitrocellulose membranes (25), and the blots were blocked with 3% (w/v) BSA in PBS and then incubated for 16 hr with primary antibody (rabbit polyclonal antiserum to human topo IIα amino acids 857-1447; a gift of Dr. D. Sullivan, University of Louisville) at a 1/2000 dilution in PBS containing 0.05% Triton X-100 and 3% BSA. After repeated washing in PBS-Triton X-100, the blots were incubated with 50,000 dpm/ml [^{35}S]Protein A (200–2000 Ci/mmol; Amersham, Arlington Heights, IL) for 1 hr and washed, and the dried blots were exposed to Kodak XAR-5 film for 3 days. The blots were also scanned using a Molecular Dynamics PhosphorImager, and band intensity was quantitated using ImageQuant NT software. Band intensity was denoted in arbitrary area units, and the ratio of these values was used to indicate the fold increase, compared with control, of topo IIα content at each time point after NaB treatment. Although a single representative blot is shown in Fig. 6A, replicate studies confirmed the reproducibility of 1.7–2.3-fold induction of topo IIα after 20–24 hr of NaB treatment.

Results

Construction of human topo IIα promoter-reporter plasmids. The human topo IIα promoter DNA sequence recently cloned from a human placental genomic DNA library (17) enabled the synthesis of oligonucleotide primers to amplify the 5′ flanking region of the topo IIα gene (from position −562 to position +90, relative to the transcriptional start site) from genomic DNA of any human cell line. Although the experiments reported here utilized the topo IIα promoter from HeLa cervical carcinoma cells, we have had success in amplifying the promoter from MCF7 human breast carcinoma DNA as well as human placental DNA (data not shown). The amplified HeLa topo IIα promoter DNA was found to differ from the published placental sequence by only two base pair substitutions, at positions −287 (cytosine to thymine) and −114 (guanine to cytosine). At present it is not clear whether these substitutions reflect PCR artifacts or are actual differences between the HeLa and placental topo IIα promoter sequences. Examination of the basal activity of HeLa promoter constructs containing these substitutions revealed no differences, compared with the activity of corresponding constructs reported by Hochhauser *et al.* (17).

Incorporation of convenient restriction enzyme sites in the PCR amplimers enabled subcloning of the position −562 to +90 promoter fragment into the luciferase reporter vector pA_3LUC (20), upstream of the luciferase cDNA (Fig. 1). This plasmid construct and reporter gene were chosen over chloramphenicol acetyltransferase vectors for several reasons, as follows: 1) pA_3LUC contains a trimer cassette of the simian virus 40 polyadenylation signal upstream of the promoter cloning region, to block spurious plasmid-initiated transcription (20), 2) luciferase reporter activity is about 100 times more sensitive than chloramphenicol acetyltransferase activity (26), 3) reporter activity detection uses a nonradioactive substrate, and 4) the luciferase assay is more amenable to replicate samples. In addition to the −562 construct (denoted −562TOP2LUC), several 5′ promoter deletions (all ending at position +90) were constructed in the context of the luciferase reporter vector, as indicated in the schematic diagram in Fig. 1, and their use is described later in this report.

***Trans*-activation of the topo IIα gene promoter in HL-60 cells by phorbol ester treatment.** The phorbol ester PMA is a well documented inducer of HL-60 leukemia cell differentiation toward the monocyte/macrophage lineage (18, 19). After 4 days of continuous PMA treatment, HL-60 cells exhibit levels of topo IIα mRNA and protein 25–50% of those of undifferentiated controls (19). Hence, historical data with this differentiating agent in HL-60 cells, combined with the relative ease of transiently transfecting this cell line, suggested that this would be an excellent system for probing the transcriptional regulation of topo IIα at the gene promoter level, using the plasmids shown in Fig. 1.

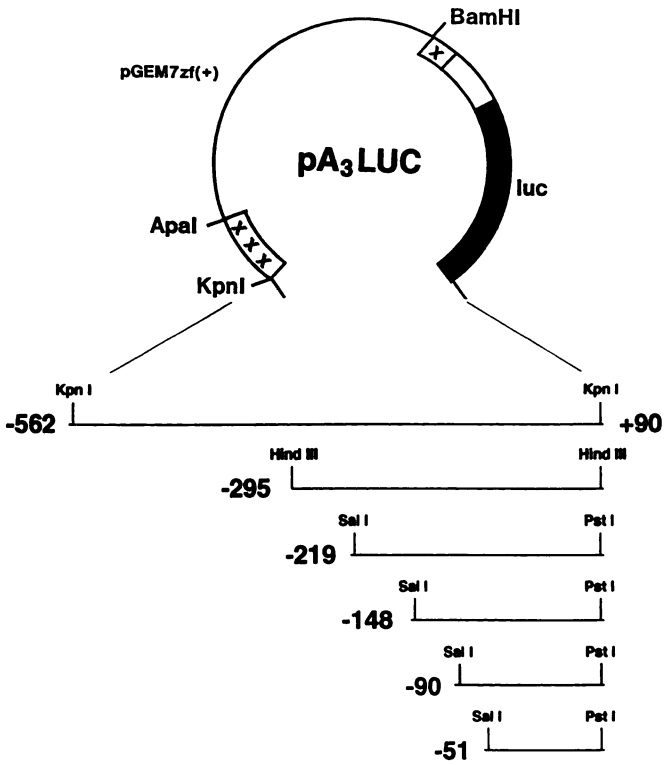

Fig. 1. Schematic representation of human topo IIα promoter-luciferase reporter constructs. Depicted is the parent luciferase reporter vector, pA_3LUC (20). *X*, *Xba*I cassette of the simian virus 40 polyadenylation signal, present as a triplet upstream of the promoter cloning site to prevent spurious promotion by plasmid sequences, as described in the text. The same cassette is also present 3′ to the firefly luciferase coding region (*luc*), to provide message stability. The position −562 to +90 human topo IIα promoter fragment was generated by PCR amplification of HeLa cell genomic DNA using the appropriate primers, as described in Materials and Methods, to enable subcloning into the luciferase reporter vector. Subsequent 5′ deletions of this promoter were then generated by PCR using a unique 5′ primer and a common 3′ primer. The only exception was the position −295 to +90 promoter fragment, which was generated by digestion of the −562 construct with *Hind*III. The convention adopted to identify each plasmid takes the name TOP2LUC preceded by the beginning 5′ position of the promoter fragment. Hence, the first construct is denoted −562TOP2LUC.

