Biochemical Pharmacology, Vol. 52, pp. 1065–1072, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00446-7

Phorbol Ester-Induced Down-Regulation of Topoisomerase IIα mRNA in a Human Erythroleukemia Cell Line

EVIDENCE FOR A POST-TRANSCRIPTIONAL MECHANISM

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ABSTRACT. Tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are reported to induce megakaryocyte terminal differentiation of the erythroleukemia cell line K562. This differentiation is accompanied by the regulation of various gene products such as γ-globin (Lumelsky and Forget, *Mol Cell Biol* **11:** 3528–3536, 1991) and platelet-derived growth factor-β (PDGF-β) (Mäkelä *et al.*, *Mol Cell Biol* **7:** 3656–3662, 1987). PMA has also been found to regulate topoisomerase (topo) IIα in other myeloid leukemia lines. The purpose of this study was to investigate whether PMA regulates topo IIα in K562 cells and, if so, to identify the mechanisms responsible for this regulation. Northern blot analysis revealed that topo IIα mRNA is down-regulated as is γ-globin. This activity was not due to a generalized decrease in mRNA, as PDGF-β message actually increased in response to PMA treatment. RNase protection assays confirmed the decline in the topo IIα message. Transfection experiments with various topo II promoter CAT constructs extending to 2200 bp upstream of the ATG start site revealed regions that enhance *and* regions that inhibit CAT expression in the absence of PMA. However, PMA did not affect this CAT expression. Run-on experiments using 5′ and 3′ human topo II cDNA probes confirmed that transcriptional initiation of the topo II gene was not affected by PMA, whereas that of c-*myc* did decrease. Therefore, the apparent decrease in topo IIα mRNA in K562 cells upon their treatment with PMA appeared to be the result of a post-transcriptional mechanism. BIOCHEM PHARMACOL 52;7:1065–1072, 1996.

KEY WORDS. topoisomerase IIα; phorbol ester; differentiation; mRNA; K562; post-transcriptional regulation

DNA topo IIα,§ a nuclear enzyme responsible for the topological change of various isoforms of DNA, has been implicated in several physiological functions including DNA replication [1, 2], recombination [3, 4], and chromosome condensation during mitosis [1, 5, 6]. Antineoplastic drugs such as epipodophyllotoxins, anthracyclines, and acridines inhibit topo IIα by blocking the enzyme-mediated religation of DNA via the formation of a DNA-cleavable complex [7].

Phorbol esters such as PMA induce differentiation of human leukemia cells by a mechanism that is as yet unknown [8, 9]. PMA has been shown also to alter the levels of topo IIα in these cells. Variant HL-60 lines that resist phorbol-induced differentiation also resist phorbol-induced down-regulation of topo IIα [10–14]. These observations suggest a mechanistic link between topo IIα and cellular differentiation.

Several examples of mammalian cells resistant to topo IIα-directed chemotherapeutic drugs have been described [for review, see Ref. 15]. Mutations within topo IIα cDNA rendered the enzyme from these cells unable to form DNA complexes in the presence of topo II-directed drugs [16–21]. Yet, to date, there is only one report of clinical resistance due to a topo IIα mutation [22]. Resistance has been linked to altered levels of topo IIα rather than to mutations within the topo IIα gene [23–27]. As with resistant forms of topo IIα, low levels of topo IIα lead to low levels of drug-induced cleavable complex formation and reduced cytotoxicity. Therefore, a system in which topo IIα is down-regulated by PMA would be useful for understanding how tumor cells acquire resistance to chemotherapeutic drugs through altered levels of topo IIα.

K562 is an erythroleukemia cell line that terminally dif-

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§ *Abbreviations:* topo IIα, topoisomerase IIα; PMA, phorbol 12-myristate 13-acetate; AP-2, activating protein II; ATF, adenovirus transcription factor; PDGF-β, platelet-derived growth factor-β; Me_2SO, dimethyl sulfoxide; SSC, 150 mM sodium chloride, 15 mM sodium citrate, pH 7; CAT, chloroamphenicol amino transferase; and CMV, cytomegalovirus.

