Characterization of the human zinc finger nfx-1-type containing 1 encoding *ZNFX1* gene and its response to 12-*O*-tetradecanoyl-13-acetate in HL-60 cells

HIROSHI HAMADA[1], MAYU YAMAMURA[1], HIROTO OHI[1], YOTA KOBAYASHI[1], KUNIYOSHI NIWA[1], TAKAHIRO OYAMA[1], YASUNARI MANO[2], MASASHI ASAI[1], SEI-ICHI TANUMA[3,4] and FUMIAKI UCHIUMI[1]

Departments of [1]Gene Regulation, [2]Clinical Drug Informatics, and [3]Biochemistry, Faculty of Pharmaceutical Sciences; [4]Genomic Medical Science, Research Institute of Science and Technology, Tokyo University of Science, Noda-shi, Chiba-ken 278-8510, Japan

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Abstract. Human promyelocytic HL-60 cells can be differentiated into macrophage-like cells by treatment with 12-*O*-tetra decanoylphorbol-13-acetate (TPA). Certain 5' upstream regions of the zinc finger protein (ZNF)-encoding genes contain duplicated GGAA motifs, which are frequently found in the TPA-responding gene promoter regions. To examine transcriptional responses to TPA, 5'flanking regions of human zinc finger CCCH-type containing, antiviral, *ZNF252*, *ZNF343*, *ZNF555*, *ZNF782* and zinc finger nfx-1-type containing 1 (*ZNFX1*) genes were isolated by polymerase chain reaction (PCR) and ligated into a multiple-cloning site of the pGL4.10[*luc*2] vector. Transient transfection and a luciferase assay revealed that the *ZNFX1* promoter most prominently responded to the TPA treatment. Deletion and point mutation experiments indicated that the duplicated GGAA motif in the 100-bp region positively responded to TPA. In addition, reverse transcription-quantitative PCR and western blotting showed that the mRNA and protein of *ZNFX1* accumulate during the differentiation of HL-60 cells. These results indicated that expression of the TPA-inducible *ZNFX1* gene, which belongs to the group of interferon-responsive genes, is regulated by the *cis*-action of the duplicated GGAA motif.

Correspondence to: Professor Fumiaki Uchiumi, Department of Gene Regulation, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba-ken 278-8510, Japan
E-mail: uchiumi@rs.noda.tus.ac.jp

Abbreviations: FCS, fetal calf serum; IFN, interferon; Luc, luciferase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TSS, transcription start site

Key words: E26 transformation-specific, GGAA motif, HL-60, TPA, zinc finger nfx-1-type containing 1

Introduction

Zinc finger proteins (ZNFs) regulate transcription (1), replication (2), and other biologically significant events, including cellular differentiation (3), and some have been suggested to be responsible for human diseases (4,5). However, the molecular mechanisms that regulate their gene expression remain unclear. Surveillance of the human genomic DNA database revealed that duplicated GGAA (TTCC) motifs, which are frequently contained in the 5' flanking regions of the DNA repair, apoptosis, anti-viral response and mitochondrial function-associated factor encoding genes (6,7), are present in the 5' flanking regions of several *ZNF* family genes (8). Interestingly, the duplicated GGAA motif responds to interferon (IFN) β (9) and *trans*-resveratrol (10-12). In the ataxia telangiectasia and Rad3-related, xeroderma pigmentosum type B, and retinoblastoma (*RB1*) promoters, the motif notably responds to 12-*O*-tetra decanoylphorbol-13-acetate (TPA) in HL-60 cells (13). These observations suggest that *ZNF* family genes could be induced by the *cis*-function of the GGAA motifs during the macrophage-like differentiation of HL-60 cells.

The GGAA is a core sequence motif that E26 transformation-specific (ETS) family proteins recognize (14). Numerous transcription factors (TFs), including GA-binding protein TF (GABP), nuclear factor erythroid 2-related factor 2, nuclear factor-κB/c-Rel, STATs and interferon regulatory factor proteins bind to the GGAA motif-containing sequences and regulate gene transcription (7). Notably, ETS family proteins play essential roles in the regulation of cellular differentiation (15,16). Duplicated GGAA motifs are contained in the promoter region of the human *CD41* (*ITGA2B*) (17) and programmed death-1 genes (18), suggesting that GGAA motif-binding proteins regulate megacaryocytic cell differentiation. ETS family TF spleen focus forming virus proviral integration oncogene (SPI1; PU.1) regulates the timing of the activation of the T-lineage developmental program (19). Other ETS family proteins, ETS variant (ETV)4 and ETV5, regulate the differentiation of embryonic stem cells (20). ETS1 regulates neural crest development via the epigenetic control of bone morphogenetic protein signaling (21). Moreover, ETS

family proteins have been suggested to affect tumorigenesis (22). The aberrant fusion protein EWS-friend leukemia integration 1 TF is known to induce osteosarcoma development (23). In addition, amplification of ETS1 is suggested to cause tumor invasion (24). These lines of evidence suggest that GGAA-binding proteins are involved in the generation and development of cancer.

In the present study, we isolated 5'upstream regions of six human ZNF-encoding genes and found that the zinc finger nfx-1-type containing 1 (*ZNFX1*) promoter is the most prominently activated in response to TPA. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting revealed that amount of *ZNFX1* transcripts and degree of translation increase during the TPA-induced differentiation of HL-60 cells. Deletion and point mutation-introducing experiments demonstrated that the duplicated GGAA motif, which is present 100-bp downstream of the transcription start site (TSS), serves an essential role in the TPA response. JASPAR analysis of the TPA-responding duplicated GGAA motif suggested that SPI1 (PU.1), ETS1, myeloid zinc finger 1 (MZF1) and STAT1/2 are involved in the regulation of *ZNFX1* gene expression during the differentiation of HL-60 into macrophage-like cells. Taken together, it was suggested that investigations in the transcription mechanism of the *ZNFX1* gene may contribute to the development of novel anti-cancer therapies.

