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Molecular Analysis of a Potentially Phorbol-Regulatable Region of the Human Topoisomerase IIα Gene Promoter[1]

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Topoisomerase IIα (topo IIα) mRNA was down-regulated to a greater extent in 2 human leukemia HL-60 cell lines sensitive to PMA-induced terminal differentiation than in their non-differentiating daughter lines following exposure to PMA (Cancer Res., 50: 7116-7122, 1990; Biochem. Pharmacol., in press). The sequence of the topo IIα promoter (ATG upstream to -650) in all four cell lines was identical to that of a human lymphocyte genomic clone and to that of the previously published sequence from a human placenta clone (J. Biol. Chem., 267: 18961-18965, 1992). Putative transcriptional start sites were identical in one sensitive/resistant pair. In the other pair, a methylated site was identified between positions -242 and -580 within the -650 bp promoter region of the resistant daughter cell only. The identity of the sequence from all four cell lines indicates that mutations in the topo IIα gene promoter of PMA-resistant cells cannot explain the absence of topo IIα mRNA down-regulation following PMA treatment. Altered methylation patterns may, however, contribute to the reduced decrease in topo IIα gene expression in one PMA-resistant line.

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The abbreviations used are: PMA, Phorbol-12-myristate 13-acetate; Topo IIα, Topoisomerase IIα; PCR, Polymerase chain reaction; AP-2, activating protein II; ATF, Adenovirus transcription factor; CRE-BP1, cAMP-response element binding protein 1.

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The differential regulation of the critical nuclear enzyme topo II in two pairs of human leukemia HL-60 cell lines has been described (1, 2). In each pair, one member terminally differentiated in response to PMA (Hall, S) and one member did not (1E3, PET). Differentiation is accompanied by a reduction in etoposide-induced topoisomerase IIα-mediated DNA cleavage and a decrease in topo IIα mRNA (3, 4). In contrast, the PMA-resistant lines, 1E3 and PET do not exhibit these same decreases in drug-induced cleavage or in mRNA levels (3, 4). These results suggest that topo IIα may play a role in leukemic cell differentiation.

The exact mechanism by which phorbol esters produce cellular differentiation is unknown. The activation of the putative phorbol receptor, protein kinase C (5) and the subsequent biochemical cascade, is associated with a host of transcriptional events (6, 7). These molecular events are in turn regulated by a number of *trans*-acting transcriptional proteins AP-1, AP-2, ATF, and PEA-3, whose binding activities and transcriptional effects have been reported to be altered by phorbol ester treatment (8-11). Furthermore, point mutations or methylation of the binding motifs for these transcriptional factors have been reported to result in reduced binding constants for the *trans*-acting factors and subsequent loss of PMA regulation (6, 7, 11). Recently, a clone for the 5' end of the topo IIα gene was isolated from a human placenta library and the sequence 650 bp upstream of the ATG start site was reported (12). There are a number of potential transcription factor binding sites within this 650 bp region. In the present work, we have sequenced the topo IIα gene promoter region from the four cell lines described above in order to identify potential point mutations responsible for the phorbol-resistance of topo IIα gene down-regulation. In addition, cytosine methylation within the promoter region was examined as yet another potential mechanism for the phorbol resistance of topo IIα-mediated processes in the phorbol-resistant 1E3 and PET cells.

Materials and Methods

Materials. Proteinase K and RNase A were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Oligonucleotides were purchased from Genosys Biotechnologies Inc. (The Woodlands, TX). Random primer kits, [^{32}P]-γ-ATP, and [^{32}P]-α-ATP were purchased from Amersham Corp. (Arlington Heights, IL). Polymerase chain reaction kits were purchased from Perkin-Elmer Cetus (Norwalk, CT). Avian myeloblastosis-virus reverse transcriptase and restriction enzymes were purchased from New England Biolabs,

Inc. (Beverly, MA). The human lymphocyte λ-Dash library was purchased from Stratagene (La Jolla, CA). All other chemicals were of the highest reagent grade.

