

# Down-regulation by retinoic acid of the catalytic subunit of protein phosphatase type 2A during granulocytic differentiation of HL-60 cells

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Activity of protein phosphatase measured in the absence of divalent cations was decreased by 50% during *all-trans* retinoic acid (ATRA)-induced HL-60 cell differentiation into the granulocytic phenotype. Treatment of HL-60 cells with ATRA led to a dramatic decrease in the amount of protein phosphatase type 2A (PP2A) protein, whereas that of protein phosphatase type 1 (PP1) protein was relatively constant, as detected by immunoblotting with antibodies specific to PP1 and PP2A. The decreased phosphatase activity may be mainly due to a decrease in the expression of the PP2A protein. The mRNA level of PP2A $\beta$  was markedly decreased within 5 h after addition of ATRA, but there was only a slight increase in the mRNA level of PP2A $\alpha$ . Selective down-regulation of PP2A $\beta$  mRNA clearly preceded the cell differentiation induced by ATRA treatment. Thus, PP2A is down-regulated during ATRA-induced differentiation of HL-60 cells into granulocytes.

HL-60 cell; Retinoic acid; Differentiation; Phosphatase

## 1. INTRODUCTION

*All-trans* retinoic acid (ATRA) induces human myelogenous leukemic HL-60 cells [1,2], as well as fresh acute promyelocytic leukemia blast cells [3,4], to differentiate terminally into mature granulocytes. ATRA binds to specific nuclear receptors (RA receptors), and the ATRA-RA receptor complex possibly regulates the transcription of target genes in a manner similar to that seen with steroid/thyroid hormones [5]. Little is known of intracellular events that initiate the process leading to cessation of proliferation and to terminal differentiation of HL-60 cells, subsequent to ATRA-RA receptor interaction. Protein kinase C activation and modulation of its isozyme expression is thought to play important roles in regulating the response of HL-60 cells to various inducers of differentiation [6]. Although critical substrates for phosphorylation by protein kinase C, which subsequently mediate differentiation, have not been conclusively identified, the regulation of the protein function by phosphorylation necessarily requires protein phosphatases in addition to protein kinases. It is therefore conceivable that protein phosphatases may also be involved in the process of HL-60 cell differentiation. The major serine/threonine protein phosphatase catalytic subunits of mammalian cells comprise four

forms which have been designated as type 1 (PP1), type 2A (PP2A), type 2B (calcineurin) and type 2C, based on a classification system proposed by Cohen [7,8]. PP1 and PP2A do not have an absolute requirement for divalent cations, whereas protein phosphatases type 2B and type 2C are Ca<sup>2+</sup>/calmodulin- and Mg<sup>2+</sup>-dependent, respectively. PP1 and PP2A are known to dephosphorylate many proteins in vitro and are likely to have pleiotropic actions in vivo [8]. Recently, we have reported that okadaic acid [9,10] and calyculin-A [11,12], both potent and specific inhibitors of PP1 and PP2A, augment the granulocytic differentiation of HL-60 cells induced by ATRA, but not the monocytic differentiation induced by 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) [13]. To further elucidate the involvement of PP1 and PP2A in HL-60 cell differentiation, we investigated changes in phosphatase activity and expression patterns of PP1 and PP2A proteins and mRNA expressions of PP2A isoforms during the granulocytic differentiation induced by ATRA.

## 2. MATERIALS AND METHODS

### 2.1. Materials

ATRA and TPA purchased from Sigma Chemical Co. (St. Louis, MO) were dissolved in ethanol (10<sup>-3</sup> M) and dimethylsulfoxide (10<sup>-4</sup> M) respectively, and stored at -20°C with protection from light.

### 2.2. Determination of cell differentiation

Procedures used for the maintenance of HL-60 cells and determination of viable cell counts were as described elsewhere [14]. The extent

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of differentiation was assessed by morphology, the ability to produce superoxide as monitored by the reduction of NBT (Nitroblue tetrazolium, Sigma). For morphological assessment, cytopsin slide preparations of the cells were stained with May-Giemsa and examined under a microscope. The extent of NBT reduction was evaluated as described elsewhere [15].

