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Involvement of CREB in the transcriptional regulation of the human GM3 synthase (hST3Gal V) gene during megakaryocytoid differentiation of human leukemia K562 cells

Hee-Jung Choi,[a,c] Tae-Wook Chung,[b,c] Nam-Young Kang,[a,c] Kyoung-Sook Kim,[c] Young-Choon Lee,[a] and Cheorl-Ho Kim[b,c,*]

[a] *Faculty of Biotechnology, Dong-A University, Busan 604-714, Republic of Korea*
[b] *Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, Kyungbuk 780-714, Republic of Korea*
[c] *National Research Laboratory for Glycobiology, MOST, Korean Government, Kyungju 780-714, Republic of Korea*

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Abstract

We studied the transcriptional regulation of human GM3 synthase (hST3Gal V) during megakaryocytic differentiation of K562 cells induced by PMA. Northern blot and reverse transcription polymerase chain reaction (RT-PCR) indicated that the induction of hST3Gal V by phorbol 12-myristate 13-acetate (PMA) is regulated at transcriptional level. To elucidate the mechanism underlying the regulation of the hST3Gal V gene expression during the differentiation of K562 cells induced by PMA, we characterized the promoter region of the hST3Gal V gene. Functional analysis of the 5′-flanking region of the hST3Gal V gene by transient expression method showed that the −177 to −83 region, which contains a CREB binding site at −143, functions as the PMA-inducible promoter in K562 cells. In addition, gel shift assay and site-directed mutagenesis indicated that the CREB binding site at −143 is crucial for the PMA-induced expression of the hST3Gal V in K562 cells.

Keywords: GM3 synthase; Promoter; K562 cell; Megakaryocyte differentiation

Gangliosides, the sialic acid (NeuAc)-containing glycosphingolipids, are found on the outer leaflet of the plasma membrane of vertebrate cells and are particularly abundant in the central nervous system [1]. They play important roles in a large variety of biological processes, such as cell–cell interaction, adhesion, cell differentiation, growth control, and receptor function [2]. GM3 is the first and the simplest of the gangliosides and is known to play important roles in the modulation of cell growth through modified signal transduction and cell differentiation [3]. GM3 induces monocytic differentiation of human myeloid and monocytoid leukemic cell lines HL-60 and U937 [4]. GM3 also induces megakaryocytoid differentiation of human erythroleukemia cell line K562 [5]. GM3 is synthesized by CMP-NeuAc:lactosylceramide α2,3-sialyltransferase (GM3 synthase, EC 2.4.99.9) which catalyzes the transfer of NeuAc from CMP-NeuAc to the non-reducing terminal galactose of lactosylceramide. GM3 synthase is a key regulatory enzyme for ganglioside biosynthesis [6] because it catalyzes the first committed step in the synthesis of nearly all gangliosides.

K562 is a human erythroleukemia cell line that upon phorbol 12-myristate 13-acetate (PMA) treatment can differentiate into megakaryocytes [7,8]; hence, they have been used extensively as a model system to study the factors involved in megakaryocyte differentiation. The amount of GM3 increases with a concomitant increase of human GM3 synthase (hST3Gal V) activity during megakaryocytic differentiation of K562 cells treated with PMA that is a megakaryocytic differentiation inducer, but not with a erythrocyte differentiation inducer, hemin [5]. Also, hST3Gal V activity is remarkably elevated in a time-dependent manner during megakaryocytic differentiation [5]. These results suggested that the

* Corresponding author. Fax: +82-54-770-2281.
E-mail address: chkimbio@dongguk.ac.kr (C.-H. Kim).

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increases of GM3 and hST3Gal V activity might not be related to the differentiated lineage but to the specific action of PMA, which is well known as an activator of protein kinase C (PKC). These results also suggested that PKC might specifically activate hST3Gal V, resulting in an increase in the content of GM3 during the differentiation of K562 cells induced by PMA, and that PKC activated by PMA might regulate the expression of hST3Gal V gene at the transcriptional level. Therefore, the regulation of hST3Gal V may be important for the expression of GM3 in K562 cells.

