Downregulation of GATA-1 Expression During Phorbol Myristate Acetate—Induced Megakaryocytic Differentiation of Human Erythroleukemia Cells

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Phorbol myristate acetate (PMA) induces the expression of megakaryocyte and/or platelet proteins during terminal differentiation of human erythroleukemia (HEL) cells. However, it is not established whether megakaryocytic differentiation is accompanied by the downregulation of the major erythroid transcription factor GATA-1 and the concomitant loss of the erythroid cytoporph. Studies of the molecular mechanism of PMA-induced differentiation in HEL cells showed that when HEL cells are treated with PMA, they dramatically decrease the expression of the erythroid-specific gene glycophorin A at the mRNA level but apparently not at the steady-state protein level. In addition, a gel mobility shift assay was used to demonstrate that GATA-1, a major erythroid transcription factor normally present at high levels in HEL cells is downregulated after treatment with PMA. In contrast, the DNA-binding activities of transcription factors AP-1 and SP-1 are upregulated by PMA treatment of HEL cells. Furthermore, Northern blot analysis shows that PMA also downregulates the steady-state level of GATA-1 mRNA in HEL cells. The coordinated negative regulation of glycophorin A mRNA and GATA-1 expression after PMA treatment suggests that downregulation of GATA-1 expression may be partially responsible for the loss of the erythroid phenotype during megakaryocytic differentiation. The reported data also suggest that GATA-1 activity may not be essential for obtaining megakaryocytic phenotype during terminal differentiation in HEL cells.

A PRIMARY DEFECT of leukemia cells is their inability to differentiate into mature blood cells. It has been observed that the genetic defect responsible for the leukemic phenotype is at least partially reversible.1,4 Several erythroid:blastic and megakaryoblastic cell lines have been shown to differentiate toward megakaryocytic phenotype following phorbol myristate acetate (PMA) treatment.4-8 In the past years, considerable study has been focused on dissecting the molecular events responsible for leukemia cell differentiation.9-15 A major biochemical effect of PMA is the activation of protein kinase C (PKC), indicating the involvement of a cytoplasmic serine/threonine kinase during leukemia cell differentiation. DNA-binding proteins or transcription factors are also known to be essential components mediating differentiation of many leukemia cells.9-12,16 For example, the tumor necrosis factor alpha (TNF-α)-induced monocyte differentiation of acute myeloblastic leukemia cells is accompanied by a drop in the c-src mRNA expression.16 Also, downregulation of c-src with an antisense oligodeoxynucleotide induces hemoglobin synthesis in Rauscher erythroleukemia cells.18 Recently, it has been reported that antisense sequence-mediated suppression of the stem cell leukemia gene, a member of the helix-loop-helix DNA-binding proteins, results in enhanced spontaneous erythroid differentiation in K562 cells.11 In addition, it is well known that PMA is an inducer for the transcription factor AP-1, a c-jun and c-fos heterodimer.10,17,18 These nuclear protooncogene products are normally expressed in leukemia cells of various lineages. However, it is unclear how the alteration of the expression of these genes affects lineage-specific differentiation of a particular leukemia cell line. One hypothesis states that modulation of the expression or activities of lineage-restricted factors and/or in particular lineage-restricted transcription factor(s) may be a key step in regulating leukemia cell differentiation.