Beginning with the −562TOP2LUC reporter construct, time course studies were initiated to establish the optimum PMA treatment and harvest times for observing down-regulation of topo II transcription, as indicated by luciferase reporter activity (27). In these preliminary experiments, however, we unexpectedly noted that 18–24-hr PMA exposure of HL-60 cells led to a 3–5-fold activation of topo IIα gene promoter activity. As depicted in Fig. 2A, this *trans*-activation occurred in a dose-dependent fashion with 30–300 nM PMA. Morphologically, these cells had already begun to take on macrophage characteristics, as evidenced by their adher-

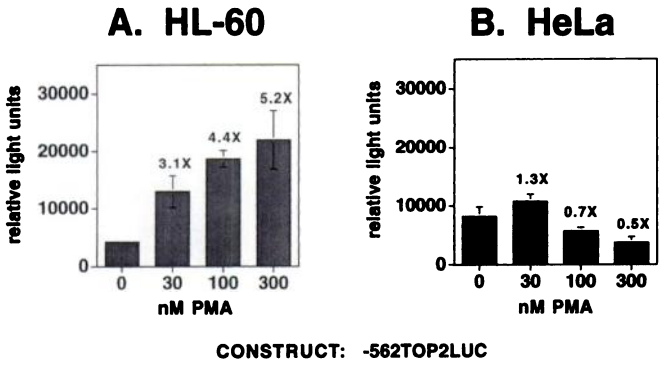

Fig. 2. *Trans*-activation of the human topo IIα promoter by continuous PMA exposure in HL-60 but not HeLa cells. HL-60 (A) and HeLa (B) cells were subcultured and harvested as described in Materials and Methods. Twenty micrograms of the topo IIα promoter-reporter construct −562TOP2LUC were then introduced into each cell line by electroporation under the following conditions: HL-60, 250 V and 950 μF; HeLa, 180 V and 500 μF. Cells were then exposed to vehicle or PMA at 30, 100, or 300 nM for the entire 24 hr after transfection at 37°. Cells were harvested and processed for the measurement of luciferase reporter activity. *Bars*, mean ± standard error of LU for triplicate plates within each group. *Numbers above treatment bars*, mean fold increase in promoter activity, compared with the respective control.

ence to substrate and extension of projections. Topo II promoter activity remained 2–3-fold elevated at 72 hr after transfection (data not shown) but efforts to examine later time points (≥4 days) were hindered by the loss of basal promoter activity, a characteristic of transient transfection assays.

We next examined whether *trans*-activation of the topo IIα promoter was a universal effect of PMA. Although PMA is known to activate protein kinase C, which in turn can phosphorylate topo II and stimulate its catalytic activity, there is currently no experimental evidence to suggest that PMA stimulates topo IIα transcription. In Fig. 2B, −562TOP2LUC was transiently introduced into HeLa cervial carcinoma cells and the cells were subjected to continuous PMA exposure (30–300 nM) for 24 hr, as was done in Fig. 2A for HL-60 cells. Interestingly, luciferase activity produced under control of the topo IIα promoter was not significantly induced in HeLa cells by any concentration of PMA and was instead slightly suppressed by 100 or 300 nM PMA. These results suggested that PMA-mediated activation of the topo IIα promoter potentially required transcription factor(s) and/or a transcriptional activation pathway present in HL-60 cells but not in HeLa cells.

Functional analysis of NaB-dependent *trans*-activation of the topo IIα promoter in HL-60 cells. Examination of the topo IIα promoter, however, did not reveal the presence of the classical phorbol ester response element, 5′-TGA(C/G)TCA-3′. This observation suggested that the effect of PMA on the topo IIα promoter was not a direct result of activator protein-1 (AP-1) *trans*-activation. We therefore hypothesized that other agents known to induce HL-60 differentiation down the monocyte/macrophage pathway might also result in topo IIα promoter *trans*-activation. To test this hypothesis we selected NaB, as an agent to stimulate monocytic HL-60 differentiation, for use in transient transfection assays. Unlike PMA, NaB is believed to induce HL-60 differentiation by altering chromatin structure and gene expression via inhibition of histone deacetylase (28). Initial time course experiments detailed in Fig. 3 confirmed that 0.4 mM NaB was at least as efficacious in inducing topo IIα promoter activity as was PMA. Furthermore, NaB-mediated induction of this promoter reproducibly occurred as early as 12 hr after transfection, with the increase achieving its maximum at 18–24 hr (Fig. 3).

In our subsequent experience with NaB the induction observed tended to increase as HL-60 cells were maintained in culture, but the peak of induction was invariably 18–24 hr after transfection. This peculiar increase in inducibility (from roughly 4-fold to >10-fold) appeared to occur with cells at passage ≥50 and was due specifically to an increase in the NaB-treated group luciferase values, rather than a decrease in the basal control luciferase values. This effect may be due to a transcription factor and/or cell cycle regulatory protein involved in the NaB effects that also confers a growth advantage to the cells during repeated subculturing. Although testing this hypothesis is one object of our ongoing studies, it became clear, for the purpose of this report, that promoter deletion studies were in order to begin at least to suggest candidate factors mediating the NaB inducibility of this promoter.

To ascertain which potential transcription factor binding sites in the topo IIα promoter might be targets for the activating effects of NaB in HL-60 cells, a series of 5′ deletions of the topo IIα promoter were constructed in the context of the luciferase reporter vector. These 5′ deletions were generated by PCR amplification using unique 5′ primers and a common 3′ primer that enabled directional subcloning into pA_3LUC. Use of the latter primer produced a 3′ end common to each promoter fragment, which ended at position +90 in the untranslated region of the topo IIα cDNA. The only exception was the −295 construct, which took advantage of an internal *Hind*III site in the topo IIα promoter. Each of these constructs is shown in Fig. 1.

