Sequential Action of Ets-1 and Sp1 in the Activation of the Human β-1,4-Galactosyltransferase V Gene Involved in Abnormal Glycosylation Characteristic of Cancer Cells*

Received for publication, December 28, 2006, and in revised form, July 24, 2007 Published, JBC Papers in Press, July 26, 2007 Published, JBC Papers in Press, July 26, 2007 DOI 10.1074/jbc.M611862200

Takeshi Sato and Kiyoshi Furukawa

From the Laboratory of Glycobiology, Department of Bioengineering, Nagoya University of Technology, Kamitomioka 1603-1, Nagaoka 940-2188, Japan

Malignant transformation is associated with increased gene expression of β-1,4-galactosyltransferase (β-1,4-GalT) V, which contributes to the biosynthesis of highly branched N-linked oligosaccharides characteristic of cancer cells. Our previous study showed that expression of the human β-1,4-GalT V gene is regulated by Sp1 (Sato, T., and Furukawa, K. (2004) J. Biol. Chem. 279, 39574–39583), and a subsequent study showed that the gene expression is also activated by Ets-1, a product of the oncogene (Sato, T., and Furukawa, K. (2005) Glycoconj. J. 22, 365). Herein we report the mechanism of β-1,4-GalT V gene activation by these transcription factors. The gene expression and promoter activity of β-1,4-GalT V increased when the ets-1 cDNA was transfected into HepG2 cells, which contain a large amount of Ets-1. Luciferase assays using deletion constructs of the promoter showed that promoter region −116 to +22 is critical for the transcriptional activation of the gene by Ets-1. Despite the presence of one Ets-1-binding site, which overlaps the Sp1-binding site, electrophoretic mobility shift assays showed that the region bound preferentially to Sp1 rather than to Ets-1. To solve this problem, we examined the transcriptional regulation of the human Sp1 gene by Ets-1 and found that the gene expression and promoter activity of Sp1 are regulated by Ets-1 in cancer cells. Functional analyses of two Ets-1-binding sites in the Sp1 gene promoter showed that only Ets-1-binding site −413 to −404 is involved in the activation of the gene by Ets-1. These results indicate that Ets-1 enhances expression of the β-1,4-GalT V gene through activation of the Sp1 gene in cancer cells.

The glycosylation of cell-surface proteins changes dramatically upon malignant transformation of cells (reviewed in Ref. 1). Such changes are brought about by the altered expression of glycosyltransferases at the transcriptional and translational levels. For example, increased amounts of highly branched N-linked oligosaccharides containing the Galβ1→4GlcNAcβ1→6Man structure, which is synthesized by N-acetylgalactosaminyltransferase (GlcNAcT)2 V and β-1,4-galactosyltransferase (β-1,4-GalT) (2, 3), are associated with the tumorigenic and metastatic potentials of cancer cells (4–6). Mink lung epithelial cells have been shown to acquire a tumorigenic potential when transfected with GlcNAcT V cDNA (7). Furthermore, tumor growth and metastasis of mammary carcinoma cells as induced in GlcNAcT V gene-disrupted mice by the polyoma virus middle T oncogene are suppressed (8). Therefore, it is very important to elucidate the regulatory mechanisms of the gene expression of the glycosyltransferases involved in the biosynthesis of the Galβ1→4GlcNAcβ1→6Man structure in cancer cells.

Seven homologous genes designated β-1,4-GaIT V–I1VII have been isolated by several groups, including us (reviewed in Refs. 9 and 10). We isolated the human β-1,4-GalT V cDNA (GenBankTM accession number AB004550) from human breast cancer cells (11). When β-1,4-GalT V is expressed in SF9 insect cells with N-linked oligosaccharides terminated predominantly with GlcNAc, the GlcNAc residues are galactosylated by β-1,4-GalT V as revealed by lectin blot analysis (12). On the other hand, transfection of the antisense β-1,4-GalT V cDNA into SW480 human colorectal adenocarcinoma cells and SH-SY5Y human neuroblastoma cells results in the reduced galactosylation of N-linked oligosaccharides (12, 13). β-1,4-GalT V has been shown to galactosylate preferentially the GlcNAcβ1→6Man group to the GlcNAcβ1→2Man group using cell lysates as an enzyme source (12, 14), suggesting that β-1,4-GalT V is involved in the biosynthesis of the outer chain moieties of N-linked oligosaccharides. When we examined changes in the expression of the β-1,4-GalT I–VI genes upon malignant transformation of cells, we observed that the expression level of the β-1,4-GalT V gene increased only in the transformed cells (14), which also correlated with that of GlcNAcT V in several human cancer cell lines (15). Quite interestingly, the tumorigenicity of B16-F10 mouse melanoma cells was reduced by the introduction of antisense β-1,4-GalT V cDNA into cells when transplanted subcutaneously into C57BL/6 mice.2 In support of