### 2.3. Preparation of cytosolic fraction and phosphatase activity

All preparative procedures were carried out at 4°C. HL-60 ( $2 \times 10^8$  cells) were homogenized in a buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 0.25 M sucrose and a cocktail of protease inhibitors [14], by a glass to glass Potter-Elvehjem homogenizer. The homogenates were immediately centrifuged at  $1,000 \times g$  for 10 min and the supernatant was centrifuged at  $100,000 \times g$  for 1 h. The resulting supernatant was used as the cytosolic fraction.

$^{32}\text{P}$ -phosphorylated myosin light chains were used as substrates of the phosphatase assay because these are good substrates for both mammalian PP1 and PP2A [16]. Phosphatase activity was determined by the liberation of  $^{32}\text{P}$  from the substrates ( $^{32}\text{P}$ -labelled 20 kDa myosin light chain) at 30°C according to the methods of Pato and Kerc [17]. The extent of dephosphorylation was restricted to below 10%. Under these conditions rates of dephosphorylation were linear with respect to time and enzyme dilution.

### 2.4. Immunoblot analysis

Antisera against PP1 or PP2A was obtained by immunizing a rabbit with the synthesized fragments of PP1 or PP2A [18,19]. HL-60 cells were incubated with ATRA ( $1 \mu\text{M}$ ) or TPA (20 nM), and the reaction was stopped by adding ice-cold trichloroacetic acid (10% final concentration). Acid-precipitated proteins were washed in acetone and subjected to SDS-PAGE using a 12.5% running gel followed by electrophoretic transfer onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was reacted with specific antibodies for PP1 and PP2A, and immunoreactive proteins were stained using the avidin-biotin peroxidase complex method (Vectastain, Vector Laboratories, Burlingame, CA). Quantitative estimation of the level of PP1 and PP2A was carried out densitometrically with a Bio-Rad video-densitometer by scanning the immunoreactive band after immediately photographing the visualized band. The area of an individual peak was measured above background in densitometric tracings and expressed as  $A \times \text{mm}$  [20].

### 2.5. RNA isolation and Northern blot analysis

Total cellular RNA was extracted using the guanidinium isothiocyanate technique [21]. Twenty  $\mu\text{g}$  of total RNA of HL-60 cells was electrophoresed through 0.8% agarose with 18% formaldehyde, and transferred to Nytran membranes (Schleicher and Schuell, Dassel, Germany). After being baked at 95°C for 2 h under vacuum, blots were hybridized at 42°C in 50% formamide,  $5 \times$  Denhardt's solution,  $5 \times$  SSPE ( $1 \times$  SSPE; 0.15 M NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , 0.1 mM EDTA), 0.5% SDS, 200  $\mu\text{g}/\text{ml}$  of denatured salmon sperm DNA, and the  $^{32}\text{P}$ -labelled cDNA probe. The probes used were the full-length cDNA of the rat PP2A $\alpha$  and PP2A $\beta$  [22] and labeled with [ $\gamma$ - $^{32}\text{P}$ ]dCTP by a multiprime labeling system kit (Amersham, Buckinghamshire, UK). Hybridized blots were finally washed with  $0.1 \times$  SSC (standard saline citrate), ( $1 \times$  SSC; 0.15 M NaCl, 15 mM trisodium citrate), 0.5% SDS at room temperature and autoradiographed. Quantification of hybridization was determined by scanning densitometry.

## 3. RESULTS AND DISCUSSION

ATRA suppressed the proliferation of HL-60 cells and induced these cells to differentiate terminally into granulocytes. Morphologic examination of Giemsa-stained cytopsin preparations showed that untreated HL-60 cells retained a blast-like phenotype throughout

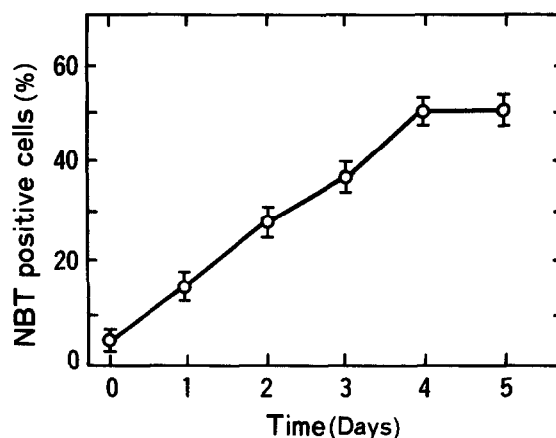


Fig. 1. Time-dependent differentiation of HL-60 cells induced by  $1 \mu\text{M}$  ATRA. Differentiation of HL-60 cells was determined by the NBT reduction test. Each point represents the mean  $\pm$  S.D. from four separate experiments.

all the experiments, whereas the ATRA-treated cells gradually acquired a differentiated morphology, exhibiting a metamyelocyte (26%), or granulocytic (38%) morphology and ability to reduce NBT from 3 to 52% after 5 days of treatment (Fig. 1).