HL-60 cells were transfected with each construct and half

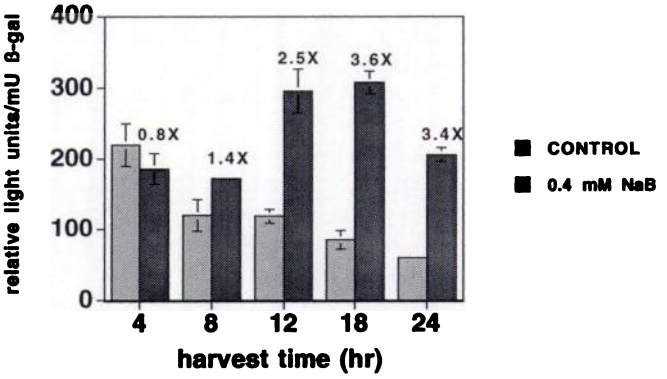

Fig. 3. Time course of NaB-mediated *trans*-activation of the human topo IIα promoter in HL-60 cells. HL-60 cells were propagated as described in Materials and Methods and transfected with 20 μg of −562TOP2LUC plus 1 μg of pCMV-β-gal internal control plasmid, in triplicate for each group. For each harvest time point, one group was treated with vehicle and the other with 0.4 mM NaB for the entire post-transfection period. At the indicated time points, cells were harvested and processed for the measurement of luciferase reporter activity, as well as β-galactosidase internal control activity. *Bars*, mean ± standard error of LU/milliunits of β-galactosidase (*β-gal*) activity for each group. *Numbers above treatment bars*, mean fold increase in promoter activity, compared with the respective control.

of the samples were exposed to 0.4 mM NaB continuously until harvest at 24 hr after transfection. Again, promoter activity was determined by quantitation of luciferase activity. In the control groups, each of the 5′ deletions exhibited basal promoter activity, although promoter activity dropped substantially when sequences between positions −148 and −90 were deleted, requiring the data to be plotted on two separate graphs in Fig. 4. Quite strikingly, NaB treatment resulted in a uniform 14–17-fold activation of promoter activity for each 5′ deletion construct down to position −90 (Fig. 4). Further deletion to create a construct beginning at position −51 resulted in reduced but still robust (3.6-fold) promoter activation (Fig. 4B). Because most of the deletion studies were conducted with HL-60 cells of late passage number, the inducibility of this promoter was greater than observed in the early time course studies (Fig. 3). Nonetheless, when HL-60 cells of early passage number (passage 20–30) were transfected with selected deletions they exhibited the same qualitative trend, with maximal induction being observed down to the position −90 promoter and some reduction in inducibility being obtained with the position −51 construct.

In all of these studies, the internal transfection control plasmid pCMV-βgal (the *E. coli* β-galactosidase gene driven by the CMV promoter) was used so that luciferase activity could be normalized to β-galactosidase activity to correct for inter- and intra-group differences in transfection efficiency. As another control, the promoterless pA_3LUC vector was shown to have very little luciferase activity when transfected into cells in the presence (58 ± 18 LU) or absence (34 ± 7 LU) of NaB. In summary, these data suggested that sequences between positions −51 and +90 of the topo II 5′ flanking region were minimally responsible for mediating the inducing effects of NaB but that complete activation also required sequences between positions −90 and −52.

Evidence that *trans*-activation of the topo IIα gene promoter may be a general feature of early monocytic maturation in culture. The fact that both PMA and NaB activated topo IIα gene promoter activity suggested that early events in monocytic differentiation, rather than the differentiating agents themselves, were a general stimulus for topo IIα promoter *trans*-activation. This more general hypothesis was then tested by examining the effects of each of these monocytic differentiating agents on topo IIα 5′-flanking region promoter activity in the promonocytic human leukemia cell line U-937. The U-937 cells differ from HL-60 cells in that they can be induced to differentiate only to monocytes, whereas the bipotent HL-60 cells can be induced to monocytes or to granulocytes. For this experiment, the −562TOP2LUC construct was introduced into exponentially proliferating U-937 cells under the same electroporation conditions as used for HL-60 cells. Transfected cells were then treated with either 30 nM PMA or 0.4 mM NaB for 24 hr. As was observed for HL-60 cells, each of these agents also stimulated basal topo IIα gene promoter activity (Fig. 5), with 0.4 mM NaB (4.4-fold) generally being a more efficacious inducer than 30 nM PMA (2.2-fold). However, raw LU are presented in Fig. 5 because the internal control reporter activity (β-galactosidase) was also stimulated reproducibly by both of these agents in U-937 cells, negating its validity as an internal control. Nonetheless, these experiments clearly demonstrate that both NaB and PMA are capable of causing topo IIα promoter *trans*-activation in two different hematopoietic cell lines capable of monocytic differentiation.

Evidence that NaB-induced promoter activity is accompanied by modest increases in topo IIα enzyme. Topo IIα mRNA or protein induction of this magnitude has

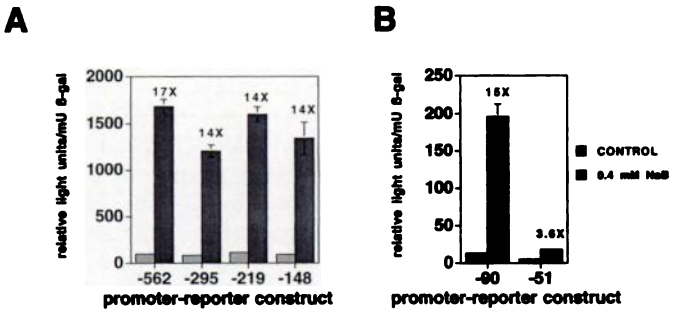

Fig. 4. 5′ deletion analysis of NaB-mediated *trans*-activation of the human topo IIα promoter in HL-60 cells. HL-60 cells were propagated as described in Materials and Methods and transfected with 20 μg of each topo IIα promoter deletion-reporter construct (A, −562-, −295-, −219-, or −148TOP2LUC; B, −90- or −51TOP2LUC) plus 1 μg of pCMV-β-gal internal control plasmid, in triplicate for each group. For each construct, one group was treated with vehicle and the other with 0.4 mM NaB for the entire 24-hr period after transfection. Cells were harvested and processed for the measurement of luciferase reporter activity, as well as β-galactosidase internal control activity. *Bars*, mean ± standard error of LU/milliunits of β-galactosidase (*β-gal*) for each group. *Numbers above treatment bars*, mean fold increase in promoter activity, compared with the respective control. The results for −90- and −51TOP2LUC promoter-reporter constructs are shown on a separate graph because of their substantially lower basal activity.