GATA-1 is a lineage-specific transcription factor predominantly expressed in erythroid cells, and expression of genes characteristic of erythroid lineage are under its control.19-21 GATA-1 gene expression appears to be developmentally controlled, and its expression correlates with the complex patterns of globin gene expression. For example, in a developing mouse embryo, GATA-1 expression precedes and parallels the levels of globin mRNA.22 In addition, in Xenopus laevis embryogenesis, GATA-1 is expressed as early as the gastrula stage. Expression of GATA-1 precedes any globin expression and blood island formation, suggesting that GATA-1 may be also involved in commitment of the mesoderm to form hematopoietic organs.23 It has been demonstrated by the gene-disruption approach that GATA-1 is absolutely required for erythroid development.24 However, it remains unclear how GATA-1 gene expression or its activity is regulated during hematopoiesis or erythroid commitment and differentiation. In addition, although mature megakaryocytes express the GATA-1 factor25,26 and the 5' regulatory region of several genes characteristic of the megakaryocytic lineage contain the GATA factor-binding motif,27 gene-disruption experiments have not documented any role for the transcription factor in megakaryopoiesis.24 Because the HEL cell line can be induced by PMA to express several megakaryocytic-specific genes and undergo a megakaryocytic terminal differentiation,3 we asked whether the PMA-induced megakaryocytic differentiation is negatively correlated with erythroid marker gene expression. We observed that following PMA treatment, glycophorin A expression in HEL cells was dramatically downregulated at the mRNA level but not at the steady-state protein level, and that HEL cells constitutively expressed GATA-1 mRNA and high levels of cytoplasmic and nuclear GATA-1 activity that were significantly down-

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regulated during PMA-induced megakaryocytic differentiation.

MATERIALS AND METHODS

Materials. HEL and K562 cell lines and cDNAs coding for human glycophorin A and platelet-derived growth factor β chain (PDGF-β) were purchased from American Type Culture Collection (Rockville, MD). Acrylamide, methylene-bisacrylamide, and protein-quantitating reagents were obtained from Bio-Rad Laboratories (Richmond, CA). Oligonucleotides containing the GATA-1-binding motif were synthesized by Oligo Etc Inc (Wilsonville, OR) and dissolved in TE buffer (10 mmol/L tris-hydroxymethylaminomethane [TRIS-HCl], pH 7.5, 1 mmol/L EDTA) at 2 mg/mL. Oligonucleotides containing the AP-1- or SP-1-binding site were purchased from Promega (Madison, WI). Poly(d1-dC), leupeptin, aprotinin, and phenylmethylsulfonyl fluoride (PMSF) were from Boehringer-Mannheim (Indianapolis, IN). Monoclonal antglycophorin IgG and antiglycoprotein IIb (gpIIb) IgG were obtained from AMAC, Inc (Westbrook, ME). Goat-anti-mouse IgG conjugated with alkaline phosphatase and the phosphatase substrates, 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium chloride (NBT), were purchased from Gibco/BRL (Grand Island, NY). Acrylamide, methylene-bisacrylamide, and protein-quantitating reagents were obtained from Bio-Rad Laboratories (Richmond, CA). The [α-32P]dCTP was purchased from Du Pont/New England Nuclear (Wilmington, DE). Fuji RX x-ray film was purchased from Fisher Scientific, Inc (Pittsburgh, PA). GATA-1 cDNA was kindly provided by Dr Stuart Orkin, the Children’s Hospital, Boston, MA. Hemin was purchased from Eastman Kodak (Rochester, NY). Antibody anclamycin (ACL) was a gift from Bristol-Myers laboratory (Wallhingford, CT) and was dissolved in 100% EtOH and diluted at least 1,000-fold when added to culture medium.

Cell culture. HEL and K562 cells were grown in suspension culture in RPMI 1640 medium containing 10% fetal calf serum and penicillin (100 μg/mL) and streptomycin (50 μg/mL). Cells were passed twice each week, seeded at a concentration of 1 to 2 × 10^6/mL. For treatment with various agents, exponential growing HEL or K562 cells were collected (1,200 g × 10 minutes) and resuspended in fresh culture medium containing PMA (100 nM), okadac acid (5 mM), hemin (60 μM), and ACL (60 μM) at a density about 5 × 10^6/mL and cultured for 2 days.