The activity of serine/threonine protein phosphatase in HL-60 cells was assayed in the absence of divalent cations, using  $^{32}\text{P}$ -phosphorylated myosin light chain of smooth muscle as substrate, a good substrate for both mammalian PP1 and PP2A [16]. Alteration of phosphatase activity was examined in cytosolic fractions because phosphatase activity was found to be most abundant in these fractions (approx. 60%) [13]. HL-60 cells were grown in the presence and absence of  $1 \mu\text{M}$  ATRA and harvested for the measurement of phosphatase activity, at various times (Fig. 2). The addition of ATRA to exponentially growing HL-60 cells resulted in a grad-

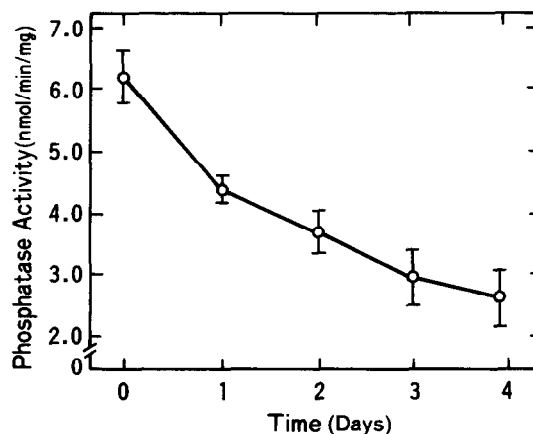


Fig. 2. Time course of protein phosphatase activity during ATRA-induced differentiation of HL-60 cells. HL-60 cells ( $2 \times 10^8$  cells) were treated with  $1 \mu\text{M}$  ATRA, and cytosolic fractions were prepared at the indicated time. Protein phosphatase activity was measured as described in section 2. Each point represents the mean  $\pm$  S.D. from three separate experiments.