Materials and methods

Materials. TPA was purchased from Sigma-Aldrich (Merck KGaA) (13).

Cells and cell culture. The human promyelotic leukemia cell line HL-60 (Institute of Medical Science, Tokyo University) (25) was cultured in RPMI-1640 medium (WAKO Pure Chemical), supplemented with 10% fetal calf serum (FCS) (Biosera), containing 120 IU/ml penicillin G (Meiji Seika Pharma) and 120 μg/ml streptomycin (Meiji Seika Pharma), at 37˚C in a humidified atmosphere with 5% CO_2.

Construction of luciferase (Luc) reporter plasmids. A 543-bp region of the human *ZNFX1* gene and other promoter regions of *ZNF252*, *ZNF343*, *ZNF555*, *ZNF782*, *zinc finger CCCH-type containing, antiviral 1* (*ZC3HAV1*) genes were obtained by PCR-amplification, which was carried out with Prime STAR (Takar Bio, Inc.), appropriate primer pairs (Table I) and HeLa S3 genomic DNA as a template. The reaction was as follows: 98˚C for 15 sec, 55˚C for 5 sec, and 72˚C for 30 sec for 30 cycles. The PCR-amplified DNA fragments were digested with restriction enzymes, then they were ligated into the *Kpn*I/*Hin*dIII or the *Kpn*I/*Xho*I site of pGL4.10[*luc*2] (Promega Corporation), to make pGL4-ZNFX1, -ZNF252, -ZNF343, -ZNF555, -ZNF782, -zinc finger CCCH-type containing, antiviral 1 (ZC3HAV1). Similarly, the deletion of the 543-bp was carried out by PCR with appropriate primer sets (Table I), and pGL4-ZNFX1 as a template. The DNA fragments were ligated into the MCS of pGL4.10[*luc*2] to make pGL4-X1-Δ1 to -Δ7 plasmids. Other plasmids, -Δ6mt1, -Δ6mt2, -Δ6mt12, -Δ7mt1, -Δ7mt2, and -Δ7mt12, were constructed by a similar procedure (13) with appropriate primer pairs (Table I) and template plasmids, pGL4-X1-Δ6 or pGL4-X1-Δ7, for the amplification of the DNA fragments (Table I). Nucleotide sequences were confirmed by a DNA sequencing service (FASMAC-Greiner Japan, Inc.) with primers Rv (5'-TAGCAAAATAGGCTGTCC CC-3') and GL (5'-CTTTATGTTTTTGGCGTCTTCC-3').

Transient transfection and luc assay. Plasmids (100 ng/1-2x10^5 cells) were transfected into HL-60 cells via the DEAE-dextran method (26) in 96-well plates. After 24 h of transfection, the culture medium was changed to RPMI/1640 containing 10% FCS with or without TPA (8.1 nM). Control cells were treated without TPA and cultured in the medium containing 0.0005% DMSO. After a further 24 h of incubation at 37˚C, cells were collected and lysed with 100 μl of 1X cell culture lysis reagent containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*',*N*',-tetraacetic acid, 10% glycerol and 1% Triton X-100, and then were mixed and centrifuged at 12,000 x g for 5 sec at 4˚C. The supernatant was stored at -80˚C. The Luc assay was performed with a Luciferase Assay system (Promega Corporation), and relative Luc activities were normalized by the protein concentration and calculated as described previously (10,11).

Western blot analysis. Whole proteins were extracted by RIPA buffer (20 mM Tris-HCl (pH 7.4), containing 0.1% SDS, 1% Triton X100, and 1% sodium deoxycholate). Proteins were separated via 8/15% SDS-PAGE. Western blot analysis was carried out as previously described (10,11) with antibodies against ZNFX1 (Abcam, cat no. ab179452) and β-actin (Sigma-Aldrich; Merck KGaA, cat no. A5441), diluted 1:2,600 and 1:10,000, respectively, followed by the addition of horseradish peroxidase-conjugated secondary antibody for ZNFX1 (Sigma-Aldrich, cat no. A0545) and β-actin (Sigma-Aldrich, cat no. A9917) diluted 1:20,000. Signal intensities of samples at 0, 1, 2, 4, 8, 16, 24, 40 and 48 h following treatment were quantified with a ChemiDoc and ImageLab System (BioRad Laboratories, Inc.).

RT-qPCR. RT-qPCR was carried out as described previously (9-11). Total RNAs were extracted by GeneElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), according to the manufacturer's protocols. First-strand cDNAs were synthesized with ReverTra Ace (Toyobo Life Science), random primers (Takara Bio, Inc.), and total RNAs extracted from HL-60 cells, which were treated with TPA (8.1 nM) for 0, 8, 24, 48 and 72 h. The reaction was carried out by incubating at 30˚C for 10 min, 42˚C for 40 min, and then heating at 94˚C for 5 min. The sequences (from 5'-3') of the primer pairs used to amplify human *ZNFX1*, integrin subunit α M (*ITGAM*; *CD11b*), and *GAPDH* cDNAs were hZX1RT: GGC CCTCAAAAGAAGCCCTG and AhZX1RT: GATGGG GAACTTCTGGAGGC; hCD11b-459: GGGCTCTGCTTC CTGTTTG and AhCD11b-758: CTGCGTTATTGGCTT CACC; and hGAPDH556: TGCACCACCAACTGCTTA GC and hGAPDH642: GGCATGGACTGTGGTCATGAG, respectively.

