

An Efficient Strategy to Identify Early TPA-Responsive Genes During Differentiation of HL-60 Cells

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We have adopted a special experimental strategy to identify early responsive genes during 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced macrophage-like differentiation of human myeloid leukemia cells (HL-60). This was performed in cells that were synchronized by nocodazole and treated with TPA in the presence of a protein synthesis inhibitor, cycloheximide, to prevent activation of secondary targets and therefore increase the probability of early transcripts in total RNA pool. The expression alteration was analyzed by microarray and the selection criteria of candidate genes were adjusted by real-time PCR validation to increase its reliability. Finally, 56 genes were identified as early TPA-responsive genes in this multiscreening step approach. Furthermore, upregulation of three candidate genes (*NFIL3*, *SKIL*, and *JMJD3*) was shown to be dosage and time dependent with TPA treatment and was found to be directly regulated by TPA through PKC-dependent signaling. These results revealed that our screenings provide a useful and efficient approach to identify early TPA-responsive genes and these genes might involve the regulation of TPA-induced differentiation program of HL-60 cells as primary targets.

Key words: Early responsive gene; TPA; Cycloheximide; Differentiation; Microarray

INTRODUCTION

The human myeloid leukemia cell line, HL-60, has been widely investigated as a model for studying the genetic and biochemical factors regulating both cell growth and differentiation. HL-60 cells undergo macrophage-like differentiation in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA or PMA) treatment (32,33), and this effect is mainly initiated through PKC signals (24,45). This differentiation program transcriptionally regulates specific target genes and induces phenotype changes in morphology, immunophenotype, and cellular maturation.

Previous studies using molecular biology approaches have identified a number of genes showing altered expression during TPA-triggered macrophage-like differentiation of HL-60 cells, such as *EGR1* (18) and the *JUN/FOS* superfamily (1), some of them might be essential for the onset of differentiation in HL-60 cells (48). With the advance of microarray that can monitor the expression of a large number of genes and the complex gene expression patterns, recent studies have been able to systematically survey cellular transcriptional regulation in TPA-induced differentiating HL-60 cells (36,44,49).

However, many microarray experiments have not

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clearly distinguished between the primary (direct) and secondary (indirect) targets of TPA. In addition, expression of mRNAs for primary target genes is often expressed at low levels or shows only slight alterations in their expression levels. It is difficult to detect these critical genes because of the limitations of current fluorescence detection system in microarray, and a large number of highly expressed secondary transcripts might mask the low-level signals of primary targets in microarray analysis (9,15,47). An alternative approach is urgently required to study such important primary genes in the transcriptome. By identifying the primary targets of TPA and using these to examine their prospective downstream target genes, it is possible to better construct transcriptional regulatory networks that link key factors to the biological function that they involve.

To overcome these problems of previous microarray study in TPA-induced cell differentiation as mentioned above, we have adopted a new strategy to discover primary TPA target genes using the HL-60 cell differentiation system. Here we have identified 56 early responsive genes as candidate genes directly upregulated by TPA with this new screening strategy. In addition to previously recognized TPA or myeloid differentiation-related genes with high expression levels, we additionally identified 32 more genes with minor changes in their expression levels and have further validated some of these genes that were transactivated by TPA directly through PKC signaling. Taking all data together, our multiscreening strategy identified the additional primary TPA target genes, which can serve as another base for better understanding the role of TPA during the differentiation of HL-60 cells.

MATERIALS AND METHODS

Cell Culture and Induction of Differentiation

HL-60 cells were cultured at a density of 2×10^5 cells/ml with RPMI-1640 (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) (GIBCO-BRL) in a humidified atmosphere of 5% CO₂ at 37°C. To induce macrophage-like differentiation, HL-60 cells were seeded at a density of 2×10^5 cells/ml and treated with 32 nM TPA (Sigma, St. Louis, MO, USA) for the indicated period; the control group was treated with vehicle (only 0.05% DMSO). Where indicated, 1 μ M Go6976 (classic PKC inhibitor) was added to culture media at 1 h before TPA treatment. These specific inhibitors used for the experiments were obtained from Calbiochem Company (La Jolla, CA, USA). In-

duction of differentiation by TPA was confirmed by transcription of the *CD11b* marker gene or by increased cell adherence (12,30). The amount of adherent cells was scored in randomly selected microscopic fields under a phase-contrast microscope observation.

RNA Extraction and RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, USA) followed by DNase I treatment (Promega, Madison, WI, USA) as described by the manufacturer. For RT-PCR, 5 μ g of total RNA was reverse transcribed into single-strand cDNA using the oligo-(dT)15 primer and PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA). GAPDH was used as the internal standard because microarray profiling showed that the signals for GAPDH probe sets were equivalent between our TPA-treated or control cells. The amplified PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide for visualization. The primer sets used for amplification were: sense 5'-CCCTAAGCTGGA GGAGATGATG and antisense 5'-TCATGCTCACT AGGCCACTGAC for *EGR1*; sense 5'-ATGGCTCT CAGAGTCCTTCTGTAA and antisense 5'-CAT CAAAGAGAACAAGGTTTTGGGA for *CD11b*; and sense 5'-TGGTATCGTGGAAGGACTCA and antisense 5'-AGTGGGTGTCGCTGTTGAAG for *GAPDH*.