Received 18 July 1995; accepted 29 April 1996.

ferentiates to megakaryocytes upon treatment with phorbol esters [28]. We analyzed the effects of PMA-induced differentiation of K562 cells on cellular topo IIα mRNA levels and found that following PMA treatment, levels decreased in K562 as had occurred previously in two other myeloid leukemia cell lines [12–14]. This work characterizes the biochemical mechanism of topo IIα regulation at the level of transcription and indicates that a phorbol ester-induced post-transcriptional process is responsible for the down-regulation of topo IIα that occurs during this type of cellular differentiation.

MATERIALS AND METHODS

Cell Culture

K562 cells of the lowest passage number possible were obtained from the Coriell Institute for Medical Research (Camden, NJ). Cells were passaged (<30) in RPMI medium supplemented with 15% fetal bovine serum at 37° in 5% CO_2. The cells were split before reaching a density of 10^6 cells/mL. Incubations with PMA were carried out with a starting cell density of 10^5 cells/mL for the indicated periods of time. PMA was dissolved in Me_2SO, and cells were treated with a final concentration of 20 nM PMA in 0.1% Me_2SO or with 0.1% Me_2SO as a control. All cells were free of Mycoplasma as determined by the American Type Culture Collection (Gaithersburg, MD).

Preparation of RNA, Northern Blots

Cells were collected by centrifugation and washed twice with phosphate-buffered saline. Total cellular RNA was isolated by a guanidinium-thiocyanante/phenol-chloroform extraction protocol as previously described [29]. Aliquots of RNA were denatured with 6% formaldehyde and fractionated by electrophoresis with a 1.5% denaturing agarose gel [30]. After staining with 0.5 mM ethidium bromide, the gels were transferred by capillary action of high salt to Hybond membranes (Amersham, Arlington Heights, IL). The membranes were then baked and prehybridized according to the manufacturer's suggestions at 42° for 16 hr. The 1.7 kb topo IIα probe used in these experiments was a gift of Dr. Leroy Liu of the Robert Wood Johnson Medical School, UMDNJ. The γ-globin probe, a 700 bp fragment encompassing the entire cDNA, was a gift of Dr. Bernard G. Forget of the Yale University School of Medicine. The probe used for PDGF-β was a full-length cDNA fragment (2.6 kb) obtained from the American Type Culture Collection (Rockville, MD). The probes (25 ng) were labeled with [α-^{32}P]dCTP using a random prime kit (Amersham). Hybridization was performed at a probe concentration of 10^6 cpm/mL of hybridization solution at 42° for 16 hr with gentle agitation. Blots were washed by successive incubations in 6× SSC, 0.5% SDS at room temperature for 30 min, 0.2× SSC, 0.1% SDS at 42° for 30 min, and 0.2× SSC, 0.01% SDS at 65° for 30 min.

Transfection Experiments

The topo IIα promoter CAT constructs were described previously [31] and are depicted in Fig. 2. K562 cells (10^8 total) were collected and washed three times with phosphate-buffered saline and then resuspended in 1 mL of RPMI without serum. An aliquot of this cell suspension (200 μL) was incubated with 15 μg of the indicated topo IIα-CAT vector and 15 μg of CMV promoted β-galactosidase vector on ice for 15 min. The suspension was then electroporated at 220 V, 960 μF in a Bio-Rad Gene Pulser. The suspension was placed on ice for 15 min before it was resuspended in 5 mL of RPMI medium with serum for 24 hr. Cells were then split in half and treated with either PMA or vehicle (0.1% Me_2SO) for 48 hr before harvesting. CAT activity and β-galactosidase activity assays were performed as previously described [30]. Quantification of radioactivity was carried out by scanning the TLC plate with a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnydale, CA).