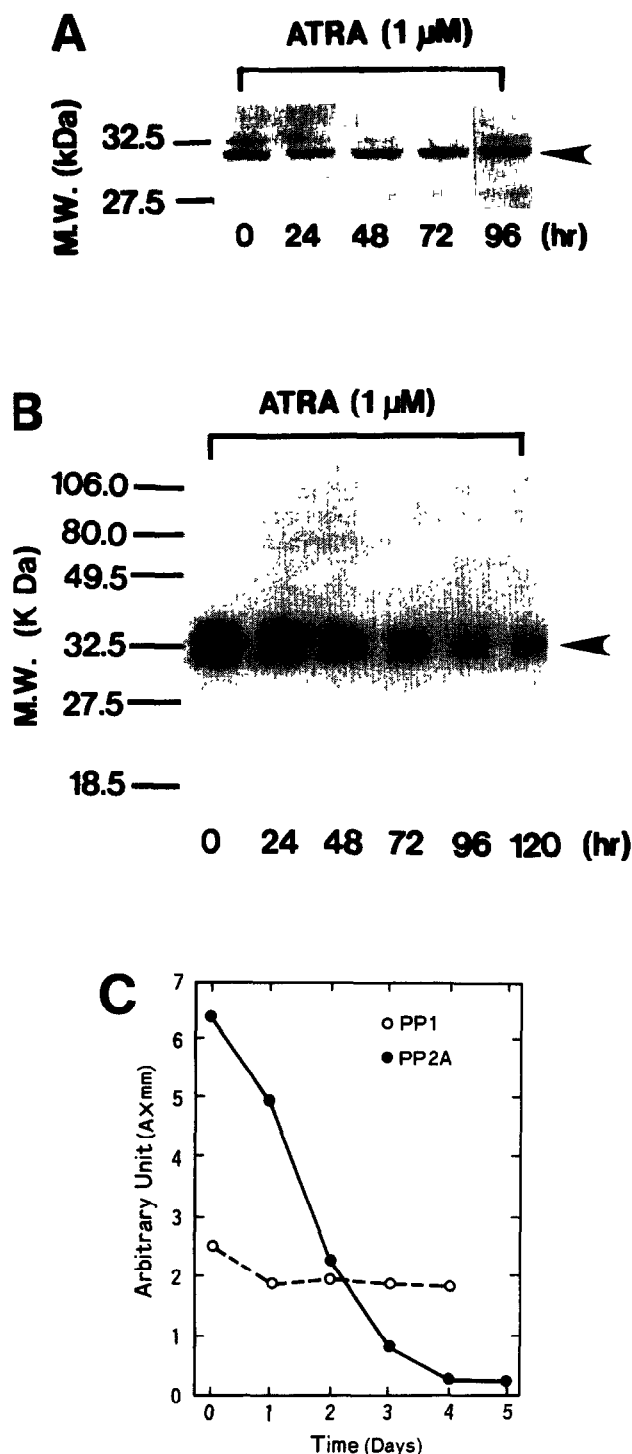


Fig. 3. Time course of changes in immunoreactive PP1 and PP2A during ATRA-induced differentiation of HL-60 cells. HL-60 cells ( $1 \times 10^6$  cells) were treated with 1  $\mu$ M ATRA and the incubation was halted by adding ice-cold trichloroacetic acid at the indicated periods of time below the corresponding lanes. The acid-precipitated proteins were analyzed by immunoblot analysis, using antisera specific for PP1 (panel A), and PP2A (panel B) as described in section 2. Molecular weight standards ( $M_r \times 10^{-3}$ ) are shown on the left. Panel C: graphic display of PP1 (○) and PP2A (●) shown in panel A and panel B, as quantitated by densitometry. Although these data are from one representative experiment, similar results were obtained in two other experiments.

ual decrease in cytosolic phosphatase activity and the activity reached the lowest level (approx. 52% decrease relative to that in untreated HL-60 cells) 3 days after ATRA treatment. Decrease in phosphatase activity appeared to be coincidental with increase in the percentage of NBT-positive cells. These data suggest that the decrease in phosphatase activity may be associated with the ATRA-induced differentiation of HL-60 cells.

To determine whether either PP1 or PP2A or both are decreased after treatment with ATRA, expressions of these proteins in whole cell extracts were investigated by Western blot analysis using polyclonal antibodies specific for PP1 and PP2A catalytic subunits. As shown in Fig. 3, PP2A was detected as a major single immunoreactive band of  $M_r = 33$  kDa and PP1 as a major single band of  $M_r = 32$  kDa, respectively. The time-dependent dramatic decrease in immunoreactive PP2A was evident after the addition of ATRA. Translocation of PP2A from the cytosol fraction to plasma membrane or nuclear fraction was not evident (data not shown). In contrast, there was only a slight decrease in the amount of PP1 (Fig. 3). Thus, the expression of PP1 and PP2A appeared to be separately modulated in the process of ATRA-induced granulocytic differentiation, and the decreased phosphatase activity might be mainly due to a decrease in the expression of PP2A protein. However, the loss of phosphatase activity of HL-60 cells following exposure to ATRA may not be fully explained by a decrease in the expression of PP2A protein and our studies do not exclude the possibility that other factors may influence the phosphatase activity. PP1 and PP2A isolated in various forms from mammalian tissues are composed of catalytic and regulatory subunits [8]. The activity or substrate specificity of the phosphatases may be altered by modification or interaction with regulatory proteins, without change in the level of expression,