Schema for Identifying Early TPA-Responsive Genes

We designed a modified screening strategy aimed at enriching early TPA-responsive genes, as outlined in Figure 1. To achieve this aim, a homogeneous cell population was first generated by synchronizing the cells with nocodazole (0.05 μ g/ml) for 24 h and then cells were washed twice with culture medium and treated for 30 min with cycloheximide (CHX, 10 μ g/ml). Differentiation was induced by incubation with TPA (32 nM) in the continued presence of cycloheximide, thus excluding the indirect transcriptional effects from the gene product of the primary target gene. Control cells were also synchronized and treated with cycloheximide, but they were treated with 0.05% DMSO instead of TPA. Cells were collected at 1 h post-TPA treatment and subjected for total RNA isolation. In order to minimize the interexperimental variations, we collected and pooled total RNA from three independent experiments for subsequent RT-PCR and microarray analysis. In the present study, microarray analysis was first used as a screening tool to get the preliminary candidate TPA target genes. A subset of these genes was further screened by quantitative real-time PCR (Q-PCR). According to the result of Q-PCR analysis, we adjusted

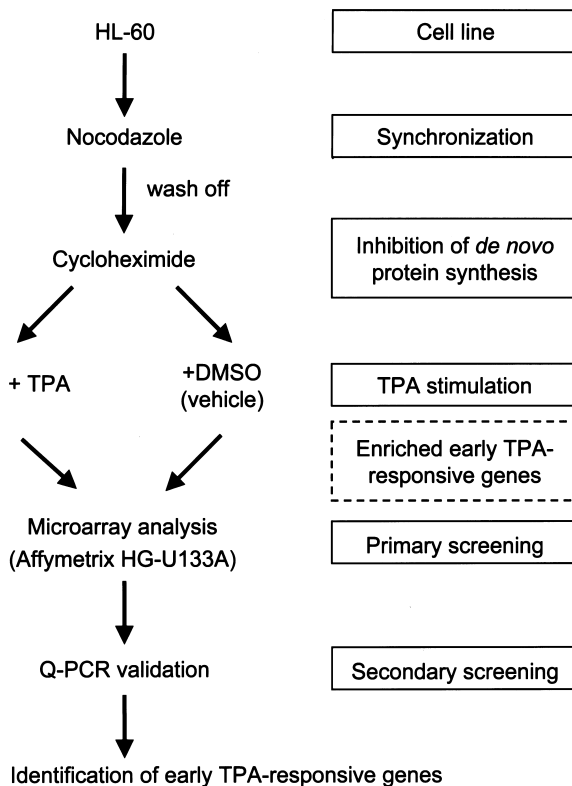


Figure 1. Schema of the experimental design for identifying early TPA-responsive genes. HL-60 cells were synchronized with nocodazole (0.05 $\mu\text{g/ml}$ for 24 h) and then cells were washed with culture medium and followed by treatment with cycloheximide (CHX, 10 $\mu\text{g/ml}$) for 30 min prior to subsequent stimulation with TPA (32 nM, 1 h) or DMSO (0.05%, 1 h) as the vehicle control. The cells were then collected and used for microarray and real-time PCR analysis.

our selection criteria for previous microarray analysis. Thus, only the early TPA-responsive genes are present in this screening, and these genes are more likely to be the primary (direct) targets of TPA in HL-60 cells.

Microarray Analysis

RNA isolated from cells treated with cycloheximide plus TPA or cycloheximide plus vehicle control as described before was used for global differential gene expression analysis using HG-U133A GeneChip (Affymetrix, Santa Clara, CA, USA). The hybridization and data analysis were performed at UC Davis School of Medicine Microarray Core Facility according to the Affymetrix technical manual. Arrays were analyzed with Affymetrix's Microarray Suite (MAS 5.0) for background subtraction, normalization, and comparison. To identify differentially expressed transcripts, comparison analysis was performed, and all data were calculated and expressed fold change as a \log_2 ratio and subjected to a statistical analysis of

significance ($p < 0.05$). The probe set information was obtained using the NetAffx online analysis tool supplied by Affymetrix (<http://www.affymetrix.com>).

Quantitative Real-Time PCR Assay

Real-time PCR was performed on a Roche Light Cycler system using gene-specific primers and DNA Fast-Start Master SYBR Green I mix (Roche, Basel, Switzerland) according to the manufacturer's protocol. For each sample, the threshold cycle C_t (or C_p) value was used as a measure of the amount of template present in the starting reaction, and the ΔC_t value was determined by subtracting the C_t value of reference gene (GAPDH) from the C_t value of target gene. The relative expression of each gene was expressed as $-\Delta\Delta C_t$ (ΔC_t value of the control sample subtract ΔC_t value of the TPA-treated sample) (29). Differences in the mean \pm SD ($n \geq 3$) between TPA-treated cells and controls were analyzed statistically using Student's *t*-test. The real-time PCR was performed using the gene-specific primers (Supplementary Table 1).