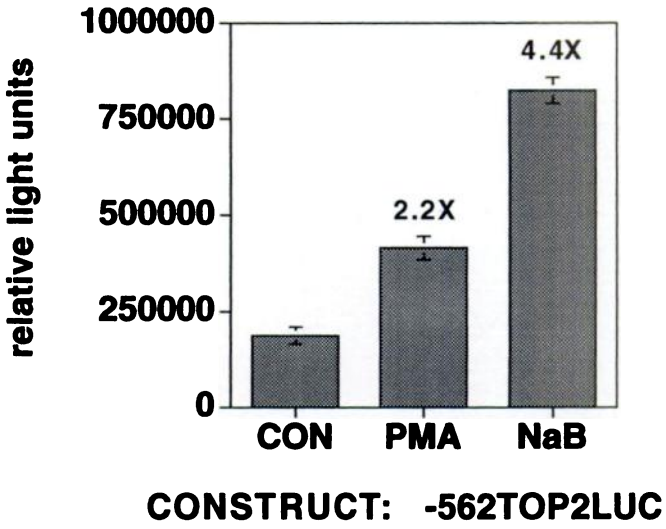

Fig. 5. PMA- and NaB-mediated *trans*-activation of the human topo IIα promoter in U-937 cells. U-937 cells were propagated as described in Materials and Methods and transfected with 20 μg of −562TOP2LUC by electroporation, under the same conditions as for HL-60 cells. Again, transfections were performed in triplicate for each group. U-937 cells are another human cell line capable of differentiation in culture, but only down the monocytic lineage. Each group was treated with either vehicle control (*CON*), 30 nM PMA, or 0.4 mM NaB for the entire 24-hr period after transfection. Cells were harvested and processed for the measurement of luciferase reporter activity. The internal control β-galactosidase reporter plasmid was responsive to PMA but not NaB and was therefore not a valid internal control. *Bars*, mean ± standard error of LU for each group. *Numbers above treatment bars*, mean fold increase in promoter activity, compared with the respective control.

rarely been observed with any treatment regimen and is usually seen only during cellular transition from quiescence to active proliferation. This raised the question of the accuracy of our plasmid promoter-reporter model as a quantitative indicator of endogenous topo IIα regulation. Therefore, the effect of NaB on the ultimate production of topo IIα protein from the endogenous HL-60 topo IIα gene was assessed by Western immunoblotting. Cells were continuously treated with either 0.4 mM NaB or sterile water control for 2–48 hr, under conditions identical to those used for transfection studies. At each time point, cells were harvested and processed for immunoblot determination of cellular topo IIα content. The autoradiogram and PhosphorImager analysis of the data are depicted in Fig. 6. Topo IIα enzyme content exhibited an initial decrease of 30–40% at 2–4 hr, followed by an increase that peaked at 1.7-fold at 20 hr, remaining slightly but reproducibly above control values to 48 hr. This trend closely paralleled the time course observed for topo II promoter-driven luciferase activity, as illustrated previously in Fig. 3, except that the magnitude of peak endogenous topo IIα content was substantially less than that observed for luciferase.

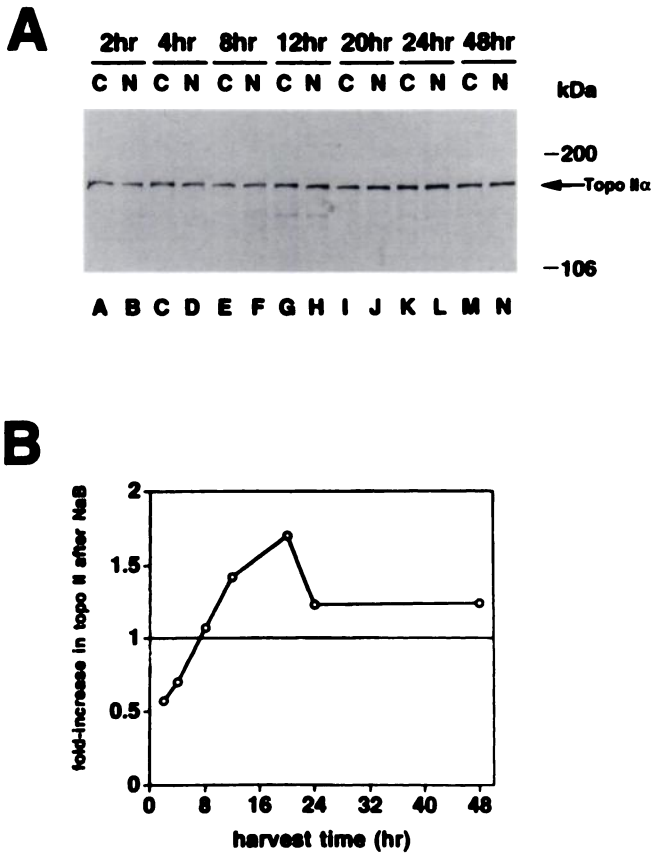

Fig. 6. Immunoblot for endogenous topo IIα protein in HL-60 cells treated with NaB. HL-60 cells were propagated exactly as described for the transient transfections, except that each sample represented a 10-ml culture. Upon seeding into fresh medium, the cells were treated continuously with either 0.4 mM NaB (*N above the autoradiogram*) or control water vehicle (*C above the autoradiogram*). Cells were harvested at times from 2 to 48 hr and processed for SDS-polyacrylamide gel electrophoresis and immunoblotting with polyclonal antiserum to the carboxyl terminus of recombinant human topo IIα, as described in Materials and Methods. Antibody binding was detected using [^{35}S]Protein A as the secondary reagent. A, The resulting autoradiogram is shown. B, After autoradiography, the blot was also analyzed with a PhosphorImager and band intensity was quantitated using ImageQuant NT graphics. The ratios of band intensity of the time-matched NaB-treated samples versus control are expressed as fold increase in topo IIα protein (*y*-axis).

