ISSN 1007-7626 中国生物化学与分子生物学报 http://cjbmb.bjmu.edu.cn 2012 年 1 月
CN 11-3870/Q Chinese Journal of Biochemistry and Molecular Biology 28(1):61~65

Binding Analysis of Transcription Factor Ets-1 with the Upstream Sequence of β1,3-N-Acetylglucosaminyltransferase Gene

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Abstract The expression of β1,3-N-acetyl glucosamonyltransferase-2 and-8 (β3GnT-2, β3GnT-8) genes, which involved in the biosynthesis of poly-N-acetyllactosamine (polyLacNAc) of cancer cells, was increased and associated with the malignant transformation. We have previously shown that the human β3GnT-2 and-8 genes were up-regulated during all-trans-retinoid acid (ATRA)-induced differentiation of human acute myeloid leukemia HL-60 cells. Here we investigated the mechanism of β3GnT-2 and-8 genes activation by the transcription factor Ets-1. RT-PCR analysis showed a significant increase of Ets-1 expression during ATRA-induced differentiation in HL-60 cells. Chromatin immuno-precipitation (ChIP) and electrophoretic mobility shift assay (EMSA) revealed Ets-1 to regulate β3GnT-2 and-8 transcription *in vivo* and *in vitro*. The results indicates that Ets-1 played an essential role in the transcriptional activation of the β3GnT-2 and β3GnT-8 genes.

Key words transcription factor Ets-1; β1,3-*N*-acetylglucosaminyltransferase; HL-60 cell lines; all-trans-retinoid acid (ATRA); chromatin immuno-precipitation(ChIP); electrophoretic mobility shift assay(EMSA)

转录因子 Ets-1 与 β1,3-N-乙酰氨基葡萄糖基转移酶基因启动子结合活性分析

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摘要 β1,3-N-乙酰氨基葡萄糖转移酶-2,-8(β3GnT-2, β3GnT-8)共同参与多聚 N-乙酰氨基乳糖($[Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3]_n$)的合成,从而使得细胞表面的相应糖链结构延长进而影响细胞的恶性转化.已有研究表明,在全反式维甲酸诱导人白血病细胞株 HL-60 分化过程中 β3GnT-2,-8 的表达上调,但其分子机制不明.本文旨在探讨 ATRA 诱导 HL-60 分化过程中,转录因子 Ets-1 对 β3GnT-2,-8 表达调控的分子机制.采用 10^{-6} mol/L ATRA 诱导人白血病细胞株 HL-60 向粒系分化,RT-PCR 检测到细胞中 Ets-1 的表达明显增加;进一步采用染色质免疫沉淀(ChIP)结合电泳迁移率变动实验(EMSA)检测证实,有活化的 Ets-1 结合至 β3GnT-2/-8 基因调控区.以上结果表明,转录因子 Ets-1 对人白血病细胞株 HL-60 分化过程中 β3GnT-2,-8 基因有表达调控作用.

关键词 转录因子 Ets-1; β1,3-*N*-乙酰氨基葡萄糖转移酶; HL-60 细胞系; 全反式维甲酸(ATRA); 染色质免疫沉淀(ChIP); 电泳迁移率变动实验(EMSA)
中图分类号 Q71

Received: September 26, 2011; Accepted: November 24, 2011
Supported by National Natural Science Foundation of China (No. 30670642)
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收稿日期: 2011-09-26; **接受日期**: 2011-11-24
国家自然科学基金(No. 30670642)**项目资助**
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N-acetylglucosaminyltransferase (GnT) including the β1→2, β1→3, β1→4 or β1→6 subfamilies, catalyzes the transfer of GlcNAc residue from UDP-β-D-GlcNAc to the acceptors of sugar chains. The β1→3GnT subfamily has eight glycosyltransferases that add GlcNAc to the Gal residue of glycoproteins and glycolipids. To date, three β1→3GnTs (β3GnT-2,-4,-8) are known to participate in the synthesis of poly-N-acetyl lactosamine (also called i antigen), which is the precursor of long chain Lewis antigens of sialyl difucosyl Lewis x, sialyl trifucosyl Lewis x and VIM-2 [1,2], etc. The β1→3 GnT-2 showed the strongest activity for poly-N-acetyllactosamine synthesis both *in vitro* and *in vivo*, whereas the β1→3GnT-4 has only a weak activity. The exact substrate of β1→3GnT-4 *in vivo* is yet unclear. The β1→3GnT-2 and-8 have been found to complement each other and synergistically activate poly-N-acetyllactosamine synthesis in cells [3].