RESULTS

Confirmation of TPA-Induced Macrophage-Like Differentiation of HL-60 Cells

TPA-induced differentiation of HL-60 cells into macrophage-like cells has been reported (32,33). We first confirmed the effects of TPA on cell adhesion and the expression of *CD11b* mRNA, which are the hallmarks of macrophage differentiation (12,30). Following 2 days of TPA (32 nM) treatment, most cells (>90%) adhered to the culture vessel and aggregated (Fig. 2A, lower panel). In contrast, control cells treated with vehicle (0.05% DMSO) morphologically resembled untreated cells and were nonadherent (Fig. 2A, upper panel). In concurrence with the cell adherent results, the maturation marker *CD11b* mRNA was increased after 8 h of TPA treatment and showed a further elevation at 24 h, but it remained low in control cells as evaluated by RT-PCR (Fig. 2B, left panel). In addition, after synchronization by nocodazole (0.05 $\mu\text{g/ml}$) for 24 h, the expressional kinetics of *CD11b* mRNA was similar in synchronized and asynchronous cells (Fig. 2B). These results indicated that nocodazole itself did not induce HL-60 cell differentiation. It showed a potential to obtain a homogenous cell population for the initiation of cell differentiation. These characteristics of macrophage-like differentiation of HL-60 cells induced by TPA were therefore consistent with those in previous reports (32,33).

SUPPLEMENTARY TABLE 1
GENE-SPECIFIC PRIMERS USED FOR REAL-TIME PCR

Gene Name	Forward Primer	Reverse Primer
<i>IL8</i>	s: TTGGCAGCCTTCCTGATT	as: AACTTCTCCACAACCCCTCTG
<i>JUN</i>	s: TTGTTTGTGGGTATCCTG	as: ATGCAGAAAAGAGGTTAGGG
<i>NFIL3</i>	s: TGGAGAAGACGAGCAACA	as: CTTTGATCTGCATGGCTT
<i>SKIL</i>	s: GAGGCTGAATATGCAGGACA	as: GCCTAGTTATCGTCATGC
<i>JMJD3</i>	s: ATCGTGCCCATGATTCAC	as: TCCACATCGCACTCGTTG
<i>C14ORF92</i>	s: CGATGTGTGAGGTCTGGT	as: GACACTTTCACAGCAGAT
<i>TNF</i>	s: ACAAGCCTGTAGCCCATGTT	as: AGACGGCGATGCGGCTGATG
<i>FOS</i>	s: GGATAGCCTCTTACTACCAC	as: TCCTGTCATGGTCTTACAACG
<i>NFKBIA</i>	s: CCAAGCACCCGGATACAG	as: GCTGGCCTCCAAACACAC
<i>BCLAF1</i>	s: GGATGGGATTGTTGAAGATG	as: GTGGGTGCAAGTTCGTCTCT
<i>PDE4BC</i>	s: TTGTATCGGCAATGGACAGA	as: GTCTCCACAATGGATGGA
<i>KIAA0286</i>	s: TTGCCCTTGCCATTATCATC	as: GCCTTTCGGGTTTCTACCTC
<i>HSPCA</i>	s: ATGAAACTGCGTCCTGTCT	as: CTTCTCCATGCGTGATGTG
<i>DDX3X</i>	s: GGTAGCAGCAGAGGATTGG	as: GCAAAGCAGGCTCAGTTACC