RNA isolation and dot blotting. Total RNA was isolated from treated HEL or K562 cells using the guanidine/HCl procedure.28 Equal amounts (10 μg) of total RNA were sequentially diluted by a factor of 2 using buffer conditions as described,28 and the diluted RNA was directly applied to nitrocellulose using a dot blot apparatus. Dot blots were hybridized overnight with a glycophorin A or PDGF-β cDNA probe labeled with 32P, washed after hybridization, and autoradiographed. Dot blots were quantitated by densitometric scanning. Northern blotting analysis was performed as described.31 Northern blots were hybridized with a GATA-1 cDNA probe labeled with 32P, washed after hybridization, and autoradiographed. Hybridization and washing conditions were as described.30

Cytosolic and nuclear protein extraction. A small-scale isolation method was adapted from the procedure described by Nicolas et al.28 Cells (1 × 10^7) were collected (2,000 g × 10 minutes) and rinsed once in 1 mL of phosphate-buffered saline (PBS). Cell pellets were then resuspended in 1 mL of lysis buffer (10 mmol/L TRIS-HCl, pH 7.5; 5 mmol/L MgCl2, 0.5% [vol/vol] triton × 100, 0.1% [vol/vol] NP-40) and left on ice for 5 minutes. Supernatants (1:2,000 g × 5 minutes) of cell lysates was discarded and the nuclear fraction resuspended in twice the pellet volume of low-salt buffer (20 mmol/L HEPES, pH 7.9; 25% [vol/vol] glycerol; 1.5 mmol MgCl2; 20 mmol/L KCl, 0.2 mmol/L EDTA; 0.2 mmol/L PMSF; 0.5 mmol/L dithiothreitol [DTT]). Following slow addition of one pellet volume of high-salt buffer (low-salt buffer with 1.2 mol/L KCl) to the resuspended nuclei, nuclear proteins were extracted on ice for 30 minutes. Nuclear suspension was centrifuged (12,000 g × 15 minutes) and the supernatant collected and dialyzed (dialysis tubing exclusion size 3,500 dalton) in dialysis buffer (20 mmol/L HEPES, pH 7.9; 20% [vol/vol] glycerol; 100 mmol/L KCl; 0.2 mmol/L EDTA; 0.2 mmol/L PMSF; 0.5 μg/mL aprotinin; 0.5 μg/mL leupeptin; 0.5 mmol/L DTT) for 3 hours. After centrifuging (12,000 g × 15 minutes), the soluble fraction was measured for protein concentration with the Bradford procedure.32 Cytosolic protein isolation was carried out as follows: Cells (5 to 10 × 10^6), rinsed once in 10 mL of the PBS saline, were resuspended in 0.5 mL of hypotonic buffer (10 mmol/L HEPES/KOH, pH 7.4; 10 mmol/L KCl, 1.5 mmol/L MgCl2; 0.1 mmol/L EGTA; 0.5 mmol/L PMSF; 1 μg/mL leupeptin; 0.1% [vol/vol] NP-40) and kept on ice for 10 minutes. One volume of 10 mmol/L CH3CN was added by homogenizing (16 strokes) and the cytosolic fraction (12,000 g × 10 minutes) was saved and dialyzed as above. Typically, 10 μg of nuclear proteins or 30 μg of cytosol proteins was used for each gel mobility shift assay.

Band shift assays. Band shift assays were performed essentially as described.25 Briefly, for each assay, nuclear (10 μg) or cytosolic (30 μg) protein extracts were incubated in a total volume of 15 μL of a buffer mixture (10 mmol/L HEPES, pH 7.9; 10% [vol/vol] glycerol; 50 mmol/L KCl; 0.1 mmol/L EDTA; 0.1 mmol/L PMSF; 0.25 mmol/L L DTT; 1 mmol/L MgCl2; 2 μg bovine serum albumin; 0.5 μg poly(dI-dC); 2.4 × 10^6 cpm of a 32P-labeled oligonucleotide with a specific activity of 1 × 10^6 cpm/μg). The 32P DNA/protein complexes were resolved from unbound DNA probe by electrophoresis in 4% polyacrylamide gels (PAGE) run in a low ionic strength buffer (8 mmol/L TRIS-HCl, pH 7.9; 3.3 mmol/L sodium acetate, pH 7.9; 1 mmol/L EDTA). The gels were dried under vacuum followed by autoradiography using Fuji RX x-ray films. The oligonucleotide used for detecting GATA-1 activity was a 34-mer DNA fragment containing the GATA-1-binding site from mouse α-globin promoter (5′GGGAGGTGGATTCAGGCAACTGATAAGGATTCCCAG-3′) as initially purified.29 Two extra guanine residues were added at the 5′ end to serve as a template for labeling with 32P-dCTP.