Nuclei Isolation and Run-on Experiments

K562 cells were treated with 20 nM PMA or with vehicle (0.1% Me_2SO) for 48 hr before nuclei were harvested according to a previously published method [32] and stored at −70° (for no more than 30 days). The transcription assay was performed by labeling and isolating the mRNA from 10^7 nuclei as described previously [33] and as modified by Sagoh and Yamada [34]. Fifteen micrograms of each of the following DNA targets was alkali-denatured and fixed to Hybond filters (Amersham): human topo IIα cDNA fragments (PCR4 and PCR1) encompassing the 5′ 1500 bp and 3′ 1200 bp, respectively [17], c-*myc* fragment containing the third exon (a gift from Dr. Mark Blick, previously of UTMDACC), and full length β-actin. Run-on products (10^6 cpm/mL) were hybridized to targets using Amersham Rapid-Hyb buffer according to the manufacturer's recommendations (Amersham). Quantification of radioactivity was carried out by scanning the blots with a Molecular Dynamics Phosphorimager (Molecular Dynamics).

RESULTS

Northern Blot Analysis

To analyze the K562 cell line as a suitable model for the study of topo IIα down-regulation by phorbol esters, mRNA steady-state levels were analyzed by northern blots as seen in Fig. 1. Previous reports observed that γ-globin mRNA decreases and PDGF-β mRNA increases in K562 cells treated with phorbol esters [35, 36]. We also observed a decrease in the steady-state level of a 0.65 kb γ-globin mRNA in PMA-treated cells within the first 24 hr (Fig. 1). A 2.7 kb PDGF-β mRNA transcript was only observed in PMA-treated cells.

A probe coding for the 5′ end of the topo IIα mRNA revealed the presence of a transcript corresponding to 6.2 kb in total cellular RNA from non-PMA-treated cells.

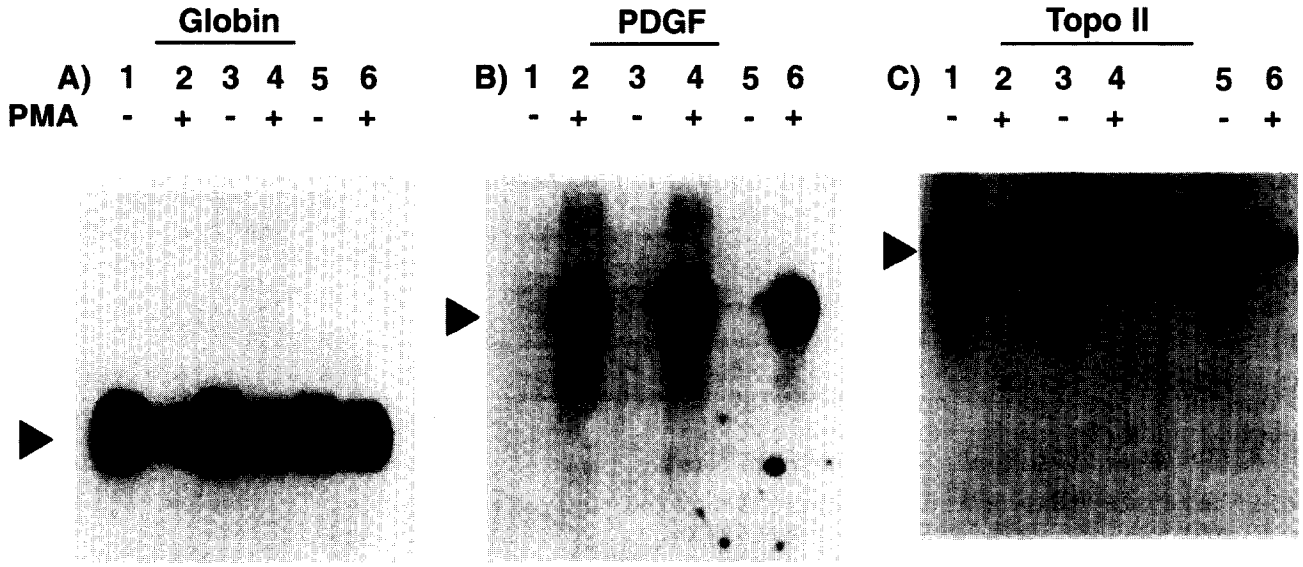

FIG. 1. Northern blot analysis of total cellular RNA from K562 cells. Total cellular RNA was isolated from cells treated with either 20 nM PMA/0.1% Me_2SO or 0.1% Me_2SO for 24 hr (lanes 1 and 2), 48 hr (lanes 3 and 4), or 72 hr (lanes 5 and 6). (A) K562 RNA analyzed with a probe coding for the γ-globin cDNA. (B) K562 RNA analyzed with a probe coding for the PDGF-β cDNA. (C) K562 RNA analyzed with a probe coding for the 5′ 1500 bp of the topo IIα cDNA. Each lane contains approximately 5 mg of total cellular RNA. The amount of RNA in each lane was standardized with a probe for β-actin.