Conditions for PCR, which was performed with BIOTAQ DNA polymerase (Bioline), were as follows: 96˚C for 10 sec, 55˚C for 5 sec, and 72˚C for 10 sec, for 30 (*ZNFX1*), 32 (*ITGAM*), and 22 (*GAPDH*) cycles. The PCR products were

Table I. Primer pairs used to amplify 5' upstream regions of the human zinc finger motif containing protein-encoding genes.

Luciferase plasmid	Primer	Sequence (5' to 3')
pGL4-ZNF252	hZNF252-6191	TCGGTACCGCAATAGGTCTGAAACCTCTC
	AhZNF252-5639	ATCTCGAGCGCGAACGCTAAATCCCGTGCC
pGL4-ZNF343	hZNF343-0212	TCGGTACCGGGATCTTAGATAAGAGGCCAG
	AhZNF343-9731	ATCTCGAGTGAAGTCTCTGTCCCCTTGGCC
pGL4-ZNF555	hZNF555-1040	TCGGTACCTCCTGTCCGGACAAGGGGTCGC
	AhZNF555-1536	ATCTCGAGCGCGACAGGAACCGGGACGCC
pGL4-ZNF782	hZNF782-1294	TCGGTACCCGGGAAGCGGTTTGGGAAGCTC
	AhZNF782-0834	ATCTCGAGAAACCTGACTCTCATCCACGTC
pGL4-ZC3HAV1	hZC3-7621	TCGGTACCACGATCTGGGGCCTGGGGACGC
	AhZC3-6933	ATCTCGAGTGCTGGCTCTGCCGCGGCGC
pGL4-ZNFX1	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AhZFX1-0666	CGATAAGCTTTCACTGCCGCCGGCGAGTGC
pGL4-X1-Δ1	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AhX1d4	GATAAGCTTGGGCGGAGCCGGGCGGGCGGC
pGL4-X1-Δ2	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AhX1d3	GATAAGCTTGGCCCAGGCACGGCCGGCGCC
pGL4-X1-Δ3	hX1d1	TCGGTACCGGGCTTGTCCGCTTCCTCGCC
	AhZFX1-0666	CGATAAGCTTTCACTGCCGCCGGCGAGTGC
pGL4-X1-Δ4	hZNFX1d3	CTAGGTACCAGGCCCTCGTGCTCTCCACCC
	AhZFX1-0666	CGATAAGCTTTCACTGCCGCCGGCGAGTGC
pGL4-X1-Δ5	hZNFX1d4	ATTGGTACCAGGGTCTGCGGGGAACGGAAA
	AhZFX1-0666	CGATAAGCTTTCACTGCCGCCGGCGAGTGC
pGL4-X1-Δ6	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AX1_WT	CGATAAGCTTCACTTTCGGTTTCCGTTCCCCGCAG
pGL4-X1-Δ6mt1	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AX1_mt1	CGATAAGCTTCACTTTCGGTTTAAGTTCCCCGCAG
pGL4-X1-Δ6mt2	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AX1_mt2	CGATAAGCTTCACTTTCGGTTTCCGTTAACCGCAG
pGL4-X1-Δ6mt12	hZX1-1208	TCGGTACCCGAAACGCTCTCTTTCCCGCCC
	AX1_mt12	CGATAAGCTTCACTTTCGGTTTAAGTTAACCGCAG
pGL4-X1-Δ7	hZNFX1d3	CTAGGTACCAGGCCCTCGTGCTCTCCACCC
	AX1_WT	CGATAAGCTTCACTTTCGGTTTCCGTTCCCCGCAG
pGL4-X1-Δ7mt1	hZNFX1d3	CTAGGTACCAGGCCCTCGTGCTCTCCACCC
	AX1_mt1	CGATAAGCTTCACTTTCGGTTTAAGTTCCCCGCAG
pGL4-X1-Δ7mt2	hZNFX1d3	CTAGGTACCAGGCCCTCGTGCTCTCCACCC
	AX1_mt2	CGATAAGCTTCACTTTCGGTTTCCGTTAACCGCAG
pGL4-X1-Δ7mt12	hZNFX1d3	CTAGGTACCAGGCCCTCGTGCTCTCCACCC
	AX1_mt12	CGATAAGCTTCACTTTCGGTTTAAGTTAACCGCAG

Shaded regions indicate mutations that were introduced into Luc reporter plasmids. Mt, mutant; WT, wild type; ZNF, zinc finger protein.

electrophoresed on 5% acrylamide gels and stained with ethidium bromide. The signal intensities were quantified using a ChemiDoc and ImageLab System.

qPCR analysis of transcripts. cDNAs were amplified using Thunderbird Real-time PCR Master Mix (Toyobo Life Science) and 0.3 μM of each primer pair. The primer pairs for amplifying human *ZNFX1* and *GAPDH* transcripts were the same as described above. qPCR was carried out using an Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.) (9-11). Amplification was performed initially for 1 min at 95˚C, followed by 40 cycles (95˚C for 15 sec and 60˚C for 30 sec). Relative gene expression values were obtained by normalizing C_T (threshold cycle) values of target genes in comparison with C_T values of the *GAPDH* gene using the $\Delta\Delta C_q$ method (27).

Surveillance of putative TSS and TF-binding sequences. The putative TSS was identified in 543-bp *ZNFX1* promoter region from the NCBI Gene database (https://www.ncbi.nlm.nih.

gov/gene/57169). The 543-bp and the 21-bp TPA responsive element were analyzed by JASPAR database (http://jaspar.genereg.net/) with threshold 97.5 and 92%, respectively.

Statistical analysis. All statistical analyses were carried out with Excel software (Microsoft Excel; version 2013; Microsoft Corporation). The experiments were repeated at least three times. All data are presented as the mean ± standard deviation. A Student's t-test was performed to evaluate the significant differences between TPA-treated and non-treated experiments. $P<0.05$ was considered to indicate a statistically significant difference. To compare multiple data sets, one-way ANOVA was performed followed by a Tukey's post-hoc test (SPSS; version 19; IBM Corp.).