32P-labeling of oligonucleotide probe. Complementary oligonucleotides (40 μg/mL in 250 mmol/L NaCl) were denatured at 95°C for 3 minutes followed by slow cooling to room temperature. 32P-labeling of the annealed oligonucleotide fragments (200 ng) was achieved by a filling-in reaction with Klenow fragment of DNA polymerase I in a 100 μL reaction mixture (7 mmol/L TRIS-HCl, pH 7.5; 7 mmol/L MgCl2; 50 mmol/L NaCl; 100 μCi α-32P-dCTP). Radioabeled DNA probe was separated from free 32P-dCTP on a 12% native polyacrylamide gel as described22 and then eluted from polyacrylamide gel by overnight incubation in 1 mL of elution buffer (0.5 mol/L ammonium acetate; 10 mmol/L magnesium acetate; 1 mmol/L EDTA; 0.1% [wt/vol] sodium dodecylsulfate [SDS]) with shaking (100 rpm). The eluted oligonucleotide probe was extracted three times with phenol/chloroform, precipitated by addition of one volume of 10 mol/L sodium acetate and three volumes of 100% ethanol, and the precipitates were collected by centrifugation (12,000 g × 15 minutes) and redissolved in TE buffer. cDNA fragments corresponding to glycophorin A, PDGF-β, and GATA-1 were labeled with 32P using the random primer procedure.34

SDS-PAGE and Western blotting. HEL and K562 cells were treated with or without PMA for 48 hours and lysed in lysis buffer (10 mmol/L TRIS-HCl, pH 7.4; 10 mol/L NaCl; 3 mmol/L MgCl2; 0.5% [vol/vol] NP-40 detergent). Cleared lysates (12,000 g × 10 minutes) were fractionated on 12% SDS-polyacrylamide gels.35 The fractionated proteins were transferred onto nitrocellulose membrane as described.36 The nitrocellulose blot was incubated with mouse monoclonal antglycophorin A or anti-gpIIb IgG (2 μg/mL) in tris-buffered saline (TBS/T) (10 mmol/L TRIS-HCl, pH 7.5; 250 mmol/L NaCl) containing 0.1% 0.1% polysorbate 20 (Tween 20), ICI Americas,
Fig 1. Total RNA was isolated from HEL cells treated with vehicle ethanol (lane 1), PMA (lane 2), okadaic acid (lane 3), PMA plus hemin (lane 4), okadaic acid plus hemin (lane 5), and hemin plus ACL (lane 6). Equal amounts (10 μg) of the isolated RNA were sequentially diluted by a factor of 2, blotted onto nitrocellulose membrane, and probed with glycophorin A cDNA (panel A) or PDGF-β cDNA (panel B) labeled with 32P. PMA, okadaic acid, hemin, and ACL were added to cell culture medium at final concentrations of 100 nmol/L, 5 nmol/L, 60 pmol/L, and 60 nmol/L, respectively.

Wilmington, DE) for 1 hour, rinsed with TBS/T buffer, and then incubated with goat-anti-mouse IgG conjugated with alkaline phosphatase. After further washing with TBS/T and TBS, the blot was developed by incubation with 5-bromo-4-chloro-3-indolylphosphate P-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) as recommended by the supplier.