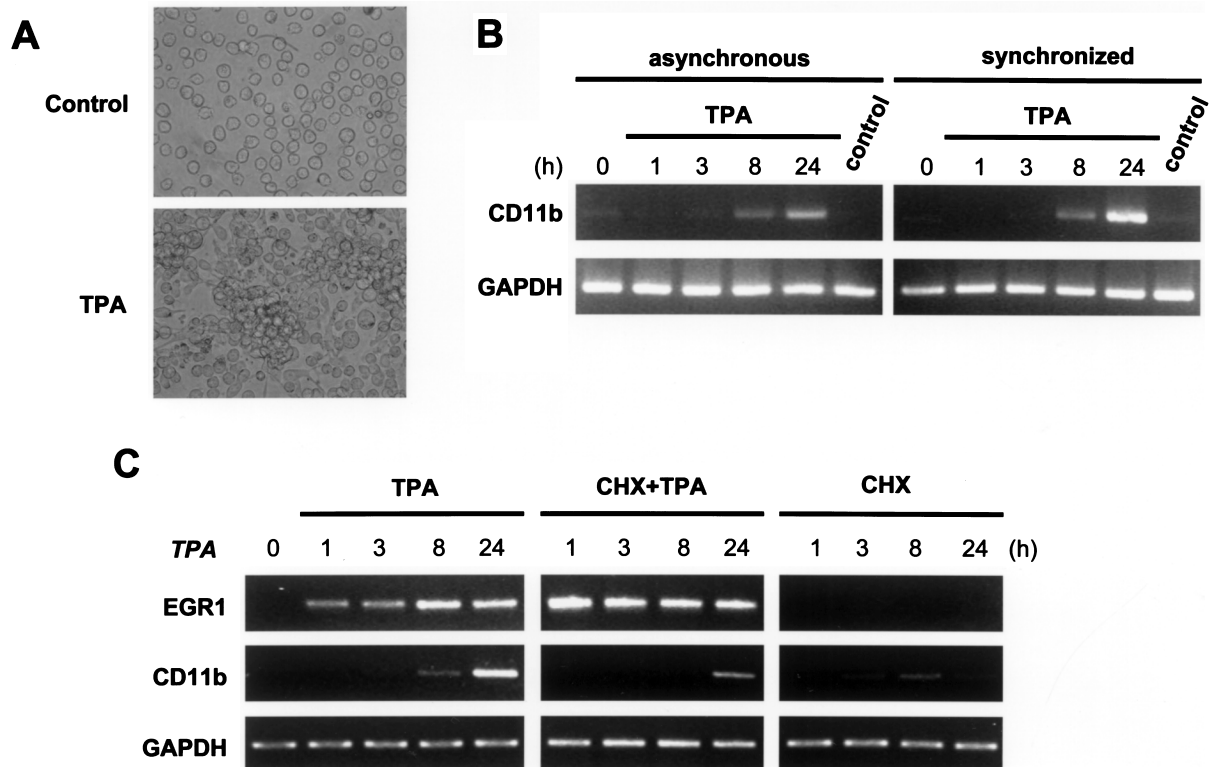


Figure 2. TPA induction of cellular differentiation and the expression of marker genes in HL-60 cells. (A) TPA induced macrophage-like differentiation in HL-60 cells. Morphological changes (aggregation and adherence) were observed in cells treated with TPA (32 nM) for 2 days. (B) The expression of the differentiation marker, *CD11b*, was increased by TPA treatment and was seen after 8 h of TPA treatment (left panel). Upregulation of *CD11b* transcription was not affected by nocodazole synchronization (right panel). The kinetic of *CD11b* transcription was analyzed by RT-PCR after the indicated time (0–24 h) of TPA treatment; control cells were treated with DMSO alone. In the right panel, the cells were synchronized with nocodazole (0.05 μ g/ml) for 24 h and then washed off before TPA stimulation. (C) The early and late TPA-responsive genes can be distinguished using the protein synthesis inhibitor cycloheximide. TPA-induced upregulation of the late responsive gene, *CD11b*, is suppressed by cycloheximide, whereas that of the early responsive gene, *EGR1*, is not. In this experiment, cells were treated with cycloheximide (CHX, 10 μ g/ml) for 30 min, then for 1 h with cycloheximide and TPA.

In addition, with only a short 20-min incubation period with TPA, it was sufficient to commit macrophage-like differentiation in HL-60 cells (33). Thus, the short period of TPA stimulation was sufficient to activate primary target genes, which are important in the initiation of cell differentiation. In order to identify such primary target genes of TPA in HL-60 cells, cells were treated with the protein synthesis inhibitor cycloheximide (CHX, 10 $\mu\text{g}/\text{ml}$) for 30 min prior to, and during, TPA stimulation to prevent the generation of the primary genes' protein product needed for induction of mRNA expression of secondary targets of TPA. To confirm the ability of cycloheximide to distinguish between primary and secondary target genes, we used the known early TPA-responsive gene, *EGR1* (26), and the late responsive gene, *CD11b*, as indicators. As shown in Figure 2C, upregulation of *EGR1* by TPA (32 nM, 0–24 h) was not inhibited by cycloheximide pretreatment, whereas transcription of *CD11b* was shut down. These results showed TPA treatment in the presence of cycloheximide did not alter the expression of the early responsive genes, but attenuated the expression of the late responsive gene, demonstrating that the use of cycloheximide can efficiently discriminate between the primary and secondary targets of TPA.

Identification of Early TPA-Responsive Genes by Microarray Analysis and Validation by Quantitative Real-Time PCR Analysis

We focused on selectively identifying early TPA-responsive genes in the present study, because we hypothesized that some of the early responsive genes might be important in initiation of differentiation of HL-60 cells, and these genes might express in low level or only slightly change in their expression patterns. Thus, we used our modified screening strategy to enrich and collect the early TPA-responsive transcripts for microarray analysis as described in Figure 1. The expression patterns of the TPA-induced genes were assessed by comparing expression in TPA plus cycloheximide-treated cells to that in vehicle plus cycloheximide-treated control using Affymetrix HG-U133A arrays. Based on our primary selection criteria for microarray analysis giving signal \log_2 ratio ≥ 0.5 and difference ≥ 30 , 83 genes were preliminary established to be upregulated by TPA. We selected 18 of the 83 genes from three categories in our primary gene list to validate our microarray screening criteria by quantitative real-time PCR analysis (Q-PCR) (Supplementary Table 2). According to the concurrence between Q-PCR and microarray result, we adjusted our microarray selection criteria and then identified 17 strongly increased genes (difference