Very recently, we isolated and functionally characterized the hST3Gal V gene promoter in human hepatoma HepG2 and neuroblastoma SK-N-MC cells [9]. To elucidate the molecular basis of hST3Gal V gene expression during the differentiation of K562 cells induced by PMA, in this study, the promoter region to direct up-regulation of reporter gene transcription in response to induced K562 cell differentiation was functionally characterized. The present results clearly indicate that the CREB binding site of the hST3Gal V promoter plays a critical role in transcriptional regulation of the hST3Gal V gene during K562 cell differentiation.

Materials and methods

Materials and cell culture. PMA was purchased from Sigma Chemical (St. Louis, MO, USA). K562 cell line was maintained in RPMI 1640 medium (Gibco-BRL, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at under 5% CO_2 at 37 °C. To induce differentiation, K562 cells were cultured for different times in the presence of 10 nM PMA, respectively. Cell differentiation was monitored by adhesion to culture plates and cell morphology.

Reverse transcription-polymerase chain reaction and Northern blot analysis. Total RNA was isolated from untreated and PMA-induced K562 cells using the Trizol reagent (Invitrogen, Life Technologies). Two micrograms of RNA was subjected to reverse transcription with the random nonamers and Takara RNA PCR kit (Takara Shuzo, Shiba, Japan) according to manufacturer's protocol. The cDNA was amplified by PCR with the following primers: hST3Gal V (413 bp), 5′-CCCTGCCATTCTGGGTACGAC-3′ (sense) and 5′-CACGATCAA TGCCTCCACTGAGATC-3′ (antisense); β-actin (247 bp), 5′-CAAG AGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCC TGTCGGCA-3′ (antisense). The PCR products were separated by gel electrophoresis on 3% agarose containing ethidium bromide with 1× TAE buffer. After electrophoresis, the intensity of the bands obtained from reverse transcription-polymerase chain reaction (RT-PCR) result was estimated using *Total*Lab software of Frog Gel Image Analysis System (CorebioSystem, Seoul, Korea). To assess the specificity of the amplification, the PCR product (413 bp) for hST3Gal V was subcloned into pGEM-T vector (Promega, Madison, WI) and sequenced and was found to be identical to the expected cDNA. Northern blot analysis was performed by the same method as described previously [10], using the [α-^{32}P]dCTP-labeled hST3Gal V fragment (413 bp obtained by RT-PCR) as a probe.

Preparation of reporter plasmids and mutagenesis. Reporter plasmids, pGL3-1600 and its derivatives (pGL3-83 to pGL3-1210), were prepared by insertion of the *Sac*I/*Bgl*II fragments of each plasmid constructed previously [9] into the corresponding sites of the promoterless and enhancerless luciferase vector pGL3-Basic (Promega, Madison, WI). pGL3-Δ177, which has a deletion from nucleotides +1 to −177, was constructed by LA-PCR using pThGM3 as template as described in the previous paper [9]. Sense primer FP1210 [9] containing *Sac*I sites, and antisense primer AP1 containing a *Bgl*II site, 5′-AA GAGATCTCCTCCGCGGCGGCCCCCGCCC-3′, were used. After the PCR fragment (1483 bp) was subcloned into pT7Blue(R) T-vector (Novagen, Madison) and sequenced, it was digested with *Sac*I and *Bgl*II and inserted into the pGL3-Basic plasmid. pGL3-177 mutCREB with base substitution of CREB binding site at position −143 to −136 was constructed using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to manufacturer's protocol. The following oligonucleotide primers: CREB-L, 5′-GTCCTCGTGTTGTCAGACCCCGCCCACGCGCCCCT-3′ and CREB-R, 5′-CGGGGTCTGACAACACGAGGACGCGGACGGC CAAT-3′ (mutated nucleotides underlined) were used, and the presence of mutation was verified by sequence analysis. pGL3-1600 mutCREB was constructed by the above method using pGL3-432. After sequence analysis, DNA fragment with mutation obtained by digestion with *Hin*dIII/*Hin*dIII was replaced by the corresponding fragment of pGL3-1600.