RESULTS

PMA downregulating glycophorin A expression at the mRNA level. It has been shown that γ-globin gene expression is negatively regulated during megakaryocytic differentiation of K562 cells treated with PMA and that this downregulation of γ-globin is caused by a decreased rate of transcription and an enhanced rate of the mRNA turnover. On the other hand, PMA-induced megakaryocytic differentiation of HEL cells is not accompanied by an alteration in glycophorin A antigen levels. We wondered if the failure to detect any change in glycophorin A antigen levels was due to the relatively slow turnover rate of the protein. Therefore, we measured the steady-state mRNA level of glycophorin A in HEL cells treated with or without PMA. Equal amounts of total RNA isolated from HEL cells using the guanidine/HCl procedure was analyzed by dot blotting. The RNA dot blot was probed with a 32P-labeled cDNA fragment of glycophorin A, washed as described in the Materials and Methods section, and autoradiographed. The results (Fig 1A) showed that compared with the untreated control (lane 1), PMA (lane 2), but not okadaic acid (lane 3), dramatically downregulated glycophorin A mRNA expression. Hemin plus ACL is known to increase erythroid differentiation for K562 cells. We observed that in the presence of ACL, hemin approximately doubled glycophorin A expression in HEL cells (lane 6) as quantitated by densitometric scanning, which is similar to that observed with K562 cells (Fig 2A, lane 6). However, hemin did not affect the PMA-induced downregulation of glycophorin A expression (lane 4), nor did it enhance glycophorin A expression in the presence of okadaic acid (lane 5). Although PMA has been shown to induce a megakaryocytic differentiation of HEL cells, it did not induce expression of PDGF-β, a megakaryocytic/platelet marker (Fig 1B, lane 2). Consistent with previous reports, PMA caused a fourfold induction in PDGF-β expression in K562 cells.

Fig 2. Total RNA was isolated from K562 cells treated with vehicle ethanol (lane 1), PMA (lane 2), okadaic acid (lane 3), PMA plus hemin (lane 4), okadaic acid plus hemin (lane 5), and hemin plus ACL (lane 6). Equal amounts (10 μg) of the isolated RNA were sequentially diluted by a factor of 2, blotted onto nitrocellulose membrane, and probed with glycophorin A cDNA (panel A) or PDGF-β cDNA (panel B) labeled with 32P. PMA, okadaic acid, hemin, and ACL were added to cell culture at final concentrations of 100 nmol/L, 5 nmol/L, 60 μmol/L, and 60 nmol/L, respectively. Arrow indicates the dot used for orientation of the blot and it is the second concentration of lane.
(Fig 2B, lane 2), whereas glycophorin A expression was essentially turned off in PMA-treated cells (Fig 2A, lane 2).

We further analyzed glycophorin A expression at the protein level by Western blotting. Equal amounts of protein lysates from HEL or K562 cells treated with or without PMA were fractionated on 12% SDS-polyacrylamide gels and the fractionated proteins transferred onto nitrocellulose membrane. The protein blot was then probed with mouse monoclonal anti-human glycophorin A IgG, followed by probing with goat-anti-mouse IgG conjugated with alkaline phosphatase. Both K562 and HEL cells expressed considerable amounts of glycophorin A antigen (Fig 3A, panel B, arrow G) and that the levels of glycophorin A antigen are not significantly affected by PMA treatment (Fig 3B, lanes 2 and 4) compared with those of the untreated controls (lanes 1 and 3). However, when the protein lysates were analyzed for the megakaryocytic marker gpllb expression, we found that K562 cells did not express any detectable levels of this antigen before (Fig 3A, lane 1) or after (lane 2) PMA treatment. On the other hand, HEL cells constitutively express gpllb antigen and levels of antigen expression were enhanced following PMA treatment (Fig 3A, lane 4) compared with that of the untreated control (lane 3).

**PMA downregulates GATA-I expression in HEL cells.** We showed that PMA-induced megakaryocytic differentiation was characterized by the downregulation of the erythroid marker glycophorin A mRNA expression (Fig 1) and by an increased expression of megakaryocytic antigen glycoprotein Iib (Fig 3A). In addition, because glycophorin A and other erythroid-specific genes contain a GATA motif(s) at their 5' flanking region,\(^{21,37}\) and because the GATA-I transcription factor is involved in regulating expression of these genes,\(^ {21}\) we asked whether the downregulation of glycophorin A expression in HEL cells by PMA was coupled with a reduction in GATA-I DNA-binding activity and/or its mRNA expression. Nuclear proteins from HEL cells treated with or without PMA were extracted and equal amounts (10 \(\mu\)g) of the extracted proteins were analyzed for GATA-I activity with a gel mobility shift assay. It was observed (Fig 4) that PMA treatment caused a significant reduction in nuclear GATA-I activity (lane 6) compared with that of the untreated control (lane 5). This detected GATA-I activity is specific, as evidenced by homologous (lane 8) and heterologous competition (lane 7). For internal controls, PMA-treated and the control nuclear extracts were analyzed for transcription factor AP-1 and SP-1 activities. It was observed that there was no detectable levels of AP-1 activity in the untreated HEL cells (lane 1), and following PMA treatment AP-1 activity was dramatically increased (lane 2), which is consistent with observations that PMA is a potent activator of AP-1 in many cell types.\(^{15,18}\) On the other hand, there existed a basal level of SP-1 activity in the untreated HEL cells (lane 3), and PMA treatment caused a significant increase in SP-1 activity (lane 4). Because K562 cells express high levels of GATA-I,\(^ {19}\) we used the nuclear extracts of K562 cells as another control (lane 9) to demonstrate authenticity of the detected GATA-I activity in HEL cells.