At present, NaB-dependent inducibility of endogenous topo IIα levels does not appear to be as passage dependent as promoter activity; to date, the maximum increase in HL-60 topo IIα content observed in response to NaB has been 2.3-fold at 20–24 hr (data not shown). However, it is recognized that promoter activity does not always precisely reflect the magnitude of endogenous protein regulation, due to a variety of factors in transcriptional regulation at the chromatin level, as well post-translational degradation of induced protein. In fact, the initial reproducible loss of topo IIα protein at 2–4 hr of NaB treatment suggests that a program of topo IIα degradation may be initiated before increased transcription of the gene. However, qualitatively, the time course of NaB inducibility of luciferase activity from a topo II promoter-reporter plasmid (Fig. 3) bears remarkable similarity to the corresponding time course for endogenous topo IIα protein content (Fig. 6B), suggesting that the proximal topo IIα promoter in the context of a plasmid is subject to many of the same regulatory processes that operate on the endogenous gene. In addition, the ~2-fold increase in topo IIα protein observed here may potentially confer a significant degree of cellular hypersensitivity to topo II-directed anticancer drugs.

[^{3}H]Thymidine incorporation early during HL-60 monocytic differentiation. Because topo IIα promoter *trans*-activation and enzyme induction early in monocytic differentiation were unexpected results, they raised questions regarding the biochemical event occurring during this time that would be consistent with increased topo IIα transcription. Although relatively few studies have investigated early biochemical events other than the appearance of cell membrane markers in monocytic differentiation, one study clearly demonstrated that HL-60 cells induced to differentiate with tumor necrosis factor-α underwent a transient 3-fold increase in [^{3}H]thymidine incorporation into DNA at 24 hr (29). This apparent increase in DNA synthesis was at control levels by 48 hr of tumor necrosis factor-α exposure and had fallen to 30% by 4 days, consistent with the appearance of several markers of terminal monocytic differentiation. In addition, a similar paradoxical increase in [^{3}H]thymidine incorporation has also been reported for HL-60 cells treated with several other differentiating agents (19).

Topo IIα has clearly been shown to be a proliferation-regulated enzyme (9) and is known to be closely associated with newly synthesized DNA (30), likely due to its crucial role in DNA decatenation before mitosis. It was therefore reasoned that PMA and NaB might create a need for increased topo IIα gene promoter activity by transiently increasing the rate of DNA synthesis early in monocytic differentiation. To test this hypothesis HL-60 cells were treated with either 30 nM PMA or 0.4 mM NaB continuously for up to 24 hr, exposed to a 1-hr pulse with [^{3}H]thymidine at various time points, and then processed for quantitation of radiolabel incorporated into TCA-precipitable material. As shown in Table 1, NaB treatment did indeed cause a modest increase in [^{3}H]thymidine incorporation (37%) after 12 hr of continuous treatment, which peaked at nearly 2 times control levels at 16 hr. This increase was, however, short-lived and fell to control levels by 24 hr. It is interesting to note that these

TABLE 1

Effects of PMA and NaB on [^{3}H]thymidine incorporation in HL-60 cells

Incorporation of [^{3}H]thymidine into TCA-precipitable material to estimate DNA synthesis rates was performed as described in Materials and Methods. Briefly, HL-60 cells were treated with either vehicle (control), 30 nM PMA, or 0.4 mM NaB continuously for 12, 16, 20, or 24 hr. One hour before harvest the cells were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine (73 Ci/mmol) and then processed for quantitation of radioactivity incorporated into TCA-precipitable material. Data are expressed as the mean ± standard deviation of the cpm for each group of four replicates and as percentage of control (mean cpm for each treatment group/cpm for the respective control × 100).

Group	[^{3}H]Thymidine incorporation	
	cpm	*% of control*
12 hr		
Control	38,744 ± 6,512	100
PMA	39,389 ± 3,349	102
NaB	53,270 ± 7,981[a]	137
16 hr		
Control	41,222 ± 6,283	100
PMA	31,973 ± 4,464	78
NaB	80,405 ± 11,336[a]	195
20 hr		
Control	96,859 ± 5,215	100
PMA	19,136 ± 1,563[a]	20
NaB	113,672 ± 5,593[a]	117
24 hr		
Control	204,340 ± 9,688	100
PMA	10,881 ± 1,162[a]	5.3
NaB	214,035 ± 10,284	105

[a] Significant differences from the respective control, $p < 0.05$.

increases temporally coincide with the initial induction of topo IIα promoter activity (Fig. 3) and protein (Fig. 6B). In contrast, PMA treatment resulted in a rapid cessation of DNA synthesis, falling to 78% of control at 16 hr and then to 5% of control at 24 hr, without ever exhibiting the transient increase observed with NaB. Hence, the robust increase in [^{3}H]thymidine incorporation observed with NaB treatment is consistent with topo IIα promoter *trans*-activation. However, PMA, which also increases topo IIα gene promoter activity, had no stimulatory effect on [^{3}H]thymidine incorporation at any of the time points tested. The significance of these disparate results for two compounds that both stimulate topo IIα promoter activity is not clear at this time. It is, however, striking that the concentration of NaB used generally stimulated topo IIα promoter activity more than did PMA. Nonetheless, it is unlikely that the transient increase in DNA synthesis with the former agent is the only mechanism driving topo IIα promoter *trans*-activation.