>200 and \log_2 ratio ≥ 1.0) and 39 weakly increased genes (difference >50 and \log_2 ratio ≥ 0.5) as listed in Table 1. These 56 early TPA-responsive genes were candidates of TPA primary target genes. Many of these genes were classified as transcription regulators, cell cycle regulators, apoptosis regulators, cytokines, signaling-related genes, and the others are metabolic, unspecified genes, or ESTs. Based on literature review, 24 of the 56 TPA upregulated genes identified in our study had been recognized as TPA-responsive genes or expressed during myeloid differentiation in previous molecular or microarray studies, such as members of the EGR family (*EGR1*, *EGR2*, and *EGR3*) (18,49), AP1 superfamily [*JUN* (1,8,23, 46), *JUNB* (5,23,46), *FOS* (25,46), *FOSB* (49)], *IL8* (42,49), *CCL2* (49), *CCL3* (37,49), *CCL4* (49), *TNF* (14), *MCL1* (20), *NFKBIA* (49), *SAT* (7,49), *HADHA* (49), *LDLR* (19), *PMAIP1* (*NOXA*) (49), *DUSP2* (35,49), *CD69* (35), *BTG2* (34), *SPRY2* (49), *CYP11B1* (10), and *IER2* (*ETR101*) (39). Among these 24 genes, 14 genes were classified as strongly increased genes (14 of 17) and 10 were weakly increased genes (10 of 39) in our screening, as shown in Table 1. We identified an additional 32 genes previously not known to be TPA-responsive genes, which exhibited lower expression. Among these, *DDX3X*, *SKIL*, *SLC7A1*, *NFIL3*, *BCLAF1*, and *JMJD3* were validated by Q-PCR (Supplementary Table 2). The result showed that the early TPA-responsive genes identified by other general approaches expressed at a higher level, and of course, were also efficiently identified by our new screening strategy. Our approach using the combination of the synchronization reagent (nocodazole) and the protein synthesis inhibitor (cycloheximide) can therefore more effectively identify TPA primary regulated genes, which expressed at lower level and/or minor alterations in this HL-60 cell differentiation model system.

Upregulation of NFIL3, SKIL, and JMJD3 by TPA Through PKC Signaling Pathway in HL-60 Cells

We selectively focused on three unreported TPA primary regulated genes (*NFIL3*, *SKIL*, and *JMJD3*), which showed significant increases in expression at lower level and/or minor alterations both in the microarray and Q-PCR analysis in this HL-60 cell differentiation system under nocodazole synchronization and cycloheximide treatment conditions. These genes with transcription regulatory activity (*SKIL* and *NFIL3*) or with chromatin remodeling activity (*JMJD3*) might be important in the regulation of TPA-dependent differentiation in HL-60 cells. First, we demonstrated their transcriptional upregulation in response to TPA was also shown in the absence of

SUPPLEMENTARY TABLE 2
VALIDATION OF MICROARRAY DATA BY Q-PCR

Gene Symbol	Microarray		Q-PCR $-\Delta\Delta Ct$	
	Difference (TPA – Control)	Log ₂ Ratio (TPA/Control)	1 h	3 h
Group I (difference >200)				
<i>FOS</i>	595.75	3.00	3.03*	4.36*
<i>TNF</i>	1399.75	2.80	1.79*	4.32*
<i>IL8</i>	1536.05	2.70	3.22*	6.61*
<i>JUN</i>	343.35	1.30	1.10*	3.04*
<i>NFKBIA</i>	1320.85	1.20	0.79*	1.13*
<i>HSPCA (Hsp90A)</i>	939.80	0.50	-0.05	-0.03
Group II (difference 50–200)				
<i>DDX3X</i>	56.80	0.80	0.05*	1.22*
<i>SKIL (SnoN)</i>	55.35	0.70	0.03	1.73*
<i>CCL2</i>	61.00	0.60	0.35*	1.23*
<i>SLC7A1</i>	60.65	0.60	-0.16*	0.85*
<i>NFIL3</i>	59.65	0.60	0.64*	2.56*
<i>BCLAF1 (BTF)</i>	55.90	0.50	-0.12*	0.80*
Group III (difference 30–50)				
<i>PDE4B</i>	31.70	0.90	0.08	0.74*
<i>C14orf92</i>	44.70	0.70	-0.29	0.13
<i>LIMD1</i>	33.45	0.70	-0.33*	-0.91*
<i>TAF1</i>	33.15	0.70	-0.43*	1.09*
<i>KIAA0286</i>	38.95	0.50	-0.16*	0.43*
<i>JMJD3</i>	34.75	0.50	0.32	2.00*

Eighteen genes showing upregulation in primary microarray analysis of CHX + TPA-treated and CHX + vehicle control were selected for quantitative real-time PCR analysis to validate the primary selection criteria. The Q-PCR is displayed as the differential cycle threshold (indicated as $-\Delta\Delta Ct$) for each gene for triplicate independent experiments.