Transfection and luciferase assay. For the reporter analysis of hST3Gal V promoter, transient transfection of K562 cells was carried out by electroporation. Briefly, the cultured cells were washed with PBS buffer (136 mM NaCl, 2 mM KCl, 0.9 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) and containing 10 μg of the luciferase reporter constructs and 5 μg of a cytomegalovirus-β-galactosidase vector (pCMVβ) as a transfection efficiency control. The 0.5 ml sample (2.5×10^6 cells) suspended in PBS buffer was placed in a cuvette. Electroporation was accomplished using a Bio-Rad Gene Pulser II at 950 μF and 250 V. The cells were then resuspended in RPMI 1640 medium containing 10% fetal bovine serum and cultured for 12 h. PMA (10 nM) was added to induce cell differentiation at 12 h after transfection and cells were cultured for another 24 h. Cells were harvested and luciferase activity was measured using the dual-luciferase reporter assay system kit (Promega, Madison, WI) and Luminoskan Ascent (Thermo Labsystems, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity.

Electrophoretic mobility shift assays. Nuclear extracts from resting and PMA-induced K562 cell were prepared as described previously [10]. Electrophoretic mobility shift assays (EMSAs) were performed using gel shift assay system kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, double-stranded oligonucleotides containing the consensus sequence for CREB (5′-TCCTCGTGA CGTCAGACCCC-3′) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and used as probes for EMSA. And also, competition was performed using the unlabeled wild-type CREB or a mutant oligomer 5′-TCCTCGTGTTGTCAGACCCC-3′ (mut CREB) with 50-fold molar excess. Nuclear extract proteins (2 μg) were pre-incubated with the gel shift binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 0.05 mg/ml poly(deoxyinosine–deoxycytosine)) for 10 min and then incubated with the labeled probe for 20 min at room temperature. Each sample was electrophoresed in a 4% non-denaturing polyacrylamide gel in 0.5× TBE buffer at 250 V for 20 min. The gel was dried and subjected to autoradiography.

Results

Induction of hST3Gal V expression during K562 cell differentiation

To determine whether hST3Gal V gene expression is regulated during K562 cell differentiation, we analyzed

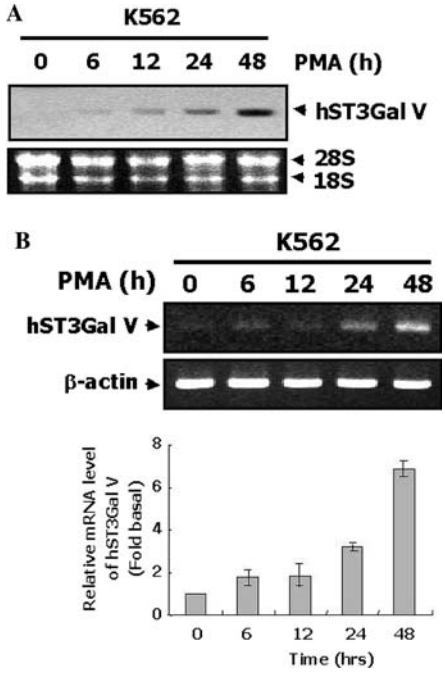

Fig. 1. Expression of hST3Gal V mRNA from K562 cells before and after PMA treatment. Total RNA from K562 cells was isolated after 0, 6, 12, 24 or 48 h of PMA treatment and hST3Gal V mRNA was detected by the Northern blot analysis (A) and RT-PCR (B). The bar graphs represent the intensity of the band obtained from RT-PCR result by densitometry. The values are means ± SD of three independent experiments.

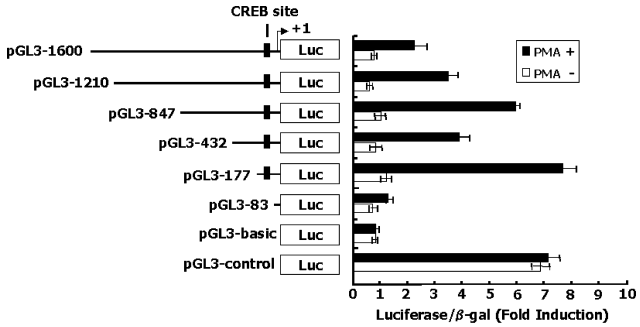

Fig. 2. Deletion analysis of hST3Gal V promoter in K562 cells before and after PMA treatment. Schematic representation of DNA constructions containing various lengths of the 5′-flanking region of the hST3Gal V linked to the luciferase reporter gene is presented, and the transcription start site is indicated as +1. pGL3-Basic without any promoter and enhancer was used as negative control. pGL3-Control with SV40 promoter and enhancer was used as positive control. Each construct was co-transfected into K562 cells with pCMVβ as the internal control. The transfected cells were incubated in the presence (solid bar) and absence (open bar) of 10 nM PMA for 24 h. Relative luciferase activity was normalized with β-galactosidase activity derived from pCMVβ. The values represent means ± SD for three independent experiments with triplicate measurements.