Results

Isolation and characterization of the 5' flanking region of the human ZNFX1 gene. The duplicated GGAA motif containing 14 nucleotide consensus sequences, which are present in the upstream region of several TPA-responding genes (13), are also contained within 500 bp from the putative TSSs of several ZNF protein encoding genes (7,8). To examine the response to TPA, we isolated ~500-bp of the 5'upstream regions of the *ZC3HAV1*, *ZNF252*, *ZNF343*, *ZNF555*, *ZNF782* and *ZNFX1* genes to construct Luc-reporter plasmids. They were transfected into HL-60 cells (Fig. 1A). As the Luc activity from the control vector-transfected cells was very low, we used the value just for a subtraction. Notable Luc activity of the pGL4-ZNF343-transfected cells was detected; however, it was not affected by TPA treatment. Therefore, we used it for the reference for the easy, reproducible, cost-effective DEAE-dex-based multiple transfection assay (28). As the *ZNFX1* promoter was significantly activated by TPA treatment compared without treatment, we focused on the regulation of the *ZNFX1* gene expression mechanism during the differentiation of the HL-60 cells. Sequence analysis revealed that the pGL4-ZNFX1 contains a nucleotide identical to NCBI Sequence ID, NC_018931.2 (nucleotide from 47799505-47798963) and that it covers the most upstream 5' end of the cDNA (Sequence ID: NM_021035.2 of the *ZNFX1* mRNA; GENE ID: 57169 ZNFX1). Interestingly, this 543-bp region also contains a 5' upstream end of the *ZFAS1* mRNA (NM_021035.2; GENE ID: 441951ZFAS1) in a reverse orientation to that of the *ZNFX1* gene (Fig. 1B). The TSS was tentatively set as +1 at the most upstream 5' end of the *ZNFX1* cDNA that is shown in the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene/57169). Sequence analysis of the 543-bp region with 97.5% relative scores suggested characteristic recognition sequences of several known TFs (Fig. 1B). Although no particular sequences similar to the TATA or CCAAT boxes were present, putative binding sites for E2F1 (-347 to -336 and -111 to -100), E2F4/6 (-347 to -336), EHF (-158 to -150), ETS1 (-208 to -202, -158 to -150, and +101 to +106), KLF5/16 and Sp1/2/4 (-257 to 241), SPI1 (-245 to -239, -208 to -202, and -157 to -151), SPIB (-158 to -150), TFAP2A (-292 to -282 and -52 to -41), and ZNF354C (+25 to +30) are contained in the 543-bp region (Fig. 1B).

Effects of TPA on ZNFX1 gene expression and its protein amount in HL-60 cells. To examine whether human *ZNFX1* gene expression is upregulated during the macrophage-like differentiation of HL-60 cells, total RNAs were extracted after TPA (8.1 nM) treatment. The *ZNFX1* gene transcripts were accumulated from 24-48 h of TPA addition (Fig. 2). This was accompanied with the induction of *ITGAM* expression (Fig. 2A), which encodes the adherent molecule integrin subunit α M/CD11b/Mac1 (29). The relative gene expression of *ZNFX1* compared with that of *GAPDH* was induced after 24 h of TPA addition (Fig. 2B). However, the amount of transcripts decreased in response to longer durations following TPA treatment (Fig. S1). Prior to the upregulation of transcription, the amount of ZNFX1 protein began to increase from 1-24 h after TPA treatment (Fig. 3), suggesting that the post-transcriptional regulation, including translation rate or stability of the ZNFX1 protein, may have been altered following TPA treatment. The accumulation of the ZNFX1 protein occurred prior to that of CD11b, which was significantly induced at 48 h after TPA treatment compared with the other time points following treatment (Fig. S2).

Effects of TPA on ZNFX1 promoter activity. To investigate the molecular mechanism as to how the human *ZNFX1* promoter is upregulated by TPA, pGL4-ZNFX1 and its derivative deletion constructs (Fig. 4A) were transiently transfected into HL-60 cells. The relative Luc activity of the pGL4-ZNFX1-transfected cells significantly increased after the addition of TPA (8.1 nM) to the cell culture compared with the corresponding control (Fig. 4A). This induction by TPA was also observed in pGL4-X1-Δ3, -Δ4, and -Δ5-transfected cells, but not in the pGL4-X1-Δ1 and -Δ2 transfected cells. The Luc activities of the pGL4-X1-Δ1 and -Δ2 transfected cells were reduced to almost background level, implying that they had lost the essential elements for transcription. In addition, a notable response to TPA was not observed from the pGL4-X1-Δ1 and -Δ2 transfected cells, suggesting that the 97 nucleotides from +87 to +183 are primarily required for the positive response to TPA in HL-60 cells.

To examine the TPA-responding elements in detail, point mutations were introduced in the pGL4-X1-Δ6 and pGL4-X1-Δ7 plasmids (Fig. 4B). The response to TPA was completely abolished when both GGAA motifs were disrupted (pGL4-X1-Δ6mt12 and pGL4-X1-Δ7mt12), suggesting that the duplicated GGAA motif serves a role in the positive response to TPA. JASPAR analysis with 92% relative scores suggested that putative binding elements for ETS1, MZF1, SPI1, STAT1, and STAT2 proteins are involved in the positive response to TPA (Table II). As expected, the disruption of the duplicated GGAA motif in the pGL4-X1-Δ6mt12 and pGL4-X1-Δ7mt12 constructs excluded the possibilities of the binding of these TFs to the 21-bp element. Taken together, these results suggested that the *ZNFX1* promoter is regulated by the duplicated GGAA motif, which is present +50 to +70 bp downstream of the putative TSS, pertaining to the positive response to TPA in differentiating HL-60 cells.