*Significant change in expression ($p < 0.05$).

nocodazole and cycloheximide in HL-60 cells (Fig. 3A, left panel). The changes of these three genes was significantly increased after 3 h post-TPA treatment, in good agreement with the Q-PCR results obtained in nocodazole- and cycloheximide-treated cells. The expression of these three genes showed a greater increase with TPA stimulation in the presence of cycloheximide than in the absence of cycloheximide treatment (Fig. 3A, right panel). They displayed as typically early responsive genes, whose expression was induced without the requirement for protein synthesis. Thus, *NFIL3*, *SKIL*, and *JMJD3* can be classified as new early TPA-responsive genes in HL-60 cells.

There is a dose dependency of the cell adherent, the terminal differentiation marker in TPA-induced HL-60 cells with only a short period TPA treatment (33). Figure 3B shows that upregulation of *NFIL3*, *SKIL*, and *JMJD3* mRNA by TPA (up to 80 nM, for 3 h) was also in a dose-dependent manner. Because TPA is a well-known PKC activator, and acute exposure to TPA can prolong activation of the classic and novel PKC isoforms (2,3,6,24,41,45). In order to determine whether this dose-dependent effect was resulting from the direct activation of their transcription

by TPA through PKC, we pretreated HL-60 cells with classic PKC inhibitor Go6976 (1 μ M) for 1 h and subsequently treated with TPA. We found that TPA-induced increase in *NFIL3*, *SKIL*, and *JMJD3* mRNA levels was significantly suppressed by treatment with classic PKC inhibitor during TPA stimulation (Fig. 3C) by Q-PCR assay. These results show that the upregulation of *NFIL3*, *SKIL*, and *JMJD3* by TPA is dose dependent and that this effect is directly mediated by activation of the PKC signaling pathway in HL-60 cells. We suggest that these TPA primary target genes may play an important role as early regulatory mediators during TPA-induced macrophage-like differentiation in HL-60 cells through the PKC signaling pathway.

DISCUSSION

Most genes regulated during myeloid differentiation are controlled at the transcription level (31), and such transcriptional regulation is the result of the comprehensive interactions of a few key transcription factors but not a single “master” regulator (31,40,48).

TABLE 1
EARLY TPA-RESPONSIVE GENES UPREGULATED IN HL-60 CELL DIFFERENTIATION

Gene Symbol	Accession No.	Microarray		Q-PCR $-\Delta\Delta Ct$		Reference(s)
		Difference (TPA – Control)	Log ₂ Ratio (TPA/Control)	1 h	3 h	
Strongly increased (TPA – control >200, Log₂ ≥1.0)						
<i>EGR4</i>	NM_001965	227.3	4.9			
<i>DUSP2</i>	NM_004418	799.1	3.3			35, 49
<i>EGR3</i>	NM_004430	421.0	3.3			18, 49
<i>EGR1</i>	NM_001964	1151.7	3.1			18, 49
<i>FOS</i>	BC004490	595.8	3.0	3.03*	4.36*	25, 46
<i>TNF</i>	NM_000594	1399.8	2.8	1.79*	4.32*	14
<i>IL8</i>	AF043337	1536.1	2.7	3.22*	6.61*	42, 49
<i>EGR2</i>	NM_000399	291.8	2.6			18, 49
<i>JUNB</i>	NM_002229	848.1	1.7			5, 23, 46
<i>FOSB</i>	NM_006732	201.7	1.7			49
<i>IER2 (ETR101)</i>	NM_004907	2729.5	1.4			39
<i>JUN</i>	BG491844	343.4	1.3	1.10*	3.04*	1, 8, 23, 46
<i>NFKBIA</i>	AI078167	1320.9	1.2	0.79*	1.13*	49
<i>TNFAIP3</i>	NM_006290	219.4	1.2			
<i>MCL1</i>	NM_021960	397.7	1.0			20
<i>TIPARP</i>	AL556438	344.4	1.0			
<i>CYP1B1</i>	AU144855	260.5	1.0			10
Weakly increased (TPA – control >50, Log₂ ≥0.5)						
<i>CD69</i>	L07555	53.1	4.0			35
<i>HNRPA1</i>	AI144007	75.5	1.7			
<i>est</i>	BC003629	59.0	1.7			
<i>IER3</i>	NM_003897	112.4	1.6			
<i>CCL3</i>	NM_002983	118.5	1.3			37, 49
<i>ZFP36</i>	NM_003407	165.3	1.0			
<i>BTG2</i>	NM_006763	101.7	1.0			34
<i>HIS1</i>	AW193511	67.3	1.0			
<i>EIF4A1</i>	U79273	180.1	0.9			
<i>est</i>	AV764378	82.1	0.9			
<i>SRPR</i>	BG474541	61.2	0.9			
<i>PPP1R15A</i>	NM_014330	128.4	0.8			
<i>NXT2</i>	AF201942	99.3	0.8			
<i>C14orf43</i>	NM_018678	98.7	0.8			
<i>EIF5</i>	BE552334	58.6	0.8			
<i>DDX3X</i>	R60068	56.8	0.8	0.05*	1.22*	
<i>SAT</i>	BE971383	103.3	0.7			7, 49
<i>KCNAB2</i>	AF044253	89.6	0.7			
<i>SKIL (SnoN)</i>	BF725121	55.4	0.7	0.03	1.73*	
<i>SFRS7</i>	AA524053	54.5	0.7			
<i>HADHA</i>	BG472176	154.0	0.6			49
<i>LDLR</i>	NM_000527	134.0	0.6			19
<i>PMAIP1 (NOXA)</i>	AI857639	131.8	0.6			49
<i>WEE1</i>	AJ277546	77.5	0.6			
<i>PPP1R10</i>	NM_002714	75.3	0.6			
<i>CCL2</i>	S69738	61.0	0.6	0.35*	1.23*	49
<i>PRKARIA</i>	M18468	60.7	0.6			
<i>SLC7A1</i>	AA527433	60.7	0.6	-0.16*	0.85*	
<i>NFIL3</i>	NM_005384	59.7	0.6	0.64*	2.56*	
<i>SPRY2</i>	NM_005842	136.9	0.5			49
<i>TXNDC</i>	NM_030755	134.9	0.5			
<i>CCL4</i>	NM_002984	119.6	0.5			49
<i>ID1</i>	NM_004508	105.8	0.5			
<i>PBEF1</i>	BF575514	76.4	0.5			
<i>RAD21</i>	BG289967	76.3	0.5			
<i>HNRPD</i>	W74620	73.0	0.5			
<i>RRM1</i>	AI692974	62.9	0.5			
<i>BCLAF1</i>	BE963370	55.9	0.5	-0.12*	0.80*	
<i>JMJD3</i>	AA521267	34.8	0.5	0.32	2.00*	