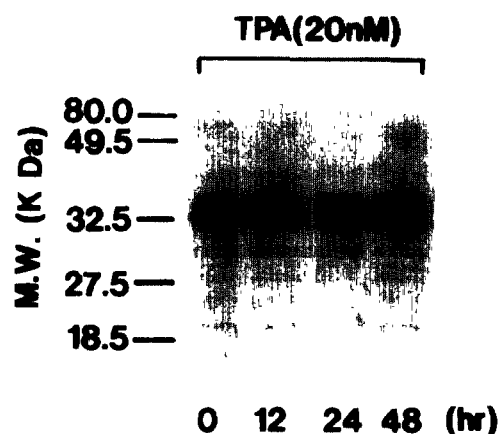
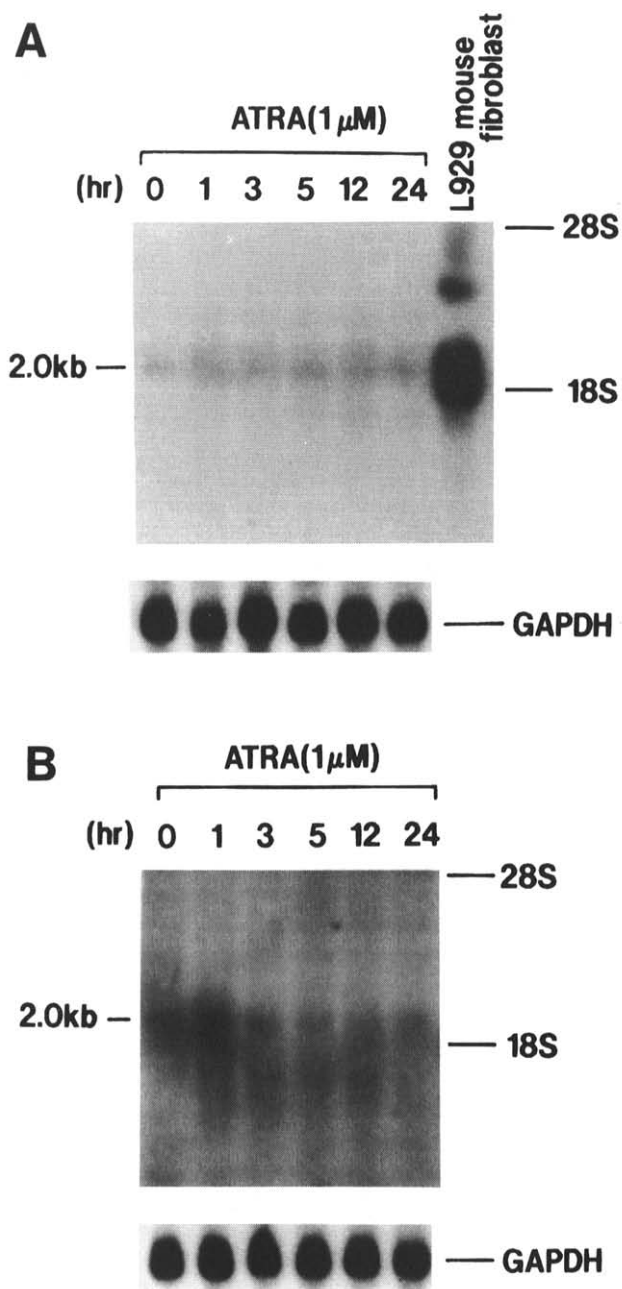


Fig. 4. Time course of changes in immunoreactive PP2A during TPA-induced monocytic differentiation of HL-60 cells. HL-60 cells ( $1 \times 10^6$  cells) were treated with 20 nM TPA for the indicated periods of time below the corresponding lanes. Immunoblot analysis were performed as in section 2. Molecular weight standards ( $M_r \times 10^{-3}$ ) are shown on the left. The result shown is representative of three independent experiments.



although these regulatory proteins remain to be fully characterized. Determination of whether these other subunits have important roles in directing the subcellular location and the modulation of specificity and regulatory behaviour of these enzymes, will be a prerequisite to understanding the control of dephosphorylation processes in HL-60 cells. The down-regulation of PP2A is, at least in part, thought to contribute to the decrease in phosphatase activity during the ATRA-induced differentiation of HL-60 cells. In contrast, the expression of PP2A protein was unaltered in the course of TPA-induced monocytic differentiation as shown in Fig. 4. Treatment of HL-60 cells with 20 nM TPA for 2 days led to acquisition of the phenotype of a mature monocyte.