the expression profile of the hST3Gal V in K562 cells, which were treated with 10 nM PMA for cellular differentiation. As shown in Fig. 1, results of Northern blots and RT-PCR showed that the induction of hST3Gal V mRNA became detectable 6 h after PMA treatment and increased up to 48 h. Morphological changes of K562 cells on the culture dish were also seen 12 h after PMA treatment and increased up to 48 h (data not shown). These results clearly show that the expression of hST3Gal V is stimulated during PMA-induced megakaryocyte differentiation.

Determination of hST3Gal V promoter activity during PMA-induced cell differentiation

To characterize the region regulating the transcription activity of the hST3Gal V during PMA-induced differentiation of K562 cells, we prepared luciferase constructs carrying 5′-deleted hST3Gal V promoter and transfected them into PMA-untreated K562 cells. Regulation of hST3Gal V promoter activity by PMA treatment was examined. In PMA-induced cells, as shown in Fig. 2, serial deletion of nucleotide −1600 to −847 resulted in enhancement of promoter activity, indicating that the region −1600 to −847 negatively regulates transcriptional activity. However, in PMA-induced cells, deletion in the region from −847 to −432 resulted in decrease of promoter activity, while deletion in the region from −432 to −177 markedly incresed promoter activity to about 50% of pGL3-432. These results suggest that potential positive and negative regulatory elements exist within the −847 to −432 and −432 to −177 regions, respectively. The maximum activity was obtained with the pGL3-177 and reached about 8-fold higher activity than the pGL3-Basic. Further deletion to nucleotide −83 caused a drastic reduction in promoter activity to the similar level of the control vector pGL3-Basic. These results clearly show that the region from nucleotides −177 to −83 functions as the PMA-inducible promoter in K562 cells.

Identification of PMA-responsive element in nucleotide −177 to −83 region of hST3al V promoter

Our previous study [9] showed that this region from −177 to −83 is GC-rich (GC content, 74%) and contains NFY, CREB, SP1, EGR3, and MZF1 binding sites. In the previous paper [11], the TransSignal Protein/DNA array with nuclear extract of HeLa cell suggested that of these sites only the consensus CREB binding site (TGACGTCA) at position −143 to −136 might be contributory to the hST3Gal V promoter activity.

To determine whether this CREB binding site contributes to PMA-induced expression of hST3Gal V in K562 cell, we performed gel electrophoretic mobility shift assay (EMSA) using a double-stranded ^{32}P-labeled oligo fragment (20 bp) containing the consensus sequence for CREB, and nuclear extract from PMA-treated K562 cells. As shown in Fig. 3, two DNA–protein complexes (I and II) that bind to this fragment were

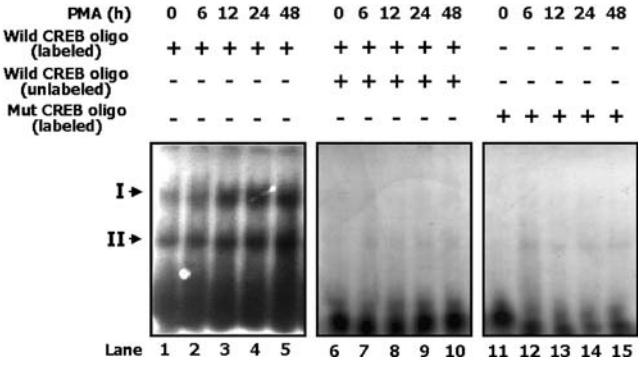

Fig. 3. EMSA with nuclear extracts and the CREB binding site sequence of the hST3Gal V promoter. Nuclear extracts isolated from K562 cells after 0, 6, 12, 24 or 48 h of TPA treatment were incubated with ^{32}P-labeled wild-type probe or unlabeled wild-type probe, or labeled mutant CREB probe. For competition experiments, 50-fold molar excess of unlabeled wild-type or labeled mutant CREB oligonucleotides was used. The DNA–protein complexes were analyzed on a 4 non-denaturing polyacrylamide gel.

detected, and the intensity of these complex increased significantly 12 h after PMA treatment and remained at a steady level for the next 48 h (Fig. 3, lanes 3–5). This binding could be competed with a 50-fold molar excess of unlabeled oligo fragment itself (Fig. 3, lanes 6–10) or mutant CREB oligo fragment (Fig. 3, lanes 11–15). These results indicated that these complexes contain CREB protein or other protein binding to this oligo fragment (20 bp).