Bypass of topo II promoter *trans*-activation by NaB treatment before transfection. Whatever the mechanism governing topo IIα promoter *trans*-activation early in HL-60 monocytic differentiation, terminal monocytic differentiation at 4–5 days is well known to be accompanied by a dramatic abrogation of topo IIα mRNA and protein levels. As mentioned earlier, however, transient transfection assays have not allowed us to examine these later time points, because basal promoter activity (as measured by luciferase activity) falls to irreproducible and barely detectable levels by 3–5 days after transfection. Although the establishment of HL-60 cells that have stably integrated the topo IIα promoter-luciferase reporter constructs would clearly be the ideal way to analyze promoter elements that ultimately govern topo IIα promoter suppression at these late time points, such cell lines are currently only in the developmental stage.

In the meantime, we reasoned that down-regulation of the topo IIα promoter late in monocytic differentiation could be effectively demonstrated by modifying the treatment-transfection protocol. As illustrated in Fig. 7A, we have previously transfected HL-60 cells and exposed them to differentiating agents simultaneously. However, in a modified approach using pretreatment of the cells with a differentiating agent 48 hr before transfection (Fig. 7A), the events that cause early activation of the topo IIα promoter would theoretically be bypassed and promoter suppression should be observed as the cells terminally differentiate. This hypothesis, based on the modified approach, is partly supported by the immunoblotting data shown in Fig. 6, which indicated that the peak of topo II protein accumulation occurred at 20–24 hr of NaB treatment and was waning by 48 hr.

For this chronic exposure experiment, NaB was chosen as

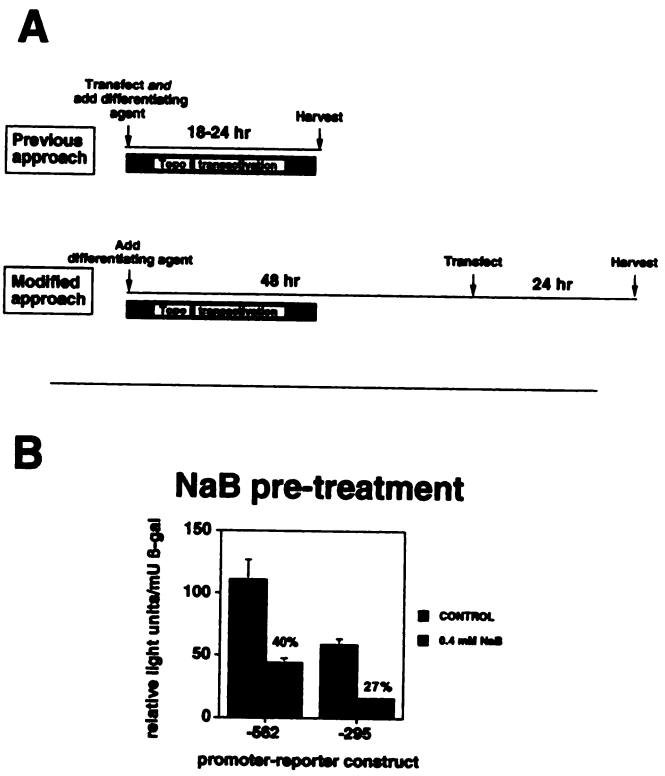

Fig. 7. Bypass of the topo IIα promoter *trans*-activation event by 48-hr NaB pretreatment of HL-60 cells. A, The experimental design for previous transfections is compared with an approach modified to observe the expected down-regulation of topo IIα promoter activity later in monocytic differentiation. Previously, cells were transfected with promoter-reporter constructs at the same time that differentiation treatment was initiated. In the modified approach, HL-60 cells were pretreated with 0.4 mM NaB for 48 hr before transfection, so that the promoter-reporter plasmid was present in the cells only during days 2–3 of differentiation. It was anticipated that this approach would bypass the initial stimulatory event (possibly DNA synthesis or Myb/Myc expression) that caused topo IIα promoter *trans*-activation early in monocytic differentiation. B, HL-60 cells were propagated as usual but in the absence or presence of 0.4 mM NaB. Equal numbers of control and NaB-pretreated cells were then transfected with 20 μg of either −562TOP2LUC or −295TOP2LUC (as well as 1 μg of pCMV-β-gal) and replated into control medium or medium containing 0.4 mM NaB. Cells were harvested 24 hr after transfection and processed for measurement of luciferase and β-galactosidase activities. *Bars*, mean ± standard error of LU/milliunits of β-galactosidase (*β-gal*) for each group. *Numbers above bars*, mean percentage of respective control basal promoter activity.

the differentiating agent, because it is far less toxic than PMA. Parallel HL-60 cultures were maintained under control conditions or in the presence of 0.4 mM NaB for 48 hr. At that time approximately 30% of the NaB cells exhibited the ability to reduce nitroblue tetrazolium, a marker of terminal differentiation. Equivalent numbers of control and NaB-treated cells were then harvested and transfected with either −562TOP2LUC or −295TOP2LUC plus the internal β-galactosidase control plasmid. Cells were then incubated for an additional 24 hr in the absence or presence of 0.4 mM NaB, and the activity of each reporter gene was than measured. In sharp contrast to what was observed early in monocytic differentiation, NaB pretreatment led to suppression of corrected −562TOP2LUC and −295TOP2LUC topo IIα gene promoter activity to 40% and 27% of control, respectively, when each promoter-reporter construct was present in the cells during 48–72 hr of NaB exposure (Fig. 7B). These data are consistent with the existence of a window during HL-60 monocytic differentiation where topo IIα gene promoter activity is stimulated, with promoter activity ultimately becoming suppressed as cells become progressively differentiated. This latter phase is clearly consistent with previous observations for the down-regulation of topo IIα mRNA and protein late in monocytic differentiation.