Nearly 30 mammalian members of the Ets family have been isolated. The Ets signals are implicated in hematopoiesis and vasculogenesis/angiogenesis at very early stages of embryogenesis and involved in tissue development later on. Most of the Ets family members are at the downstream nuclear targets of Ras-MAPK signals and play important roles in cell development, differentiation and proliferation, as well as in apoptosis and tissue remodeling. The deregulation of Ets resulted in malignant transformation in various cells [4]. Several Ets genes are rearranged to generate chimeric oncoproteins in human leukaemia, Ewing's tumor and prostate cancer. The aberrant expression of several Ets genes was observed in different human malignant tumors. Ets-1, in particular, involved in malignant transformation and tumor progression (including invasion, metastasis and neoangiogenesis) transactivate cancer related genes, and was proposed as a potential target for selective cancer therapy [5].

HL60 cells can be induced for granulocytic differentiation by all-trans-retinoid acid (ATRA), an active physiological derivative of retinoid acid. We reported that β3GnT-2 and-8 were up-regulated and increased polyLacNAc at the cell surface during ATRA-induced differentiation of HL60 cells [5]. However, the underling mechanism for the transcriptional regulation remained unknown. We determined the expression of Ets-1 in HL-60 cells by RT-PCR during the myelocytic differentiation induced by ATRA. The interaction between Ets-1 and β3GnTs were examined by chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA).

1 Materials and Methods

1.1 Materials

HL60 leukemia cell line was obtained from Shanghai Institute of Cell Biology. Fetal calf serum (FCS), RPMI-1640 and IMEM mediums were purchased from GIBCO/BRL. Random primer (6 bases) was purchased from Promega Company. *Taq* E polymerase, dNTP mixture and PCR buffer were purchased from MBI Company. TRIzol, RNase Inhibitor, Superscript II RNase H Reverse Transcriptase and pUC Mix DNA Marker were from Invitrogen Company. Anti-Ets1 was the product of Abcam. ChIP Assay Kit, PCR Clean Up Kit/DNA Purification Kit, PMSF and SDS were from Beyotime Institute of Biotechnology. EMSA Kit was purchased from Viagene Biotech. The other reagents were commercially available in China.

1.2 Cell culture and treatment of ATRA

HL-60 cells were cultured in RPMI-1640 complete medium at a 37 ℃ humidified atmosphere containing 5% CO_2. The mediums are renewed every two and three days for the cells cultured in RPMI-1640. The cells were harvested when they were in logarithmic growth phase, and adjusted to $2 \sim 3 \times 10^6$ cells/mL, then subjected to RNA extraction, ChIP and EMSA. In the experiments using differentiation-agents, the cultured cells were adjusted to 5×10^5/mL. ATRA was added to the culture medium in a final concentration of 10^{-6} mol/L for 2 hours before the cells were harvested. In non-treated control cells, an equal volume of the ethanol was added instead of the differentiation-agents.

1.3 RNA extraction and RT-PCR for determination of Ets-1 expressions

Total RNA was extracted from cells using TRIzol/chloroform/isopropanol method according to the protocol provided by Promega. cDNAs were synthesized from 4 μg of the total RNA with 150 ng random primer, 25 mmol/L dNTP Mix 1 μL, 40 U RNase inhibitor, and 200 U Superscript II RNase H reverse transcriptase in 20 μL reaction mixture. The mixture was incubated at 37 ℃ for 60 min and 95 ℃ for 5 min. After the addition of 2 U RNase H, the PCR was performed in a volume of 25 μL containing 1 μL cDNA, 10 pmol/L primer pair of Ets-1 or GAPDH (loading control), 0.2 μmol/L of each dNTP, 5 IU *Taq* E polymerase and PCR buffer. The cDNA was subjected to denaturation at 94 ℃ for 5 min, followed by 25 cycles of PCR. Each cycle included denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 45 s and elongation at 72 ℃ for 75 s. Finally, the samples were further incubated for elongation at 72 ℃ for 7 min. After completion of the RT-PCR, 10 μl products or pUC Mix DNA marker were applied to 1.5% agarose gel for electrophoresis and stained by ethidium bromide. The intensities of the amplified DNA bands were scanned by ImageMaster System (UVP), including the analysis of the screened photos with NIH Image software. The quantification of the DNA bands was also performed with ImageMaster System. The

primers of Ets-1 and GAPDH were designed by using Genetyx software as well as GenBank Blast, and synthesized by Sheng-gong Bioengineering Company in Shanghai. The sequences of the forward (F) and reverse (R) primers were as follow, (1) Ets-1, F: 5′-GGGTGACGACTTCTTGTTTG-3′, R: 5′-GTTAAT GGAGTCAACCCAGC-3′. The length of the PCR product was predicted to be 512 bp; (2) GAPDH, F: 5′-AGAAGGCTGGGGCTCATT TG-3′, R: 5′-AGG GGCCATCCACAGTCTTC-3′. The length of the PCR product was predicted to be 258 bp. Three independent and reproducible experiments were performed and the mean ± SD was calculated.