Cell Culture. The Hall and 1E3 lines used in these experiments were generous gifts from Dr. Robert Hall, University of Tennessee Medical School (Memphis, TN) (2). The HL-60 Beran cells were a generous gift of Drs. M. Beran and B. Andersson, MD Anderson Cancer Center (Houston, TX) (13, 14). The S, and PET lines were generously provided by Dr. Donald MacFarlane, University of Iowa (Iowa City, IA) (1, 15). The cells were propagated in Iscove's modified Dulbecco's medium, JRH Biosciences (Lenexa, KS) and 10% fetal calf serum at 37°C in 5% CO_2. All cells were mycoplasma free as determined by the American Type Culture Collection (Gaithersburg, MD).

Polymerase Chain Reactions (PCR) and DNA Sequencing. Genomic DNA was extracted from freshly harvested cells by the procedure of Davis *et al.* (16). PCR reactions were carried out as described previously (17) with the following modifications. The DNA was denatured at 94°C for 1 min, annealed at 52°C for 1 min, and then extended at 72°C for 1 min. This cycle was repeated 29 times. To insure that all products were complete, a final cycle was performed consisting of 94°C for 1 min, 54°C for 1 min, then 72°C for 10 min. PCR products were isolated on a low-melt agarose gel and purified on an Amicon microcon centrifuge apparatus (Beverly, MA) with successive washings in water. The samples were then directly sequenced on an ABI automated sequencer, according to the manufacturer's procedures. The topo IIα promoter extending upstream from the ATG start site to the -650 region was amplified in two reactions. Reaction 1 encompassed the ATG start site and extended to -380 bp. Reaction 2 encoded the promoter region from -350 to -650 bp.

Analysis of Methylation Patterns. Genomic DNA from all four cell lines was isolated, as previously described (16). The DNA was digested with either 100 units Hpa II per μg of DNA or 100 units Msp I per μg of DNA. Southern blotting was performed according to a previously published protocol (18) and probed with a fragment of the topo IIα promoter, extending from the ATG start site to position -650 bp (see Fig. 3B).

Primer Extension. A 30-mer oligonucleotide was synthesized corresponding to positions 10 to 41 bp of the topo IIα cDNA (19) in the antisense orientation 5'-GCATATTTTCATTTACAGGCTGCAATGGTG-3'. The oligonucleotide was end-labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase, as previously described (20). Primer extension reactions were carried out using avian myeloblastosis-virus reverse transcriptase as previously described (12).

Results and Discussion

Sequence analysis of the PCR-amplified region in the topo IIα promoter extending from the ATG translational start site upstream to -650 bp revealed 100% identity among all samples analyzed (Fig. 1). Furthermore, 2090 bp of the topo IIα promoter were sequenced from a human lymphocyte genomic DNA library confirming the previously published 650 bp sequence from a human placental topo IIα clone (12). Therefore, the resistance of topo IIα RNA to down-regulation following phorbol treatment of 1E3 and PET cells

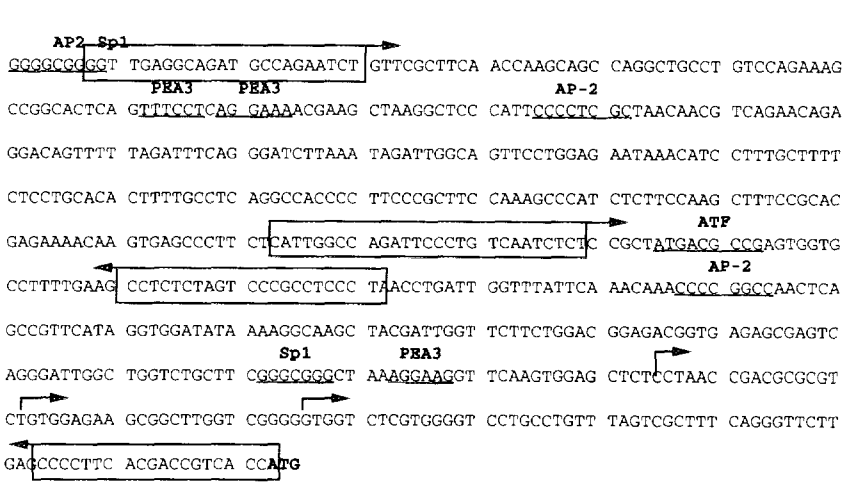

Fig. 1. Structure of the human topo IIα promoter from the ATG start site to -650 bp. The transcriptional start sites mapped by primer extension are indicated by horizontal arrows. Oligonucleotides for PCR reactions are boxed and the direction of extension is indicated. Possible regulatory binding sites are underlined and identified.

is not due to the presence of a sequence mutation in the promoter region from the ATG start site to position -650.