A potential discrepancy in the data should be noted, specifically that in preliminary studies we observed that PMA exposure concurrent with −562TOP2LUC transfection still yielded elevated luciferase activity at 72 hr (data not shown) but in Fig. 7 we observed a sharp decrease in luciferase activity expressed from the same plasmid at essentially the same time after treatment. However, the nature and goals of these two different experiments account for these seemingly disparate findings. In the PMA experiment, plasmid and differentiating agent were present simultaneously. Although the rate of promoter activity likely dropped off hours before harvest, the 3–4-hr half-life of the accumulated luciferase would have given the impression that transcription was still elevated at 72 hr. In contrast, the NaB pretreatment experiment (Fig. 7) was performed with promoter-reporter plasmids present in the cells only during the 48–72-hr period of NaB exposure, providing a relatively brief snapshot of transcription occurring only during this later 24-hr period after the initial *trans*-activation was complete. Hence, these two observations are consistent with a *trans*-activation event occurring early in the differentiation pathway induced by both of these compounds.

Discussion

The cloning of the human topo IIα gene promoter by Hochhauser *et al.* (17) has set the stage for understanding the transcriptional regulation of this clinically useful anticancer drug target. To our knowledge, the current report details the first attempt to identify the transcriptional control mechanisms governing the proliferation-dependent regulation of topo IIα at the gene promoter level. Our approach of using a classically studied cell line in the topo II field, the HL-60 human leukemia line, not only has allowed us to begin to characterize the expected suppression of topo II during terminal monocytic differentiation (Fig. 7B) but also has led to the novel observation that the topo IIα gene promoter is activated early in this differentiation pathway.

It was not entirely unexpected that, whereas NaB induced topo IIα promoter activity 4–17-fold, endogenous topo IIα enzyme levels were increased only roughly 2-fold. The impact of overexpressed luciferase protein versus overexpressed topo IIα in a cell likely carries very different consequences. Although several hundred-fold overexpression of luciferase is very well tolerated even in higher eukaryotic cells, the topoisomerases in general are challenging enzymes to overexpress and stably maintain in many cells. In support of this hypothesis is the fact that topo IIα content is reproducibly decreased 30–40% 2–4 hr after NaB treatment (Fig. 6), suggesting that degradation of topo IIα protein may have been initiated by the same stimulus that mediates topo IIα promoter *trans*-activation several hours later. This observation is provocative in light of our previous work demonstrating a normal 27-hr half-life for topo IIα protein in HeLa cells (31). The current data may indicate that topo IIα is degraded at a rate that limits its steady state increase to 2-fold, whereas the rate of topo IIα mRNA production may approach the magnitude observed with luciferase reporter protein. Detailed studies of NaB-stimulated topo IIα transcription rate are underway to address this interesting alternative.

Other explanations to account for the disparity between the magnitude of induction of luciferase activity produced under control of the topo IIα promoter and endogenous topo IIα protein levels might focus specifically on the nature of promoter-reporter assays as a model for endogenous transcriptional regulation. Most obvious is that the −562TOP2LUC topo IIα gene promoter may lack a negative regulatory element that might be present upstream and might mediate transcriptional down-regulation during monocytic differentiation. Although promoter activity from a −1200TOP2LUC construct was also recently shown to be induced 3–5-fold by PMA or NaB treatment of HL-60 cells at 24 hr,[1] we are currently unable to rule out the influence of linearly distant promoter sequences or even intronic regulatory sequences, which may nonetheless be in close proximity to the minimal promoter in the context of chromatin. By similar reasoning, the −562TOP2LUC promoter on a naked plasmid likely displays different accessibility to transcriptional activators and/or suppressors than when constrained in chromatin. The development of cell lines with stably integrated topo IIα promoter-reporter genes may aid in addressing this hypothesis. Promoter activation does not currently appear to be dependent on the choice of reporter enzyme, because PMA also increases chloramphenicol acetyltransferase activity under the control of the topo IIα promoter in HL-60 cells.[2]

Regardless of the mechanism for topo IIα promoter *trans*-activation, it is our contention that even a 1.7–2.3-fold increase in HL-60 topo IIα protein is of significance, from several viewpoints. It is striking that topo IIα levels rarely vary by >2–3-fold in proliferating cells in culture. Hyperthermia, one of the few known positive effectors of cellular topo IIα levels, results in only a 2-fold stimulation of enzyme expression (32). Even attempts to select cells hypersensitive to topo II poisons have resulted in the establishment of a Chinese hamster ovary line with topo II overexpression of no more than 3-fold, compared with the parental wild-type line

[1] D. Fraser and D. Kroll, unpublished observations.
[2] D. Hochhauser, personal communication.

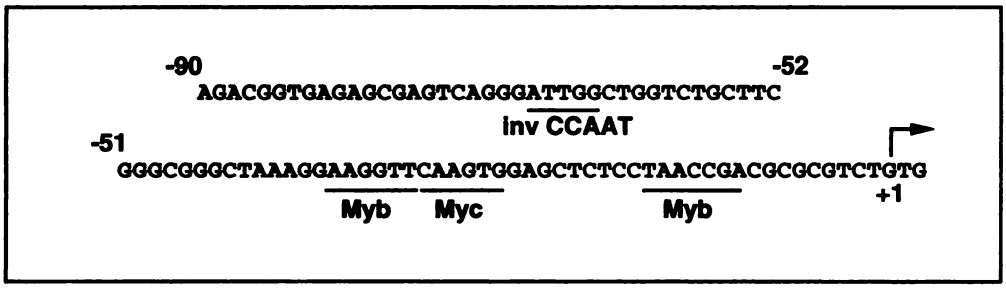

Fig. 8. Potential transcription factor consensus binding sites within the position −90 to position +1 segment of the human topo IIα gene promoter. The upper DNA sequence was derived from the report of Hochhauser *et al.* (17), as well as our own sequencing of this region amplified from HeLa cell genomic DNA. Potential binding sites for Myc and Myb, as well as the inverted CCAAT box, are detailed by *underlining*, based upon references described in Discussion. Our current hypothesis is that, whereas sequences within the position −51 to position +1 segment of the promoter can partly mediate topo IIα *trans*-activation early in HL-60 differentiation, there is a requirement for cooperation with sequences between position −90 and position −52 to confer maximal inducibility, as indicated by our data. The documented role of inverted CCAAT boxes (present at positions −68 to −64 of this promoter) in the cell cycle regulation of several genes suggests that this element may be one candidate for mediating maximal topo IIα promoter *trans*-activation.