However, the 6.2 kb topo IIα mRNA was barely observed in K562 cells following PMA treatment. The decrease in the level of topo IIα mRNA continued over the entire 72-hr PMA treatment period. These results indicated that PMA treatment of K562 cells resulted in the down-regulation of the steady-state level of topo IIα mRNA.

Transfection Experiments

To determine whether the decrease in the level of topo IIα mRNA was due to transcriptional modulation by PMA, a series of transfection experiments was performed. Constructs in which the CAT gene was under the control of various deletion mutants of the topo IIα promoter are depicted in Fig. 2. Transient transfection of these constructs in K562 cells revealed that the highest level of CAT expression was observed when only 295 bp proximal to the ATG start site of the topo IIα promoter was used (Fig. 2). However, addition of topo IIα promoter sequences immediately upstream of this 295 bp region decreased the basal level of expression; this suggested the presence of inhibitory sequences within a region extending from 295 to 2200 bp. The inhibition was not the result of variations among transfection experiments, since a construct containing the β-galactosidase under control of the CMV promoter was co-transfected in each experiment and used as an internal control to correct the data observed.

The constructs were then used to examine the effect of PMA on the topo IIα promoter expression of CAT (Figs. 3 and 4). The CAT levels of different constructs in PMA-treated cells could not be standardized to each other by β-galactosidase because of the effect of PMA on the CMV promoter in this construct. Therefore, cells transfected with an individual topo IIα promoter construct were split equally for treatment with or without PMA. The level of expression from PMA-treated cells was then normalized against that of non-PMA-treated cells. Once the PMA level of expression was standardized against the non-PMA-treated cells, β-galactosidase expression from the non-PMA-treated cells could then be used to standardize the data observed from one construct to another. Although the pattern of basal level expression observed was similar to that of non-treated cells, in no case did we observe a decrease in CAT expression by PMA over that of non-treated cells. Therefore, these data suggest that an effect of PMA on the topo IIα promoter may not be the mechanism responsible for the down-regulation of the steady-state level of topo IIα mRNA.

Run-on Experiments

To further study the effect of PMA on the transcriptional rate of the topo IIα promoter, we performed run-on assays with nuclei from PMA-treated and non-treated K562 cells. When labeled transcripts from these nuclei were used to probe topo IIα cDNA, no difference in signal was found between PMA-treated and non-treated cells (Fig. 5). Furthermore, there was no difference in signal from either the 5′ (PCR4) or 3′ (PCR1) end of the topo IIα gene independent of whether the cells were treated with PMA. Results with β-actin, used as an internal control, were similar to the results observed for topo IIα. The validity of the measurements was substantiated by the observation that the number of transcripts initiated for the c-*myc* gene *did* decrease ($P < 0.05$) upon phorbol ester treatment in accord with previously published observations [37].