---

2 The abbreviations used are: GlcNAcT, N-acetylgalactosaminyltransferase; β-1,4-GalT, β-1,4-galactosyltransferase; RT, reverse transcription; DN, dominant-negative; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation GA2 (asialo-GM2), GalNAcβ1→4Galβ1→4Glcβ1→1ceramide; GM2, GalNAcβ1→4(NeuAcα2⇒3)Galβ1→4Glcβ1→1ceramide; GD2, GalNAcβ1→4(NeuAcα2⇒8)NeuAcα2⇒3Galβ1→4Glcβ1→1ceramide; GT2, GalNAcβ1→4(NeuAcα2⇒8)NeuAcα2⇒8 NeuAcα2⇒3Galβ1→4Glcβ1→1ceramide.

3 K. Shirane, T. Sato, S. Furukawa, Y. Kobayashi, K. Wada, N. Takahashi, K. Kato, and K. Furukawa, manuscript in preparation.
this, the invasion of SHG44 human glioma cells has been shown to be suppressed by transfection with antisense β,1,4-GalT V cDNA (16). These findings indicate that the expression of the β,1,4-GalT V gene is associated with the tumorigenic and invasive potentials of cancer cells. Therefore, it is of interest to investigate the regulatory mechanism of β,1,4-GalT V gene expression in cancer cells.

The expression of the GlcNAcT V gene has been shown to be regulated by Ets family transcription factors, including Ets-1 and Ets-2, in cancer cells (17–19). Our previous study showed that the expression of the GlcNAcT V gene in cancer cells (47). However, it remains unknown how these two transcription factors are involved in the transcriptional activation of the β,1,4-GalT V gene. In this study, we address the regulatory mechanism of the β,1,4-GalT V gene in cancer cells with Sp1 and Ets-1.

**EXPERIMENTAL PROCEDURES**

Cell Cultures—A549 human lung carcinoma cells and HepG2 human hepatocarcinoma cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. SH-SY5Y human neuroblastoma cells were grown at 37 °C in a mixture of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1.0 mg/ml glucose.

**Plasmids**—The human ets-1 cDNA was amplified by reverse transcription (RT)-PCR as described previously (12). In brief, a total RNA preparation was obtained from SH-SY5Y cells with an RNaseq total RNA system (Qiagen Inc., Hilden, Germany), from which single-strand cDNAs were prepared using a hexadecyloxyribonucleotide mixture (oligo(dN)6, TaKaRa, Shiga, Japan) as a primer and Ready-To-Go™ You-Prime First-Strand Beads (Amersham Biosciences). The cDNA encoding the full-length coding sequence of the human ets-1 gene was amplified by PCR (95 °C for 10 min; 40 cycles at 94 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 1.5 min; and at 72 °C for 15 min) with AmpliTaq GoldTM (PerkinElmer Life Sciences) using the single-strand cDNAs as templates and 5’- and 3’-primers specific to the ets-1 gene. The following 5’- and 3’-primer pairs were designed based on the sequence of the human ets-1 gene (GenBank™ accession number J04101) (22): TSET-1, 5’-GGG-GTACCCACATTAGGGGCCGCCGT-3’; and TSET-2, 5’-GGGGGGCCCGCCATCAGTGCGGAGCTG-3’. For amplification of the cDNA encoding dominant-negative (DN) Ets-1, which contains a DNA-binding domain corresponding to amino acids 306–441 but lacks a transcriptional activation domain (see Fig. 2), TSET-5 (5’-GGGGTGACCATGGACTATGTGCGGGACCGTG-3’) and TSET-2 were used for PCR. TSET-5 was designed to contain an initiation codon for the appropriate translation of DN-Ets-1. The newly synthesized KpnI and Apal sites in the primers are underlined.