To further confirm whether this CREB binding site played an important role for PMA-induced expression of hST3Gal V in K562 cell, we used pGL3-177 mutCREB and pGL3-1600 mutCREB constructed by mutating CREB binding site from pGL3-177 and pGL3-1600, respectively, and pGL3-Δ177 constructed by deleting −177/+1 region containing CREB binding site on pGL3-1600 as evidenced by luciferase promoter assay. It was obviously observed that this change abolished the CREB binding to this CREB binding site since mutant CREB oligomer (containing TG*TT*GTCA) could not compete for the binding while wild-type CREB oligomer (containing TGACGTCA) could (Fig. 3). In PMA-treated K562 cells, this mutation caused a reduction in promoter activity to about 58%, as compared with pGL3-177 (Fig. 4). pGL3-1600 mutCREB also showed a reduction in promoter activity to about 56%, as compared with pGL3-1600. In addition, deletion from +1 to −177 resulted in the similar reduction with pGL3-1600 mutCREB (Fig. 4). These combined results indicate that this CREB site is crucial for the PMA-induced expression of hST3Gal V, and that the CREB binding to this site is involved in the induction of hST3Gal V by PMA.

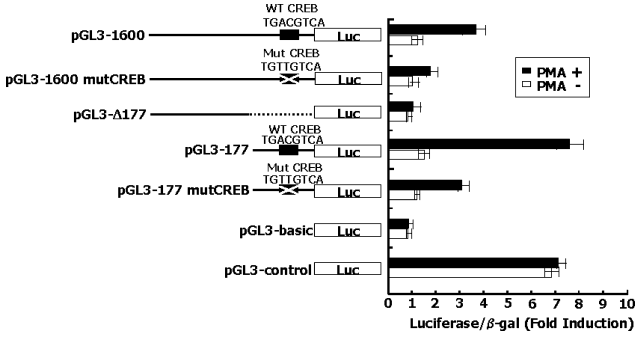

Fig. 4. Effect of mutation in the CREB binding site on hST3Gal V promoter activity. pGL3-Basic without any promoter and enhancer was used as negative control. PGL3-Control with SV40 promoter and enhancer was used as positive control. Each construct was co-transfected into K562 cells with pCMVβ as the internal control. The transfected cells were incubated in the presence (solid bar) and absence (open bar) of 10 nM PMA for 24 h. Relative luciferase activity was normalized with β-galactosidase activity derived from pCMVβ. The values represent means ± SD for three independent experiments with triplicate measurements.

Discussion

Previous study has shown that the hST3Gal V activity and the amount of GM3 ganglioside increase in a time-dependent manner in K562 cells treated with PMA that is a well-known megakaryocyte differentiation inducer [5]. That result has also suggested that GM3 increase is the consequence of the up-regulation of hST3Gal V. However, transcriptional regulation of the hST3Gal V during PMA-induced megakaryocyte differentiation of K562 cells has not been examined at the molecular levels. Here, we report for the first time that the expression of hST3Gal V is up-regulated during PMA-induced megakaryocyte differentiation of K562 cells. PMA treatment increased hST3Gal V mRNA levels in a time-dependent fashion: the induction of hST3Gal V mRNA became detectable 6 h after TPA treatment and increased up to 48 h. The hST3Gal V activity also increased in a time-dependent manner by 10 nM PMA treatment (data not shown). The hST3Gal V activity and GM3 levels in PMA-induced K562 cells have been also elevated in a time-dependent manner [5]. Therefore, these results clearly indicate that the relative levels of hST3Gal V mRNA and its protein are increased in a time-dependent fashion by PMA treatment in K562 cells.