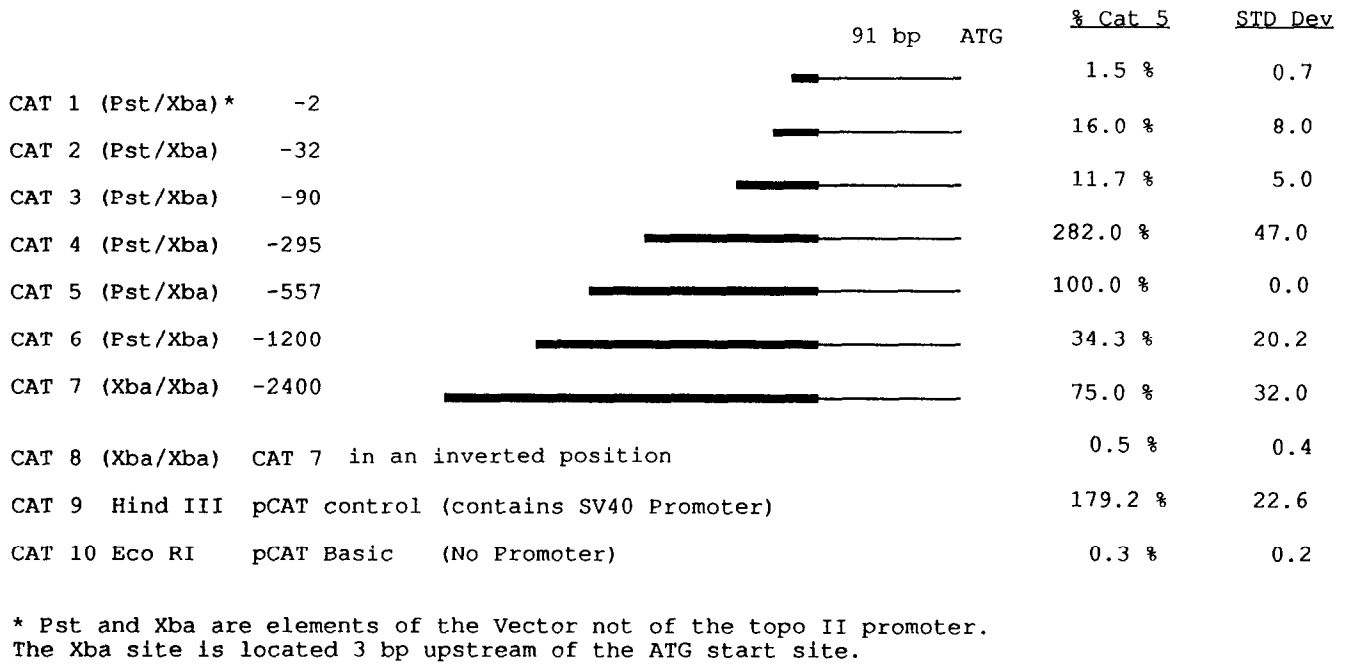

FIG. 2. Deletion mutants and activity of the topo IIα promoter. Constructs were used to promote pCAT activity in a transient transfection of K562 cells by electroporation. The numbers on the left are the size of the deleted promoter fragments. Fifteen micrograms of DNA was used in each transfection and co-transfected with 15 μg of a vector containing β-galactosidase under the control of the CMV promoter. The β-galactosidase activity is used to normalize the CAT activity expressed between successive transfection experiments. CAT activity of the various deletion mutants are expressed as a percentage of CAT 5 activity after 72 hr of transfection. CAT 5 was included in all experiments and could thus serve as an internal control against which to reference the results with the different constructs. Data are the means of at least 3 independent experiments.

In conclusion, the differentiation of K562 erythroleukemia cells to megakaryocytes by phorbol ester is associated not only with regulation of various mRNAs such as those of γ-globin and PDGF-β but also with the down-regulation of topo IIα. The regulation appears to be at the level of a post-transcriptional mechanism because two independent measurements showed no effect by PMA on the topo IIα promoter.

DISCUSSION

K562 is a human cell line that undergoes megakaryocytic terminal differentiation upon treatment with phorbol esters. We have shown that the terminal differentiation of K562 cells is accompanied by a decrease in the steady-state level of topo IIα mRNA and would be predicted to result in a subsequent decrease in the level of protein as observed for HL-60 cells [13]. The down-regulation of topo IIα mRNA was also analyzed by RNase protection assays (results not shown) and essentially confirmed the results observed in the northern analysis. However, the down-regulation is clearest in low-passage cells either as a result of the spontaneous differentiation of K562 cells or of various subtypes within these cells [38]. Therefore, it appears that PMA-induced differentiation of low passage K562 cells is an ideal system for studying the regulation of topo IIα mRNA steady-state levels.