1.4 ChIP assays and PCR amplification of β3GnT-2,-8

The ChIP assay was performed according to the kit instructions. Soluble chromatin from 2×10^6 HL-60 cells was incubated with 2 μg of anti-Ets1 affinity gel to get immunoprecipitation complexes. Purify and concentrate the complexes to 20 μL by PCR Clean Up Kit/DNA Purification Kit. The PCR was performed in a volume of 25 μL containing 1 μL purified ChIP product, 10 pmol/L primer pair of β3GnT-2, β3GnT-8 or β-actin (loading control), 0.2 μmol/L of each dNTP, 5 IU *Taq* E polymerase and PCR buffer. The target genes were amplified by 35 cycles of PCR. Each cycle included denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and elongation at 72 ℃ for 30 s. After completion of the PCR, 10 μL products or pUC Mix DNA Marker were applied to 1.5% agarose gel for electrophoresis and stained by ethidium bromide. The intensities of the amplified DNA bands were scanned by ImageMaster System (UVP), including the analysis of the screened photos with NIH Image software. The quantification of the DNA bands was also performed with ImageMaster System. The primers of β3GnT-2, β3GnT-8 and β-actin were designed by using Genetyx software as well as GenBank Blast, and synthesized by Sheng-gong Bioengineering Company in Shanghai. The sequences of the forward (F) and reverse (R) primers were as follow, (1) β3GnT-2, F: 5′-CCAGAAGGCAAGCAAT-3′, R: 5′-TCCCGATGAGGTCCAG-3′. The length of the PCR product was predicted to be 385 bp; (2) β3GnT-8, F: 5′-CCCTGACTTCGCCTCCTAC-3′, R: 5′-GGTC TTTGAGCGTCTGGTTGA-3′. The product was 362 bp; (3) β-actin, F: 5′-GAGCTACGAGCTGCCTGA CG-3′, R: 5′-CCTAGAAGCATTTGC GGTGG-3′. The product was 416 bp.

1.5 Electrophoretic Mobility Shift Assay

The sequences of the oligonucleotides used as probes in EMSAs were 5′ biotin-AACTTTGGGGGA GGAAGTCAGGGCTGGA (consensus Ets-1 probe) and 5′ biotin-AACTTTGGGGGAGGAAATCAGGGC TGGA (mutated Ets-1 probe). Cells (5×10^6) were detached from cultures by brief treatment with PBS, washed twice with PBS, then we used non-radioactive EMSA Kits (Viagene Biotech) to detect the activated Ets-1. Nuclear protein extracts were incubated with 1 μg poly (dI-dC) at 4 ℃ for 20 minutes in binding buffer. Then 0.5 μL biotin-labeled oligonucleotide probe was added and the reaction was incubated at 4 ℃ for 20 minutes. In competition studies, nuclear protein extracts were preincubated with 50-or 100-fold unlabeled probes at room temperature for 15 minutes and incubated with labeled probe at room temperature for 20 minutes. The reaction mixtures were electrophoresed on 4% polyacrylamide gels at 180 volts for 60 minutes and subjected to autoradiography.

2 Results

2.1 Expressions of Ets-1 in HL-60 cells during myelocytic differentiation

We performed RT-PCR to determine the Ets-1 expression during differentiation after the treatment with ATRA in HL-60 cells. The results showed that, Ets-1 was up-regulated at 2 hours by 3 folds following ATRA (Fig. 1, $P < 0.05$), suggesting that the induction of β3GnT-2,-8 might be regulated by Ets-1.

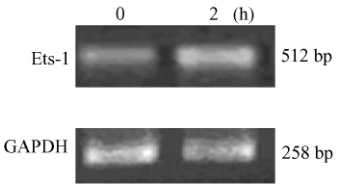

Fig. 1 RT-PCR analyses of Ets-1 expressions in HL-60 cells during myelocytic differentiation HL-60 cells were treated with or without ATRA for 2 hours. Total RNA was extracted using TRIzol/chloroform/isopropanol. GAPDH was used as a loading control for PT-PCR. Left lane: Untreated cells; Right lane: ATRA (10^{-6} mol/L) treated cells

2.2 Ets-1 protein regulates *β3GnT-2* and *β3GnT-8* genes

To determine whether Ets-1 transcription factor targeted to both β3GnT-2 and β3GnT-8 promoters, ChIP assay was performed using an anti-ETS1 antibody. As showed in Fig. 2, Ets-1 was acted on the promoters of both β3GnT-2 and β3GnT-8 genes. The results suggested that the Ets binding sites at the proximal region of both β3GnT-2 and β3GnT-8 promoters were functional.