Our results in this study show that the region between −177 and −83 in the hST3Gal V promoter functions as the core promoter essential for transcriptional activation of hST3Gal V in PMA-induced K562 cells. This region was found to be also needed for the enhancer activity of the hST3Gal V promoter in SK-N-MC and HepG2 cells [9]. The endogenous hST3Gal V gene expression was clearly detected in both cells by RT-PCR [9], but not in PMA-uninduced K562 cells. This indicates that this region functions as the PMA-inducible promoter in K562

cells. Recent report has suggested that only the consensus CREB binding site (TGACGTCA) at position −143 to −136 in this region might be contribute to hST3Gal V promoter activity [11]. Our present result by site-directed mutagenesis indicates that this CREB element mediates PMA-dependent up-regulation of hST3Gal V expression. By EMSA, we demonstrate that CREB binds to this site of the hST3Gal V promoter in a time-dependent manner by PMA induction, as shown in the increases of hST3Gal V expression, enzyme activity, and GM3 levels. Thus, our data strongly suggest that CREB may play an important role during PMA-induced megakaryocyte differentiation of K562 cells.

CREB is a transcription factor that is the target of a variety of signaling pathways mediating cell responses to extracellular stimuli, involving proliferation, differentiation, and adaptive responses of cell process [12,13]. Several signal pathways, such as PKA, PKC, Ca^{2+}/CaMKs, SAPK/JNK, P38, and ERK MAPKs could activate CREB binding to CRE, and the capacity of CREB to activate transcription is regulated by phosphorylation at serine 133 [12,13]. Previous study has shown that the activation of PKC by PMA in human fibroblasts stimulates CREB phosphorylation and subsequent CREB-mediated gene transcription [14]. Other studies have also indicated that CREB phosphorylation in oligodendrocytes may be mediated by PKC and MAP kinase-dependent signal transduction pathways [15–17]. It has been suggested that protein kinase C (PKC) may specifically activate hST3Gal V, resulting in an increase in the content of GM3 ganglioside during the differentiation of K562 cells induced by PMA, and that PKC activated by PMA may regulate the expression of hST3Gal V gene at the transcriptional level [5]. It is possible, therefore, that the expression of hST3Gal V gene may be activated by PKC and MAP kinase-dependent signaling pathway. It is of interest to dissect these pathways for clarifying the up-regulation-associated mechanisms of hST3Gal V expression in PMA-induced K562 cells. The present result of EMSA using oligo fragment (20 bp) containing the consensus sequence for CREB and nuclear extract from PMA-treated K562 cells showed two DNA–protein complexes (I and II), which could be competed with a 50-fold molar excess of unlabeled oligo fragment itself, or mutant CREB oligo fragment, indicating that these complexes contain CREB protein or other proteins binding to this oligo fragment.

Interestingly, computer program-based analysis revealed the presence of CREBP1CJUN, which is recognized by CRE-binding protein and c-Jun heterodimer, at the same position with CREB binding site [9]. Macrophage differentiation of HL-60 cells induced by PMA resulted in marked increase in c-Jun mRNA [18], and c-Jun mRNA expression with PKC activation increases during PMA-induced monocytic differentiation of U937 cells [19]. c-Jun has been described in several studies to heterodimerize with CREB [20–22]. These results suggest that one of these complexes may contain c-Jun heterodimer with CREB. Therefore, gel supershift assay using anti-CREB and anti-c-Jun antibodies is required to clarify which complex contains CREB or c-Jun heterodimer with CREB.

On the other hand, pGL3-83 and pGL3-Δ177 showed a drastic reduction in promoter activity to the similar level of the control vector pGL3-Basic, while pGL3-177mutCREB revealed a reduction in promoter activity to about 58%, as compared with pGL3-177. This suggests that there are other transcription factors involved in the basic transcription of hST3Gal V gene in the region from nucleotides −177 to −83. This region also contains NFY, SP1, EGR3, and MZF1 binding sites. NFY is a ubiquitous transcription factor which binds to CAAT elements in the proximal promoters of a wide variety of mammalian genes [23]. Although NFY is modulated during monocyte to macrophage differentiation [24], it is not reported to be a substrate for any protein kinase. SP1 is known to play a crucial role in the tissue-specific expression of CD14 in monocytic cells [25]. The myeloid zinc finger gene, MZF1, is a hematopoietic transcription factor expressed in developing myeloid cells and is necessary to a granulocyte differentiation of human promyelocytic leukemia cell line HL-60 induced by retinoic acid (RA) [26]. Identification of these transcription factors may facilitate understanding of the mechanism for transcriptional regulation of hST3Gal V gene during PMA-induced megakaryocyte differentiation of K562 cells.

Acknowledgments

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