A number of genes such as the interferon-α/β [39], mouse collagen I [40], c-*myc* [37], SV40 viral genes [41], somatostatin [42], and urokinase plaminogen activator [43] are known to be transcriptionally regulated by phorbol esters. *Trans*-acting factors like AP-2, ATF, and PEA3 have been shown to be involved in the phorbol ester induction of these various genes. Furthermore, previous work in our laboratory [44] and in others [31] have identified putative binding sequences for these factors in the promoter for topo IIα. Therefore, PMA treatment of K562 cells could lead to the down-regulation of topo IIα transcription, which would then result in the observed decrease in mRNA levels. Transfection assays of the CAT gene under the control of various deletion mutants of the topo IIα promoter resulted in a high basal level of transcription with the first 295 bp of the promoter, followed by inhibition of this basal level by 5′ sequences 296 to 2200 bp. These results are similar to those observed from the human topo IIα promoter in HeLa cells reported previously [31]. However, the transfection assays did not reveal an ability of the topo IIα promoter to be down-regulated by PMA. Therefore, if phorbol esters mediated transcriptional control in lowering the steady-state level of topo IIα mRNA in K562 cells, either (1) the *cis*-acting sequences of induction are not contained within these CAT constructs, (2) the phorbol sequences are present, but inhibitory sequences within the 2200 bp promoter prevent their detection, or (3) the decrease in the steady-state level of topo IIα mRNA is not due to an altered rate

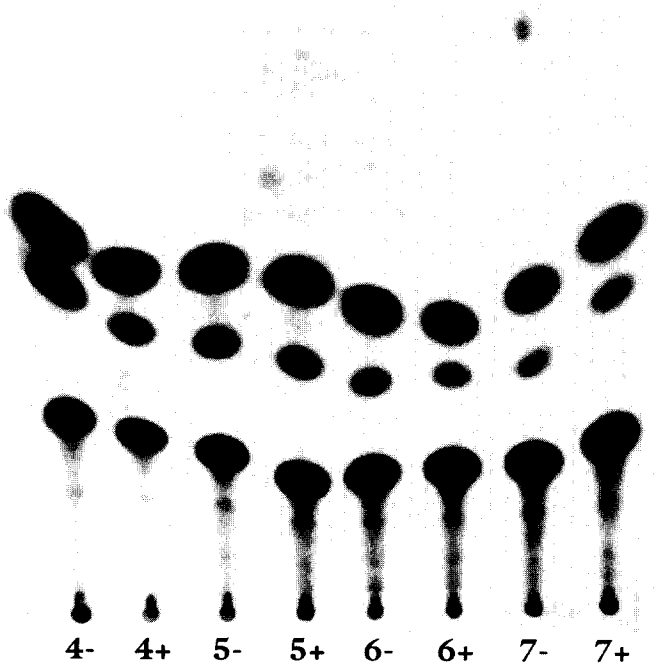

FIG. 3. Thin-layer chromatography of PMA-treated topo IIα deletion mutants. Representative thin-layer chromatography of CAT activity was performed on ± PMA-treated K562 cells after transfection with the topo IIα deletion constructs. The numbers on the figure represent topo IIα deletion constructs depicted in Fig. 2, either treated (+) or non-treated (-) with PMA. Fifteen micrograms of construct DNA was electroporated into K562 cells and co-transfected with 15 μg of a vector containing β-galactosidase under the control of the CMV promoter. Twenty-four hours after transfection, the cells were split and either treated for 48 hr with 20 nM PMA/0.1% Me_2SO or with 0.1% Me_2SO as a control.

of transcription but rather to an alternative mechanism of post-transcriptional control.

Transcriptional regulation of the topo IIα gene by PMA was examined more closely by run-on experiments using probes from both the 5′ and 3′ end of the cDNA. The results of these experiments indicted that the number of initiated topo IIα transcripts was not affected by treatment of K562 cells with PMA. The *c-myc* gene has been shown previously to be down-regulated by phorbol esters through the attenuation of transcriptional elongation [45, 46]. Yet, since no difference can be observed between the 5′ and 3′ cDNA probes used for topo IIα, it is unlikely that attenuation is the means by which the steady-state level of topo IIα mRNA is down-regulated by phorbol esters. Therefore, we propose that the down-regulation of topo IIα mRNA in terminally differentiated K562 cells is solely the result of a post-transcriptional mechanism. The post-transcriptional PMA-induced down-regulation of the topo IIα mRNA may be the result of a destabilization of the mRNA; however, aberrant mRNA processing or interference with mRNA transport from the nucleus is also possible. Furthermore, the destabilization of the topo IIα mRNA does not appear to be the result of an overall nonspecific effect of PMA on all mRNA species in K562 cells, since there was no decrease in β-actin and PDGF-β was shown to increase upon treatment with PMA.