Discussion

Although, it might be worth to analyze expression of the selected ZNF family genes, their responses to TPA were not greater than that of *ZNFX1*. In addition, as epigenetic

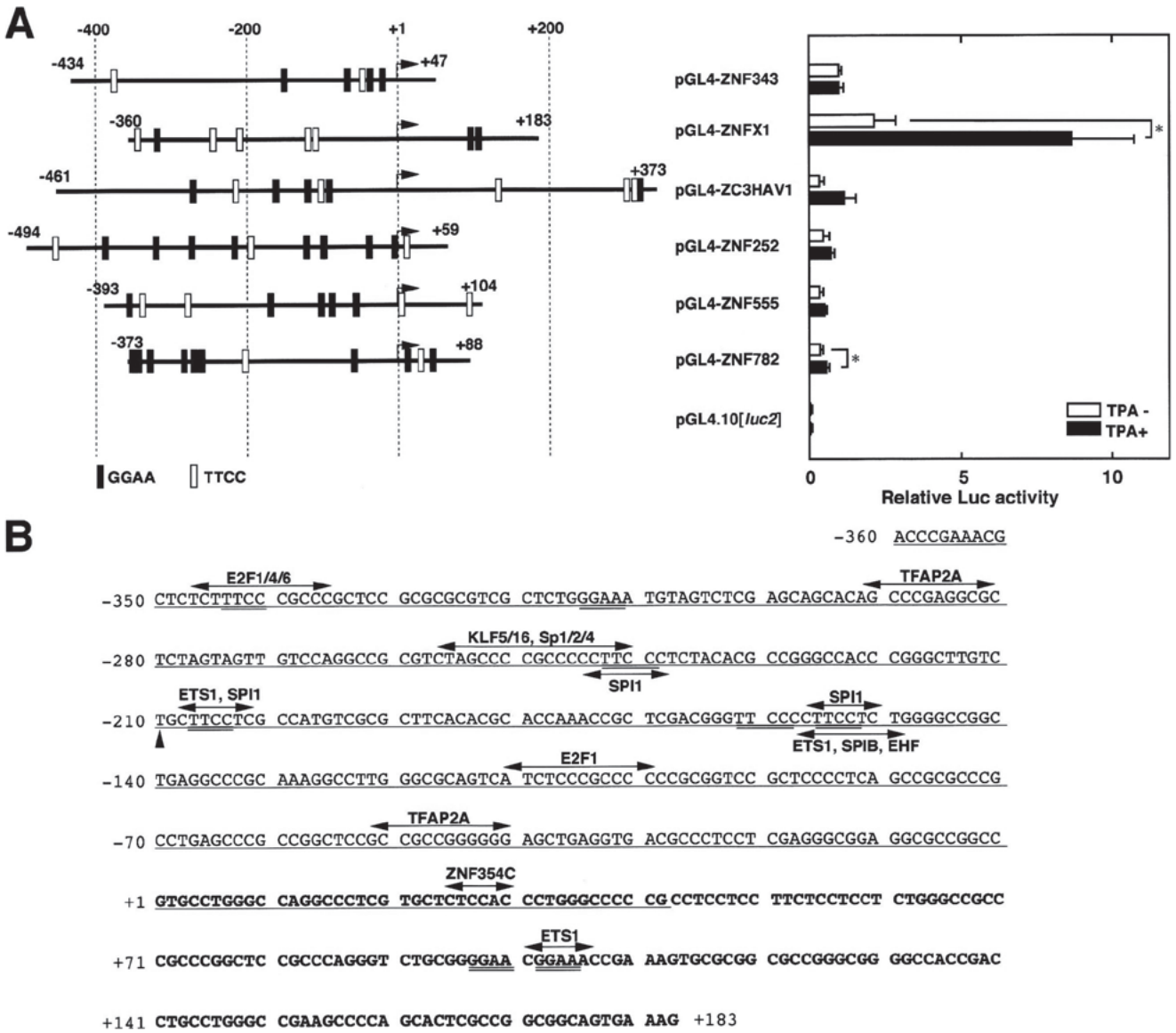

Figure 1. Promoter activities of the 5' upstream region of the human ZNF family protein encoding genes. (A) (Left panel) The 5' flanking sequence of the human ZNF protein-encoding genes, which has been ligated upstream of the Luc gene of the pGL4.10[luc2] vector, is shown. The most upstream 5'regions of the cDNA reported are designated +1. HL-60 cells were transiently transfected Luc reporter plasmids and they were treated with or without TPA (8.1 nM) for 24 h. Luc activities were normalized to that of the pGL4-ZNF343-transfected cells. Results are presented as the mean ± standard deviation from four independent experiments. (B) Nucleotide sequence of the 5' flanking sequence of the human *ZNFX1* gene. The nucleotide sequence of the 543-bp fragment that was obtained from PCR is shown. The most upstream 5' end of the *ZNFX1* cDNA (Gene ID: 57169, NM_021035.2, NC_018931.2, and NC_000020.11), which is typed in bold, is designated as nucleotide +1. Putative transcription factor binding sites that are predicted by JASPAR analysis with 97.5% relative scores are indicated by arrows. The GGAA and TTCC motifs are doubly underlined. The substituted nucleotide T at -210, which is identical to the Single Nucleotide Polymorphisms database (rs 3818066 A/G), is indicated by the arrowhead. Underlined characters represent overlap with the human *ZFAS1* gene (GENE ID: 441951), which is transcribed in the opposite direction to that of the *ZNFX1* gene. Statistical analysis was performed with a Student's t-test. *P<0.05. E2F1, eukaryotic translation termination factor 1; EHF, ETS homologous factor; ETS1, E26 transformation-specific 1; KLF5, Kruppel like factor 5; Luc, luciferase; SPI1, spleen focus forming virus proviral integration oncogene; SPIB, Spi-B transcription factor; TFAP2A, transcription factor AP-2 α; ZC3HAV1, zinc finger CCCH-type containing, antiviral 1; ZNF, zinc finger protein; ZNFX1, zinc finger nfx-1-type containing 1; ZFAS1, ZNFX1 antisense 1.