There are at least two genes encoding isoforms of PP2A, PP2A $\alpha$  and PP2A $\beta$ , identified at various ratios in mammalian tissues [23]. The isoforms of PP2A have a > 97% identity of amino acids and there is currently no information on possible functional differences. To determine whether the ATRA-induced down-regulation of PP2A of HL-60 cells was reflected at the level in RNA transcripts, Northern blot analysis using radiolabeled rat PP2A isoform-specific cDNA probes were made on total cellular RNA obtained from ATRA-treated HL-60 cells (Fig. 5). After hybridization to the PP2A $\beta$  probe, two bands of approx. 2.0 kb and 1.2 kb were observed, and the 2.0 kb mRNA was a major transcript in HL-60 cells (Fig. 5B). In HL-60 cells, the down regulation of the PP2A $\beta$  was evident within 3 h after the addition of ATRA. The level of PP2A $\beta$  mRNA was further decreased to approx. one-third of the basal level of untreated HL-60 cells within 5 h, as normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression (Fig. 5C). Decreased PP2A $\beta$  expression continued throughout the course of ATRA treatment. Rehybridization of these blots with the GAPDH cDNA probe confirmed that relatively equal amounts of RNA were loaded in each lane. The change in PP2A $\beta$  mRNA was complete within 5 h, while the decrease in immunoreactive PP2A occurred over 2 days, suggesting

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Fig. 5. Time course of PP2A isotype PP2A $\alpha$  and PP2A $\beta$  mRNA expression in HL-60 cells stimulated with ATRA. HL-60 cells ( $2 \times 10^6$  cells) were treated with 1  $\mu$ M ATRA. Total RNA (20  $\mu$ g) was isolated from HL-60 cells at the indicated periods of time below the corresponding lanes. Northern blots of total RNA were hybridized to  $^{32}$ P-labeled PP2A $\alpha$  (panel A) and PP2A $\beta$  (panel B) cDNA probes. The same RNA blots were hybridized to GAPDH cDNA probe for assessment of RNA quantities in each lane (bottom). The positions of the 28 S and 18 S ribosomal RNAs are indicated. Panel C: graphic representation of relative intensity of hybridization signals of PP2A $\alpha$  (▲) and PP2A $\beta$  (■) obtained from densitometric scanning of the autoradiogram pictured in panels A and B. The ratio of PP2A $\alpha$  and PP2A $\beta$  specific signals to the GAPDH signal was determined for each time point following ATRA addition and the resulting value is designated as relative intensity. Similar results were obtained in two independent experiments.

the long half-life of PP2A protein. The down-regulation of PP2A $\beta$  mRNA expression clearly preceded the ATRA-induced HL-60 cell differentiation as monitored by NBT reduction. The PP2A $\alpha$  probe also detected a major band of 2.0 kb and minor band of 3.0 kb in L929 mouse fibroblast RNA (Fig. 5A). In HL-60 cells, the 2.0 kb transcript was weakly expressed, and the expression of the PP2A $\alpha$  2.0 kb transcript was relatively unaltered by ATRA treatment. Other investigators found that the expression of PP2A $\alpha$  mRNA is also unaltered after addition of the monocyte inducer TPA to HL-60 cells [24], although expression of PP2A $\beta$  mRNA was not examined. The present data suggest that the two isoforms of PP2A could be differently regulated during ATRA-induced differentiation.

A growing body of evidence suggests important roles of protein phosphatases as well as protein kinases in the growth control of cells [25]. Differentiation of HL-60 cells by chemical agents such as ATRA, 1,25-dihydroxyvitamin D<sub>3</sub> and phorbol diester is always accompanied by withdrawal from the cell cycle [2]. The expression of many genes are known to be regulated during HL-60 cell differentiation [26]. The change in PP2A $\beta$  mRNA may be the result of, rather than the cause of, differentiation. However, we reported that two potent inhibitors of PP1 and PP2A, okadaic acid and calyculin-A, augment ATRA-induced granulocytic differentiation of HL-60 cells, but have no effect on the monocytic differentiation induced by phorbol diester [13]. PP2A is thought to regulate multiple functions in vivo, including several metabolic pathways, protein synthesis, DNA replication and the cell cycle [8]. Consistent with this hypothesis, abundant transcripts of PP2A $\beta$  are present in untreated HL-60 cells, and it is of interest that transcripts of PP2A $\beta$  are decreased at the initial steps of ATRA-induced differentiation. It is possible that down-regulation of PP2A may be involved in ATRA-induced differentiation of HL-60 cells toward granulocytes. In ongoing studies, we are examining modulation/expression of phosphatases by other differentiation inducers in order to establish the regulatory roles of phosphatases in cellular signalling of HL-60 cell differentiation.

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