One can predict several possible models that may explain the destabilization of topo IIα mRNA upon phorbol-induced differentiation of K562 cells. One model assumes that the topo IIα mRNA in erythroid cells is specifically stabilized by a putative *trans*-acting factor. PMA-induced differentiation to a megakaryocyte cell would then inactivate the stabilization factor and result in a decrease in the half-life of the topo IIα mRNA. A second model would predict that a *cis*-acting sequence within the topo IIα mRNA is responsible for stabilization within the erythrocyte environment. PMA and the subsequent megakaryocytic differentiation would then activate a specific nuclease, which could interfere with the stabilizing activity of the *cis*-acting element. A third model would not involve a stabilization factor or sequence but rather depend solely on a PMA-induced transcript-specific nuclease that acts upon targeted mRNAs including topo IIα. Previous reports have

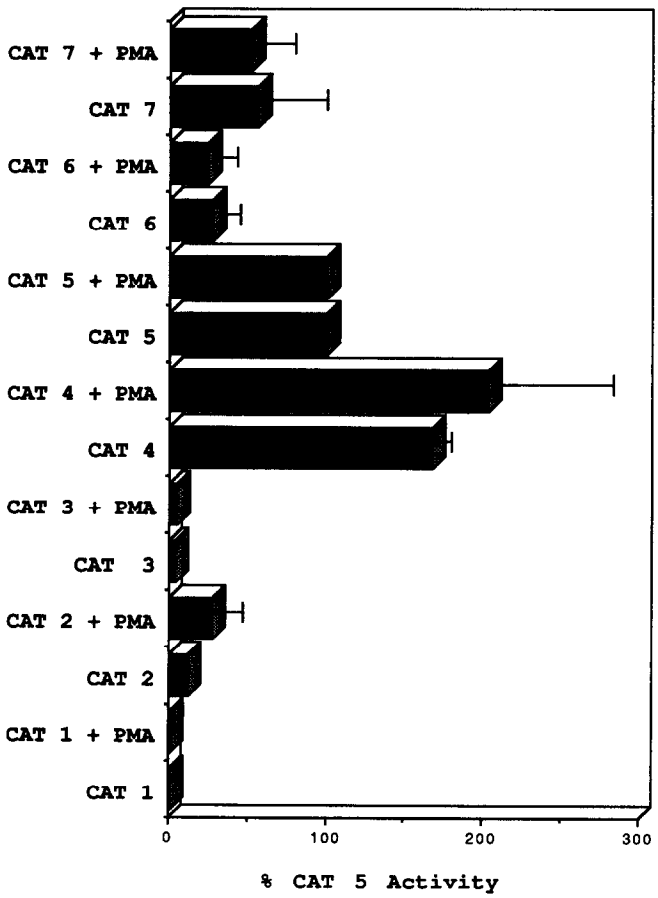

FIG. 4. Effect of PMA treatment on the topo IIα promoter. Graphical summary of all CAT activity from the ± PMA-treated K562 transfections. β-Galactosidase was used to standardize the ratio of CAT activity from PMA-treated vs non-treated cells against the activity of CAT 5. Data are the means ± SD of at least three independent experiments.