or post-transcriptional regulation could serve a role in the transcription of these genes, expression may not always be associated with promoter activities. Therefore, in this study, the 543-bp region upstream of the human *ZNFX1*, which is head-head oriented with the *ZFAS1* gene, was characterized. IFN treatment is known to affect HL-60 cell differentiation (30). Additionally, duplicated GGAA motifs are frequently found in 5'-flanking regions of the human interferon stimulated genes (ISGs) (9). It should be noted that TPA-responding duplicated GGAA motif is present 0.1-kb downstream of the putative TSS of the *ZNFX1* that belongs to the ISGs (31). We observed upregulation of the human *ZNFX1* gene and protein expression during HL-60 differentiation into macrophage-like cells. JASPAR analysis indicated that ETS1, MZF1, SPI1, and STAT1/STAT2 are candidate proteins to bind to the TPA-responding sequence, 5'-CTGCGGGGAACG GAAACCGAA-3'. Although MZF1 was listed, it may not be involved in the TPA response as its overexpression was reported to exhibit no effect on TPA-induced monocyte/macrophage differentiation of HL-60 cells (32). Given that STAT1 and ETS

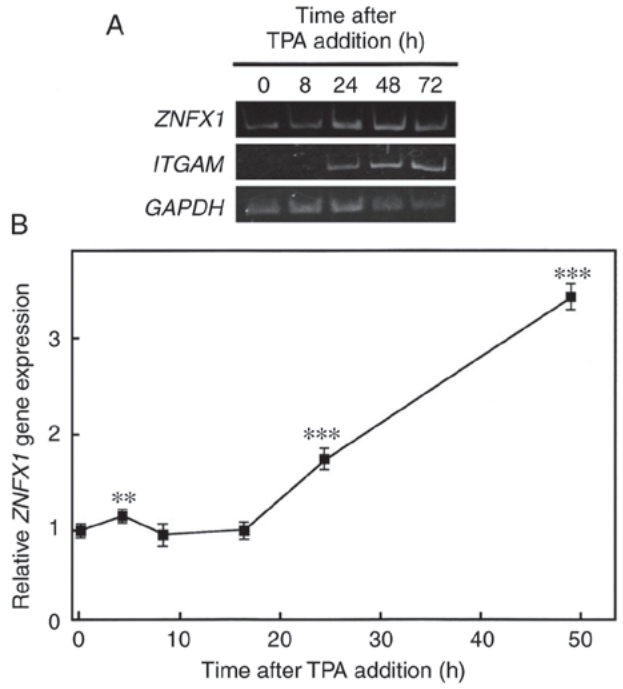

Figure 2. Effects of TPA on *ZNFX1* gene expression in HL-60 cells. (A) The culture medium of HL-60 cells was replaced with RPMI-1640 medium containing 10% fetal calf serum with 8.1 nM of TPA, and cells were harvested after 0, 8, 24, 48 and 72 h. Total RNAs were extracted from cells, and synthesized cDNAs were subjected to PCR with primer pairs to amplify *ZNFX1* (upper panel), *ITGAM* (middle panel) and *GAPDH* (lower panel) cDNA. (B) Quantitative PCR was conducted to analyze *ZNFX1* and *GAPDH* gene expression in HL-60 cells after 8.1 nM of TPA treatment for 0-48 h. The results indicated the relative *ZNFX1*/*GAPDH* gene expression ratio compared with that of TPA non-treated cells. Results are presented as the mean ± standard deviation from at least three independent experiments. One-way ANOVA was conducted followed by a Tukey's post-hoc test. $^{**}P<0.05$ and $^{***}P<0.01$ vs. 0 h sample. ITGAM, integrin subunit α M; TPA, 12-*O*-tetra decanoylphorbol-13-acetate; ZNFX1, zinc finger nfx-1-type containing 1.

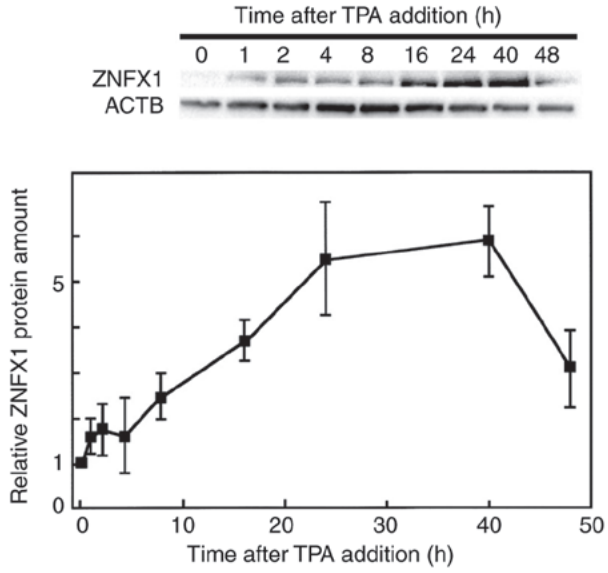

Figure 3. Effects of TPA on ZNFX1 protein levels in HL-60 cells. The culture medium of HL-60 cells was replaced with RPMI-1640 medium containing 10% fetal calf serum with 8.1 nM of TPA, and cells were harvested after 0, 1, 2, 4, 8, 16, 24, 40 and 48 h of TPA treatment. The extracted proteins were then separated by 15% SDS-PAGE, and western blotting was performed with primary antibodies against ZNFX1 and ACTB. Each band was quantified, and results show the relative ZNFX1/ACTB protein expression ratio. Results are presented as the mean ± standard deviation from three independent experiments. ATCB, β-actin; TPA, 12-*O*-tetra decanoylphorbol-13-acetate; ZNFX1, zinc finger nfx-1-type containing 1.