The genes showing upregulation detected in this comparison microarray analysis of TPA-treated and vehicle control in the presence of cycloheximide in synchronized HL-60 cells are listed. The TPA induction schema and detailed microarray analysis are described in Materials and Methods.

*Significantly ($p < 0.05$) upregulated by TPA-treated over vehicle-treated cells.

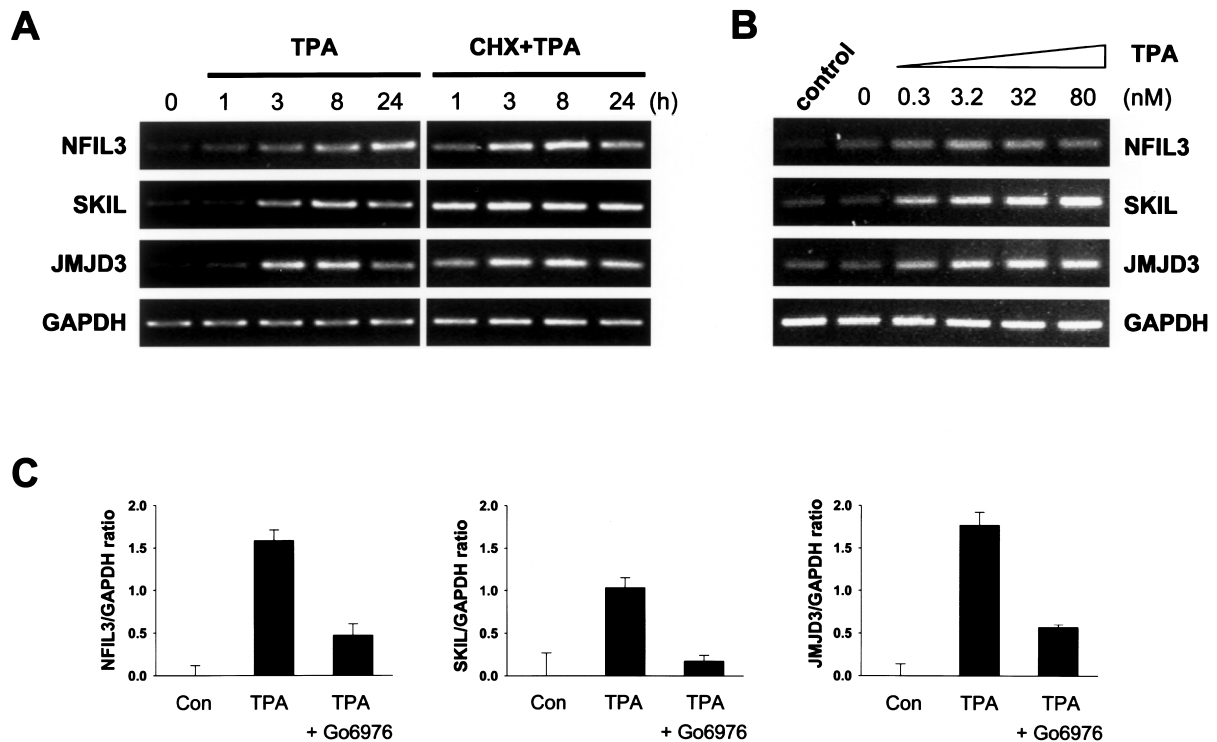


Figure 3. Expression pattern of TPA upregulated genes in HL-60 cells in the absence of cycloheximide. (A) Upregulation of *NFIL3*, *SKIL*, and *JMJD3* is not suppressed by cycloheximide treatment. Cells were pretreated with cycloheximide (CHX, 10 μ g/ml) for 30 min and then were treated with TPA (32 nM) for 0–24 h, and gene expression was determined by RT-PCR. (B) Upregulation of *NFIL3*, *SKIL*, and *JMJD3* by TPA is shown to be dose dependent. Cells were treated for 3 h with 0–80 nM of TPA and gene expression was determined by RT-PCR. *GAPDH* was used as control for normalization. (C) The TPA-induced upregulated expression of *NFIL3*, *SKIL*, and *JMJD3* is decreased by Go6976 treatment. Cells were pretreated with classic PKC inhibitor, Go6976 (1 μ M) for 1 h prior to TPA stimulation to block PKC signaling. Q-PCR assay was used to determine the altered expression of the genes in the TPA-treated cells relative to that in untreated cells, normalized with *GAPDH* and calculated by $\Delta\Delta C_t$ method. The results are the mean \pm SD for three independent experiments.

Therefore, it is beneficial to identify these unrecognized regulators in order to determine the molecular mechanisms of controlled myeloid differentiation. Genes that show immediate expression alterations might be important in the initiation stage of cell differentiation, whereas genes that show delayed expression changes might be involved in the progression of differentiation, maturation, and/or apoptosis (22). Because the early responsive genes might play key regulatory roles, they deserve much more attention. In addition, it is helpful to construct transcriptional regulatory networks by using these primary targets to examine their prospective downstream target genes, which could link these key factors to their biological function. Therefore, we tried to identify additional key regulatory factors that have not been found in previous studies and might be involved in the initiation stage of differentiation.

The early responsive genes are likely to be primarily regulated at the transcription level, as their induction does not require protein synthesis, but only the modification of preexisting transcriptional modulators. In contrast, the delayed responsive genes require