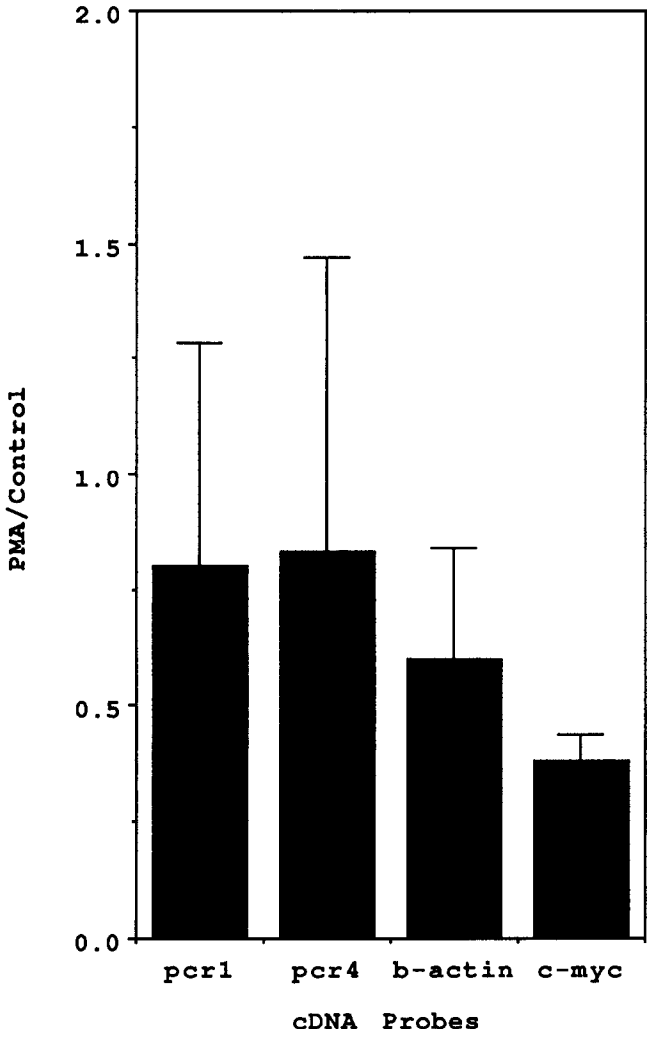

FIG. 5. Nuclear run-on experiments: Effect of PMA treatment on the transcription of the endogenous topo IIα promoter in K562 nuclei. K562 cells were treated with 20 nM PMA/0.1% Me_2SO or with 0.1% Me_2SO for 48 hr before isolating nuclei. Nuclei (10^7) were incubated with [^{32}P]UTP followed by isolation of the labeled transcripts. The labeled mRNA was hybridized to dot blots containing 15 μg of *isolated* fragments coding for c-*myc* (3 exon), 5′ topo IIα cDNA (PCR4, 1–1500 bp), 3′ Topo IIα cDNA (PCR1, 4200–5200 bp), and β-actin cDNA. The blots were scanned and standardized against the value for β-actin. Values are the means ± SD of seven independent experiments.

supported the second model for a *cis*-acting stabilizing element acted upon by a phorbol-induced *trans*-acting factor [for review, see Ref. 47]. One type of *cis*-acting element is the adenosine-uridine rich domains (AUUUA) found in the 3′ untranslated region (3′-UTR) of various cDNAs for granulocyte/macrophage colony-stimulating factor [48, 49], c-*myc* [50], preproendothelin-1 [51], and plasminogen-activator inhibitor type 2 [52]. Sequence analysis of the topo IIα 3′-UTR reveals only one copy of AUUUA rather than a repeated AU-rich domain which is commonly observed [53]. Therefore it is unlikely that the single AU-rich sequence in the topo IIα 3′-UTR is involved in the destabilization by PMA. A second *cis*-acting element found to be responsible for mRNA stabilization is the characteristic stem-loop structures identified in histone [54] and transferrin receptor mRNA [55, 56]. This structure is also believed to be involved in the PMA-induced destabilization of the m1 muscarinic acetylcholine receptor mRNA by 3-UTR regions [57, 58]. We have analyzed the 2° structure of the 3′-UTR for the topo IIα mRNA and found the possibility for similar stem-loop structures. Therefore, one could speculate that PMA-induced differentiation of erythroleukemia cells to megakaryocytes induces a sequence specific nuclease that, in turn, would act on the 3′-UTR sequences leading to destabilization of the topo IIα transcript and a decrease in the steady-state level of mRNA. However, there are reports of sequences within the 3′ coding region in addition to the 3′-UTR that can also influence the stability of a mRNA species, i.e., c-fos [59, 60]. Therefore, one should analyze the entire 3′ region of the topo IIα mRNA for domains involved in stabilization and PMA-induced destabilization.

This work was supported by Grant RO1CA40090 from the National Cancer Institute and Grant DHP-39H from the American Cancer Society.

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