The transcription start site of topo IIα in phorbol-sensitive S cells was compared with that in the respective resistant line (PET) by primer extension. Figure 2 shows that when a 30-mer oligonucleotide corresponding to positions 10 to 41 bp in the topo IIα cDNA was used as a primer, a doublet of 146 bp and 132 bp can be observed as can a smaller band of 106 bp. These bands were observed in an unrelated cell line, (HL-60 Beran) as well as in S, and PET cell lines. These bands correspond to transcription start sites of 105, 91, and 65 bp upstream of the ATG translational start site. The initiation sites are consistent with the previous observations of Hochhauser *et al.* (12). Therefore, these results appear to indicate that an alternate initiation site is not the means by which topo IIα mRNA persists in PET cells upon phorbol treatment.

Sequence analysis of the -650 region of the topo IIα promoter revealed the presence of binding sites for several putative *trans*-acting transcriptional factors. These include AP-2, ATF, and PEA3, which have been shown to be involved in the phorbol ester induction of various mammalian genes (21, 22, 23). Homology studies of the topo IIα promoter region indicate that these sites

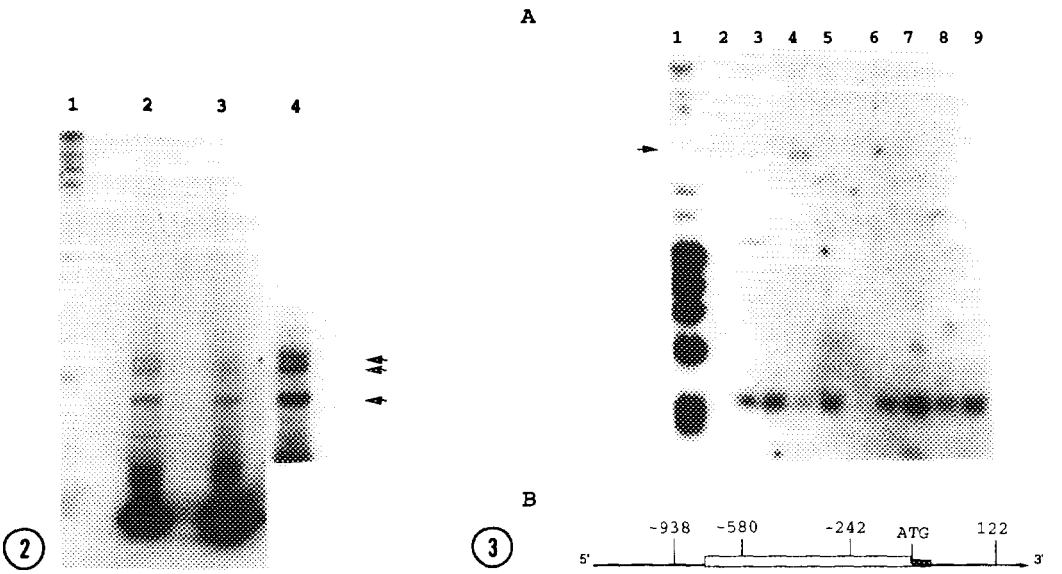

Fig. 2. Total cellular RNA from human leukemia HL-60 cell lines was hybridized with an excess of end-labeled oligonucleotide, and the primer extension reaction was performed as described in Materials and Methods. The products were analyzed on an 8% polyacrylamide-7 M urea gel. Lane 1 is end-labeled Hind III Lambda-DNA. Lane 2 depicts products from S cell RNA. Lane 3 depicts products from PET cell RNA. Lane 4 depicts products from HL-60 Beran RNA. Arrows indicate the predominant extension products.