2.3 The binding of nuclear proteins to the Ets-1 sequence in the *β3GnT-8* promoter by EMSA

Nuclear extracts from HL-60 cells resulted in specific EMSA bands (arrow in Fig. 3), whose specificity was determined by the ability of a 50 or 100-fold excess of unlabeled probe to compete for its binding(Fig. 3B). It should be noted that in Figure 3B, the mutated probe present in the β3GnT-8

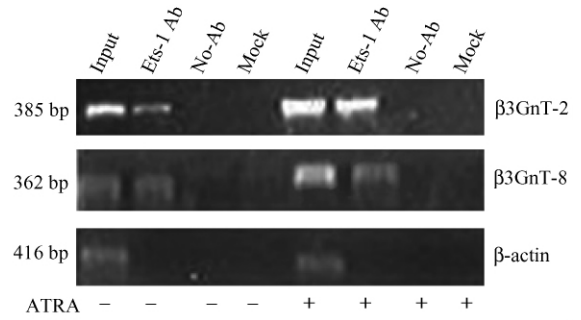

Fig. 2 ChIP assays to detect the effect of ATRA on the Ets-1 binding to the β3GnTs promoter in HL-60 cells
HL-60 cells were treated with or without ATRA for 2 hours. β-Actin was used as a control

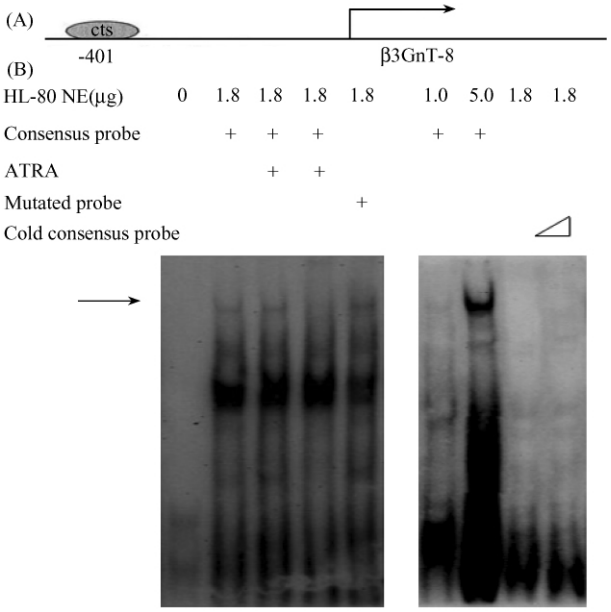

Fig. 3 Binding of HL-60 nuclear proteins to the EBS-containing β3GnT-8 promoter (A) Ets-1 binding elements in the β3GnT-8 gene promoter; (B) After different treatment, HL-60 nuclear proteins were incubated with biotin-labeled probes or non-labeled probes. The reaction mixtures were subjected to electrophoresis on 4% polyacrylamide gels

promoter did not intefere with the activated Ets-1 protein binding.

3 Discussion

The mRNA expression of both β3GnT-2 and β3GnT-8 were up-regulated in HL-60 cells during the ATRA-induced myelocytic differentiation [6], which was detectable as early as in 2 hours.

Glycosyltransferases were expressed in a tissue- and cell-specific manner and were regulated at the level of transcription under multiple promoters [7]. By transient cotransfection, Ets-1 was shown to bind the two Ets cis-acting elements and trans-activate the promoter activity of the human GnT-V (N-acetylglucosaminyl transferase V) gene in HuCC-T1 cells [8]. GnT-V catalyzes the transfer of N-acetyl glucosamine from UDP-N-acetylglucosamine to α-6-D-mannoside to produce the β1-6 linked branching of N-glycan oligosaccharides for the polylactosamine content [9]. Sato et al reported that Ets-1 similarly enhanced the expression of β-1,4-GalT V through the activation of Sp1 in cancer cells [10, 11]. We confirmed that Ets-1 regulated the expression of β3GnT-2, and-8 in ATRA-induced differentiation of HL-60 cells by ChIP and EMSA assays. With our previous study [12], Ets-1 is suggested to activate GnT-V, β-1,4-GalT V, and β3GnT-2,-8 genes, increases in polyLacNAc chain formation in differentiated HL-60 cells, thus to affect the biological characteristics and functions of cells with the change sugar chain structures.

Acknowledgements We thank Dr. YR Liu for his excellency of the technical support.

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