The GATA-I factor has been characterized as a nuclear protein that participates in regulating erythroid specific gene expression during erythroidic and perhaps megakaryocytic differentiation. However, we have recently observed that this transcription factor is also present in a relative high level in cytosol of K562 cells and the detected cytosolic GATA-I activity is not the result of nuclear protein contamination, as determined by tracking other protein markers in both cytosolic and nuclear compartments (W. Dai, unpublished data, June 1992). We asked whether the downregulation of nuclear GATA-I in HEL cells after PMA treatment was simply caused by the translocation of GATA-I. To answer the question, the cytosolic fraction of HEL cells treated with or without PMA was isolated and equal amounts (30 \(\mu\)g) of total proteins were analyzed on a 12% SDS-polyacrylamide gel. The fractionated proteins were then transferred to a nitrocellulose membrane and probed for gpllb (panel A) or glycophorin A (panel B) expression with a specific monoclonal IgG. Arrow indicates the position of gpllb or glycophorin A, respectively. PMA was added to a final concentration of 100 nmol/L.
Fig 4. HEL cells were treated with vehicle or PMA (100 nmol/L) and nuclear proteins were extracted as described in Materials and Methods section. Equal amounts (10 µg) of extracted proteins were analyzed for activities of AP-1 (lanes 1 and 2), SP-1 (lanes 3 and 4), or GATA-1 (lanes 5 to 8) with gel mobility shift assay. Lanes 1, 3, and 5 represent DNA-binding activities in vehicle-treated HEL cell extracts. Lanes 2, 4, and 6 to 8 represent DNA-binding activities in PMA-treated cell extracts. Lane 9 represents GATA-1 activity detected in K562 cell nuclear extracts (10 µg). Lanes 7 and 8 represent DNA binding activity in the presence of 100-fold molar excess of the unlabeled 34-bp DNA fragment (lane 8, homologous competition) or an unrelated DNA fragment of similar size (lane 7, heterologous competition).

Fig 5. (A) HEL cell cytoplasmic (lanes 1 and 2) or nuclear (lane 3) proteins were isolated from HEL cells treated with vehicle (lanes 1 and 3) or PMA (lane 2) as described in the Materials and Methods section and analyzed for GATA-1 activity with gel mobility shift assay. Arrow indicates the position of GATA-1. (B) HEL cell cytoplasmic (lane 1) or nuclear (lanes 2 to 4) proteins were isolated and analyzed for SP-1 activity with gel mobility shift assay. Arrow indicates the position of SP-1. Lanes 3 and 4 represent DNA-binding activities in the presence of 100-fold molar excess of unlabeled DNA fragment (lane 3, homologous competition) or an unrelated DNA fragment of similar size (lane 4, heterologous competition).
of many transcription factors, we wondered if PMA downregulated only GATA-1 activity in HEL cells. We next determined if PMA also affected steady-state levels of GATA-1 mRNA. Total RNA (15 μg) isolated from HEL cells treated with or without PMA for 2 days were fractionated on a formaldehyde agarose gel and transferred to a nitrocellulose membrane. The RNA blot was then hybridized with a GATA-1-specific cDNA probe labeled with 32P, washed following hybridization, and autoradiographed. It was shown (Fig 6) that HEL cells constitutively expressed considerable levels of GATA-1 mRNA (lane 1) that were enhanced in the presence of hemin plus ACL (lane 3). However, in the presence of PMA, the GATA-1 mRNA level was significantly reduced (lane 2), correlating very well with the downregulated GATA-I activity in HEL cells. We next determined if PMA also affected steady-state levels of GATA-I mRNA. Total RNA fractionated on a formaldehyde agarose gel and stained with ethidium bromide before being transferred to the nitrocellulose membrane, indicating that approximately same amounts of RNA were used for analysis.