family proteins, which co-operatively regulate transcription of the *CD40* gene in microglia/macrophages (33,34), they may regulate *ZNFX1* gene expression. The JAK/STAT signaling pathway serves a role in the TPA-inducible expression of 2'-5'-oligoadenylate synthetase 1 (*OAS1*), which belongs to the ISGs, in HL-60 cells (35). Of note, the *OAS1* promoter possesses a duplicated GGAA motif responsive to the ETS family TF E74 like ETS transcription factor-1 in HeLa S3 cells (9). The TPA-responsive 21-bp region harbors similar sequences to the consensus binding motifs for ETS family type III proteins, including SPI1, SPIB, and SPIC, and type I proteins, including ETS1/2 and GABPα (14). GABP α/β protein targets the GGAA motif in the promoter region of mouse *RB1*, which is regulated during myogenesis (36). Spi1 (PU.1) and SPIB proteins occupy similar regions in the genome, suggesting they co-operatively regulate the expression of genes that regulate B cell development (37). An electrophoretic mobility shift assay (EMSA) with antibodies indicated that SPI1 (PU.1) binds to the TPA-responding regions of the human poly(ADP-ribose) glycohydrolase (38) and eukaryotic translation termination factor 4 (39) gene promoters. Although JASPAR analysis in our study suggested candidate, it has not been shown which TFs bind to the TPA-responding sequence. *ZNFX1* expression could be induced by combinations or the expression profiles of the duplicated GGAA-recognizing TFs, which include ETS family proteins and several DNA binding proteins (8). This should be elucidated in future experiments, such as EMSA, Footprint analysis, and chromatin immunoprecipitation assay, along with overexpression/knockdown experiments.

Binding of transcription suppressor ETS2 repressor factor to the GGAA motif is hindered by upregulation of the ETS transcription factor ERG in prostate cancer (40). Occupation or combinations of the GGAA motif binding proteins near TSSs play a role in both positive and negative regulation of cellular differentiation regulator encoding gene expression (8). In other words, if the binding of strong *trans*-activating TFs were replaced by weak ones after TPA treatment, the GGAA motif could be a suppressive element (8). This may partly explain the reason why the *ZC3HAV1*, *ZNF343*, *ZNF252*, *ZNF555* and *ZNF782* promoters exhibited marked activation in response to TPA treatment. Alternatively, specific elements near the TSSs could hinder the positive response of the duplicated GGAA motifs. Furthermore, TPA-inducible microRNAs (41) should be taken into account for regulation of gene expression.

TPA-inducible genes in HL-60 cells have been studied previously (42). The induction profile of the expression of the *RB1* gene, with a 5' flanking region has duplicated GGAA motifs (13), resembles that of the *ZNFX1* gene. Although, in this study, *RB1* gene expression was not analyzed, it has been reported to be induced at 10 h after TPA treatment (13). The expression of these two genes continued to increase after the TPA treatment for at least for 48 h when almost all cells attach to the culture dish (13). RB1, in association with E2F proteins, regulates the expression of various genes to control cell cycle

Table II. Putative transcription factor-binding sites in the TPA-responsive 21-bp element in the *ZNFX1* promoter.

Matrix ID	Gene	Score	Relative score	Start	End	Strand	Predicted sequence
MA0098.1	ETS1	7.79718	1	11	16	-	TTTCCG
MA0056.1	MZF1	8.50962	0.973735	4	9	+	CGGGGA
MA0080.1	SPI1	7.72714	0.958724	6	11	+	GGGAAC
MA0517.1	STAT1::STAT2	16.9526	0.927577	6	20	-	TCGGTTTCCGTTCCC

The sequence, 5'-CTGCGGGGAACGGAAACCGAA-3', which covers the TPA-responsive duplicated GGAA motif in the human *ZNFX1* promoter, was subjected to JASPAR database analysis, and the obtained data with 92% relative profile score threshold is shown. ETS1, E26 transformation-specific 1; MZF1, myeloid zinc finger 1; SPI1, spleen focus forming virus proviral integration oncogene; STAT1, signal transducer and activator of transcription 1; STAT2, signal transducer and activator of transcription 2; TPA, 12-*O*-tetra decanoylphorbol-13-acetate; ZNFX1, zinc finger nfx-1-type containing 1.