(12). This modest increase in topo IIα content conferred a significant degree of cellular hypersensitivity to amsacrine, reducing the IC_{90} 4-fold and, more importantly, increasing cell killing by 1 log unit at each concentration (12). In this light, our findings become more interesting, because several agents, including NaB, have been investigated clinically for their ability to cause differentiation of human leukemias, as they do in various leukemias of myeloid and erthyroid origin in culture (33–35). Differentiation therapy, particularly down the granulocytic lineage, is also a new indication for certain retinoids, including all-*trans*-retinoic acid and 9-*cis*- and 13-*cis*-retinoic acid (34–36). It appears that conventional cytotoxic chemotherapy must ultimately be coupled to these regimens, because resistance develops very rapidly to the differentiating agent given alone (36). Although clearly preliminary, our results might have clinical implications, in that the carefully timed combination of low doses of a topo II-targeted drug with a monocytic differentiating agent might act in an additive or synergistic fashion to eradicate responsive leukemias.

It is recognized that a variety of stimuli, including growth factors, proliferative fraction in culture, and even hyperthermia, trigger intracellular events that all converge to increase topo IIα expression. In the HL-60 system described here, it appears that topo IIα promoter *trans*-activation can also be triggered by early biochemical events involved in monocytic differentiation, because both monocytic differentiating agents tested produced the same effect in two different promonocytic cell lines (HL-60 and U-937). In contrast, the only effect PMA had on a nonhematopoietic line (HeLa) was to suppress basal promoter activity. A very recent report has suggested the potential for the topo IIα promoter to be positively regulated by phorbol esters (37). Although those investigators were puzzled as to why such positive regulatory elements were present in a promoter expected to be down-regulated in HL-60 cells, our report now provides experimental evidence that the topo IIα promoter does indeed contain regulatory regions through which PMA can exert positive transcription-activating effects, but these effects may be cell type dependent.

The 5′ promoter deletion analysis may provide valuable information regarding the factor(s) mediating this transcriptional stimulation. Within the minimal promoter fragment displaying activation during early monocytic differentiation (positions −51 to +90), there is at least one and possibly two consensus binding sites (Fig. 8) for c-Myb, a relatively hematopoietic-specific transcription factor whose expression is closely linked to cellular proliferation. In support of a role for this factor, recent co-transfection studies with a CMV-driven c-*myb* expression plasmid and −90TOP2LUC suggest that Myb stimulates the topo IIα promoter with great potency and efficacy.[3] In addition, the position −51 to +90 region also includes a potential CANNTG E-box binding site for helix-loop-helix factors such as c-Myc (38). Within the upstream sequences necessary for maximal *trans*-activation is also an inverted CCAAT box (from position −68 to position −64) (Fig. 8). The presence of this element near transcriptional start sites has been observed for the cell cycle-regulated gene thymidine kinase (39), as well as several of the histone genes. It has also been suggested previously that at least one of these inverted CCAAT boxes may be involved in the proliferation-dependent regulation of the topo IIα promoter (17).

However, our original and ultimate goal in defining potential regulators of topo IIα transcription derives from the fact that down-regulation of topo IIα transcription and cellular topo IIα enzyme content is a known mechanism of resistance to antitumor drugs that target this enzyme. In the simplest of models, topo IIα transcription could be attentuated in resistant cells either by mutations in *cis*-acting topo IIα promoter sequences where transcriptional activators or suppressors bind or by altered regulation of these *trans*-acting factors themselves. Any combination of these possibilities could represent a common mechanism of resistance to topo II-directed drugs that ultimately converges on the down-regulation of topo IIα transcription. The results presented in Fig. 7B indicate that we now have the tools to examine how topo IIα transcription is normally down-regulated during HL-60 differentiation and should be able to ask whether these normal mechanisms can also be exploited by topo II-directed drug-resistant tumor cells.

Lastly, the results presented here indicate that the use of topo IIα promoter-reporter constructs may be one relatively rapid method with which to begin to understand the genetic regulation of this major anticancer drug target. Nonetheless,

[3] T. Brandt, D. Fraser, S. Leal, and D. Kroll. C-myb transactivation of the human DNA topoisomerase IIα promoter in HL-60 cells. Manuscript in preparation.

caution should be exercised in ascribing significance to the absolute magnitude of promoter inducibility until endogenous topo IIα levels are also quantitated. Nevertheless, use of this method in the context of well characterized systems in the topo II field, such as HL-60 cell differentiation (10, 19), not only will facilitate the elucidation of the classically described down-regulation of topo IIα transcription but also may lead to unexpected results that have been previously overlooked, such as the early activation of the topo IIα promoter in monocytic differentiation. Given the known relationship between cellular topo II levels and tumor cell sensitivity to topo II-directed antineoplastic agents, the promoter-reporter approach provides another tool for our efforts to enhance the selective cytotoxic efficacy of antitumor drugs that target this enzyme.

Acknowledgments

The authors wish to acknowledge Drs. Carlos Enrique Catalano and James P. Hoeffler and the members of their respective laboratories for insightful input during the course of these studies and Angela Kroll, Diane Wegman, and Dylan P. Hartley for reading and commenting on the manuscript. Dr. Scott Kaufmann (Mayo Clinic, Rochester, MN) provided invaluable assistance in developing the HL-60 lysis protocol. The authors also wish to acknowledge Dr. Daniel Hochhauser for sharing unpublished data and providing comments on the manuscript. The authors appreciate the generous plasmid gifts from Drs. Ian Hickson (Oxford University), Bill Wood (University of Colorado Health Sciences Center), and David Gordon (University of Colorado Health Sciences Center) and the gift of polyclonal anti-topo II rabbit serum from Dr. Dan Sullivan (University of Louisville).

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