**DISCUSSION**

Several human erythroblastic or megakaryocytic leukemia cell lines can be induced by PMA to acquire multiple markers of the megakaryocyte/platelet phenotype. The induction of these marker genes is accompanied by an increase in their cytoplasmic content and cause cells to exhibit features of morphologic maturation. In the present study, we have shown that in HEL cells, the increased expression of megakaryocytic marker (Fig 3) is associated with negative expression of both the glycoporphin A mRNA (Fig 1) and the GATA-I mRNA and its DNA-binding activity (Figs 4, 5). Lanes 4 to 6 were total RNA fractionated on a formaldehyde agarose gel and stained with ethidium bromide before being transferred to the nitrocellulose membrane, indicating that approximately same amounts of RNA were used for analysis.

**Fig 6.** HEL cells were cultured in presence of vehicle ethanol (lane 1), PMA (lane 2), or hemin plus ACL (lane 3) for 2 days. Equal amounts (15 μg) of total RNA isolated from cells with various treatments were analyzed for GATA-1 mRNA expression using the Northern blotting method. Arrow indicates the position of GATA-1. Lanes 4 to 6 (vehicle, PMA, and hemin plus ACL, respectively) represent total RNA fractionated on a formaldehyde gel stained with ethidium bromide.
karyocytes, the low levels of GATA-1 mRNA and its DNA-binding activity in HEL cells undergoing megakaryocytic differentiation may mediate the expression of genes shared by these two lineages.

It is interesting to observe that GATA-1 expression and its DNA-binding activity is inversely correlated with that of AP-1 activity during HEL cell differentiation (Fig 5). It has been long speculated that a relationship exists between these two transcription factors in regulating erythroid gene expression because the GATA motif and 12-o-tetradecanoyl-phorbol-13-acetate (TPA)-responsive cis-acting element (TRE) are consistently present at loci known to be important for establishing the erythroid program. Recently, in vivo dimethyl sulfate footprinting analysis by Orkin’s group showed that a single AP-1/NF-E2 site as well as four sites of GATA within the hypersensitive site 3 of the &globin locus control region were recognized by protein factors present in erythroid cells, strongly suggesting that the AP-1 and GATA-1 are essential trans-acting factors involved in regulating globin gene expression during erythroid development. Because NF-E2 is not proteolytic product of AP-1, and because AP-1 activity is upregulated during megakaryocytic differentiation of HEL cells (Fig 4, lane 2), we propose that NF-E2 is one of the essential factors for upregulating erythroid-specific gene expression, and that AP-1 may compete for AP-1/NF-E2 site with NF-E2 and negatively regulate their gene expression.

Consistent with previous studies, we did not observe a significant decrease in steady-state levels of glycophorin A antigen in HEL cells treated with PMA. This relatively constant level of glycophorin A protein is very likely caused by its high expression and a slow turnover rate of the antigen in HEL cells. On the other hand, one predicts that the PMA-induced decrease in the glycophorin A mRNA level should be coupled with a drop in the rate of new protein synthesis. In fact, using metabolic labeling with [35S]methionine and immunoprecipitation, it has been demonstrated that a threefold reduction is observed in glycophorin A biosynthesis in K562 cells treated with PMA for only 4 hours. A similar reduction is also found in glycophorin A protein synthesis in PMA-treated HEL cells.

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Downregulation of GATA-1 expression during phorbol myristate acetate-induced megakaryocytic differentiation of human erythroleukemia cells

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