protein synthesis for induction and might depend on the prior induction of other genes (11,13). Such concerns are usually addressed by blocking protein synthesis using cycloheximide. There have been reports of the use of cycloheximide in the microarray analysis of primary targets of specific genes, such as p53 (17) and *c-myc* (27). In this study, we aimed to identify early TPA-responsive genes in treated HL-60 cells in the presence of cycloheximide. Our data showed that a subset of genes showing a minor change in transcription might be more effectively identified in the presence of cycloheximide than in its absence. If we take *JUN* as an example, it is immediately induced during monocytic differentiation (23), but it has never been detected at an earlier time period of TPA stimulation by previous microarray approaches. In our study, *JUN* mRNA was induced in TPA-treated HL-60 cells in the presence of cycloheximide. The transcription of *JUN* was not affected by cycloheximide treatment alone, but the half-life of the *JUN* transcript was prolonged by TPA treatment, resulting in its mRNA accumulation (38). We therefore suggest that it is beneficial to adopt cycloheximide treat-

ment in our experimental strategy, so cycloheximide can reduce/block signals generated from late responsive genes and therefore enhance the probability of early responsive gene detection, which might be expressed at lower levels and with a shorter half-life. Therefore, this new approach can identify additional factors that have never been reported in early TPA-induced HL60 cells.

This is the first report to show that the expression of three transcription-regulated genes, *NFIL3*, *SKIL*, and *JMJD3* is immediately upregulated during TPA-induced cellular differentiation in HL-60 cells. We demonstrated that the TPA-induced upregulation of these three genes was slightly increased and the expressional alteration can be further enhanced by cycloheximide cotreatment and that this expression pattern was very similar to that of the prototype early responsive genes, *EGR1* (26) and *JUN* (23), in TPA-induced HL-60 cell differentiation. Except for the novel gene *JMJD3*, *NFIL3*, also known as *E4BP4*, is a member of the bZIP transcription factor superfamily, and it acts as a transcription activator of *IL3* and also is upregulated by *IL3* and therefore plays an important role in *IL3*-mediated survival (16,21). The

other gene, *SKIL*, also known as *SnoN*, has also been found to involve in the regulation of cell growth and differentiation through TGF- β signaling pathway (4,43) and its expression level was also enhanced in the differentiation of murine tri-lineage early myeloid progenitors (28). *SKIL* might act as a transcription repressor of the *CD11b* gene, because the differentiation marker *CD11b* expression was significantly increased in 1,25-dihydroxyvitamin D₃-induced monocytic differentiation in HL-60 cells with *SKIL* shRNA knock down transduction (data not shown). Although these transcription-related genes were recognized in this study as primary TPA-regulated genes, their exact biological roles in regulating HL-60 cell differentiation is not yet clear and further investigations are needed.

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