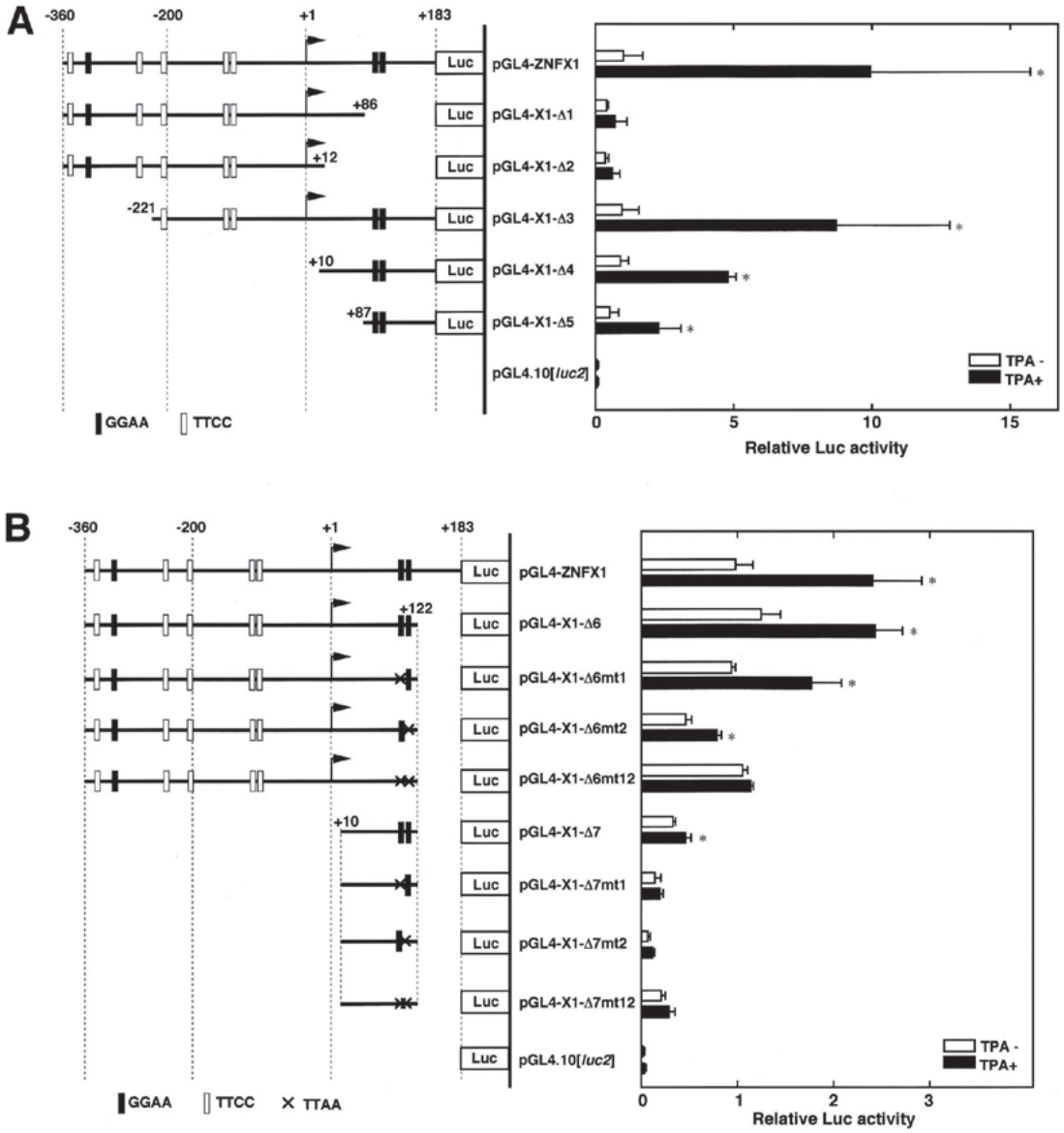

Figure 4. Deletion and mutation analysis of the 543-bp human *ZNFX1* promoter region. The 5' flanking sequence of the human *ZNFX1* gene, which has been ligated upstream of the *Luc* gene of the pGL4.10[*luc*2] vector, was presented (left panel). The putative 5' end of the cDNA is designated +1. The GGAA and TTCC sequences and the transcription factor binding elements that were predicted from the JASPAR program (score >97.5) are schematically shown as shaded and unshaded rectangles. (A) Luc reporter plasmids were transiently transfected into HL-60 cells and treated with or without TPA (8.1 nM), and then they were collected after 24 h incubation. (B) The transient transfection and Luc assay experiments were performed with pGL4-ZNFX1, pGL4-X1-Δ6, pGL4-X1-Δ6mt1, pGL4-X1-Δ6mt2, pGL4-X1-Δ6mt12, pGL4-X1-Δ7, pGL4-X1-Δ7mt1, pGL4-X1-Δ7mt2, and pGL4-X1-Δ7mt12. Histograms show relative Luc activities of deletion construct-transfected cells; results are presented as the mean ± standard deviation from at least three independent experiments. Statistical analysis was performed with a Student's t-test. *P<0.05 vs. TPA-. Luc, luciferase; TPA, 12-*O*-tetra decanoylphorbol-13-acetate; ZNFX1, zinc finger nfx-1-type containing 1.

progression (43,44). Interestingly, the human *ZNFX1* and *RB1* genes are under the control of bi-directional promoters, having *ZFAS1* and *LINC00441*as partner genes, respectively. The expression of the *ZFAS1* (*ZNFX1-AS1*) gene is downregulated in breast tumors (45), and its overexpression inhibits proliferation to induce the apoptosis of cancer cells (46), implying that ZFAS1 serves as a tumor suppressor. ZNFX1 protein expression increased until 40 h after treatment with TPA I our study, suggesting that it plays a role in sustaining the macrophage-like state of the HL-60 cells. Certain ZNFs, including zinc finger E-box binding homeobox 1, ZFP36, broad-complex, tramtrack and bric-à-brac-zinc finger, and ectopic virus integration site 1 are known to regulate cell differentiation (5,47-49). Additionally, epigenetic regulation should be considered in this process. In *Caenorhabditis elegans*, ZNFX1 functions as an RNA helicase that directs transgenerational epigenetic inheritance (50).

A potential cancer therapy with low toxic effect, or differentiation/apoptosis-inducing therapy, has been proposed (51,52). Providing that upregulation of both *ZNFX1* and *ZFAS1* genes affect the differentiation process, further investigations into the regulation of their expression may contribute to the establishment of differentiation-inducing gene therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HH, MY, HO, YK, and KN constructed the Luc reporter plasmids. HH and HO performed experiments and analyzed the data (transfection assay, RT-qPCR, western blotting, and statistical analysis). YM performed ANOVA. FU interpreted the data and wrote the manuscript. ST collected and analyzed/interpreted the data. TO and MA interpreted the data and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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