Fig. 3. **A)** Southern blots of genomic DNA were digested with either Hpa II or Msp I, ethidium bromide stained after agarose electrophoresis, transfered to nytran paper and probed with the -650 bp region of the topo IIα promoter as described in Materials and Methods. Lane 1, Hind III digested end-labeled Lambda DNA and Hae III digested end-labeled ϕ-X174 DNA. Lane 2, Hall cell DNA digested with Hpa II. Lane 3, Hall cell DNA digested with Msp I. Lane 4, 1E3 cell DNA digested with Hpa II. Lane 5, 1E3 cell DNA digested with Msp I. Lane 6, S cell DNA digested with Hpa II. Lane 7, S cell DNA digested with Msp I. Lane 8, PET cell DNA digested with Hpa II. Lane 9, PET cell DNA digested with Msp I. **B)** Genomic line graph depicting the Hpa II sites located within and around the -650 promoter sequence. Open box indicates the 650 bp area of the probe used, while the ATG and black box designate the first exon.

could possibly act as putative binding sites for *trans*-acting factors, for they agree well with derived consensus sequences (24). Although there are one or two nucleotide degenerations among these sequences, these changes have been shown to minimally affect the binding constants and the steady state *in vitro* rates of transcription (6-8, 11, 25). The AP-2 site of the topo IIα promoter shows the highest degree of homology when compared with the human metallothionein-IIA AP-2 element. However, in the topo IIα promoter, AP-2 is not found in a tandem array with itself, AP-1 or PEA3 as is seen in a variety of phorbol-responsive genes

indicating the possible novel use of this *trans*-acting factor (9, 10, 17). The PEA3 site at position -127 has non-homology at both its 3' and 5' ends to the consensus sequence (24). The significance of these nucleotide changes is unclear. However, both PEA3 sites seen at position -564 show the highest degree of homology with known sites such as the urokinase plasminogen activator gene (23), yet the tandem array is unique to the topo IIα promoter.

Previous studies indicate that AP-2, PEA-3, and ATF binding lead to increased transcriptional activity upon phorbol ester treatment rather than to the decrease observed in topo IIα mRNA (6, 7, 8). Perhaps the unique arrangement of these putative binding sites in the topo IIα promoter dictates a novel physical array of these factors that produces down-regulation following phorbol ester treatment. Alternatively, the unique cellular response of HL-60 cells to phorbol treatment may result in down-regulation of many proliferation-associated genes, one of which is topo IIα. The sequence of the topo IIα promoter shows no homology with genes whose expression is known to be attenuated by phorbol esters such as the interferon-α/β (26), mouse collagen I (27), or c-myc (28). However, the mechanism by which down-regulation of topo IIα mRNA by phorbol esters is accomplished may be through an as yet unidentified "repressing" *trans*-acting factor.

The effect of CpG methylation on transcriptional activity has been reported for a number of phorbol-responsive genes (6, 11, 25). Southern blot analysis was performed on genomic DNA from the four cell lines, as described in Materials and Methods. One can observe the appearance of bands corresponding to 350 bp in all Hpa II and Msp I genomic DNA digestions (Fig. 3A). However, a band of approximately 3 kb is present in the Hpa II digested 1E3 DNA but not in the Msp I digest (Fig. 3A), indicating a methylation site in the topo IIα promoter of 1E3. If this contributes to the reduced topo IIα gene down-regulation in 1E3, it would distinguish 1E3 cells from the PET cells. This distinction could be added to others that we have reported previously. For example, the two cell lines showed different proclivities toward phorbol ester-induced modulation of etoposide cytotoxicity (3, 4). Methylation of an AP-2 binding site in the human proenkephalin promoter led to a decreased affinity of AP-2 binding, as well as to a decreased ability of the gene to be induced by phorbol esters (11). It can be seen that one of the possible methylation sites is within the -242 AP-2 putative-binding site. However, the effect of

methylation must await the identification of the phorbol-responsive element. Understanding the differences by which the topo IIα gene in 1E3 and PET resist phorbol-induced down-regulation will aid not only in understanding the transcriptional regulation of topo IIα but also in identifying factors involved in the cellular differentiation of leukemic cells.

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