**Activation of β,1,4-GalT V Transcription by Ets-1**

**Cell Transfection**—One day prior to transfection, the cell lines (2 × 10⁵ cells each) were seeded into 60-mm tissue culture dishes. A549 cells were transfected with 10 μg of pcDNA3.1 or pcDNA/ets-1 and FuGENE 6 transfection reagent (Roche Applied Science). Similarly, HepG2 cells were transfected with 10 μg of pcDNA3.1 or pcDNA/DN-ets-1 and FuGENE 6 transfection reagent. Cells were harvested 48 h after transfection. Total RNA preparations were obtained from the cell lines using an RNaseq total RNA system. Northern blot analysis was performed with a [32P]-labeled human ets-1, DN-ets-1, β,1,4-GalT V, or Sp1 cDNA fragment as described previously (11, 14). The gene expression levels of Sp1 were analyzed by RT-PCR (60 °C for 30 min; 94 °C for 2 min; 24, 26 and 28 cycles at 94 °C for 1 min and 60 °C for 1.5 min; and 60 °C for 7 min) using the total RNA (1 μg each) and oligonucleotide primers specific to the Sp1 gene (TS127, 5’-GCCGAGCAGCAAC-GACTCTCAAC-3’; and TS128, 5’-GTCTTGCCATA-CCTTCCCACAG-3’) as described previously (15).

**Western Blot Analysis**—Goat anti-Sp1 (PEP 2) and rabbit anti-Sp1 (H-225) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Western blot analysis using anti-Sp1 and anti-β-tubulin antibodies was performed as described previously (24, 25). The band intensity was quantified with NIH Image) analysis software (Version 1.37v).
Activation of β-1,4-GalT V Transcription by Ets-1

Luciferase Assay—To assay the promoter activity of the β-1,4-GalT V gene, a 2.3-kb genomic fragment containing the 5′-region of the human β-1,4-GalT V gene was isolated from a human placenta genomic library (13) and inserted into a firefly luciferase reporter vector, pGL3-Basic, which contains neither a eukaryotic promoter nor an enhancer element. The unaltered plasmid (pGL3-Basic) was used as a promoterless control, and the plasmid pRL-TK (Promega Corp.), which contains the Renilla luciferase gene driven by the herpes simplex virus thymidine kinase promoter, was used as a normalization control to correct for variable transfection efficiencies. One day prior to transfection, 1 × 10⁵ cells from each cell line were seeded into 35-mm tissue culture dishes and transfected with 1 μg of the reporter plasmid, 0.1 μg of pRL-TK, and FuGENE 6. Cells were harvested 48 h after transfection, lysed in 200 μl of lysis buffer, and subjected to freeze-thaw lysis. The firefly or Renilla luciferase activity was determined as described previously (13).

The results show the means ± S.E. of three experiments.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA were performed according to the instructions for the LightShift chemiluminescent EMSA kit (Pierce) as described previously (13). In brief, a nuclear extract was prepared from SH-SY5Y cells with NE-PER nuclear and cytoplasmic extraction reagents (Pierce) containing multiple protease inhibitors according to the manufacturer’s instructions. The following oligonucleotides were used for EMSAs: probe 5′-CTGGCCCGCCGCTCCCCCGGTCGGCCC-3′; Ets-1 consensus, 5′-CTCGATCTCGAGCAAGAAATCCGATG-3′; and Sp1 consensus, 5′-CCTTGGTGGGGCGCGGGCCTAAGCCTG-3′. The synthesized oligonucleotides were 3′-end-labeled with biotin-N4-[32P]ATP and terminal deoxynucleotidyltransferase according to the instructions for the biotin 3′-end DNA labeling kit (Pierce), and the biotinylated complementary oligonucleotides were then annealed to generate the double-stranded oligonucleotides as probes. The binding reaction was performed by preincubating 4 μl of nuclear extract with 50 ng of poly(dl-dC) in buffer solution containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, and 1 mM dithiothreitol for 10 min at room temperature. Approximately 30–50 fmol of probe was added, and the reaction mixtures were incubated for 20 min at room temperature. Subsequently, the samples were separated from the free probes by electrophoresis on 6% nondenaturing polyacrylamide gels and then electrophoretically transferred to Hybond N⁺ membranes (Amersham Biosciences). To detect DNA-protein complexes, the membranes were incubated with streptavidin-conjugated horseradish peroxidase and then visualized with LightShift luminol/enhancer solution and LightShift stable peroxide solution (Pierce) according to the manufacturer’s instructions. For competition analyses, unlabeled Ets-1 or Sp1 consensus oligonucleotides were added at 100-fold molar excess prior to addition of the biotinylated probes. To identify the transcription factor comprising the DNA-protein complexes, supershift assay was performed as described previously (13).

Enzyme-linked Immunosorbent Assay—The following Ets-1 consensus oligonucleotides were used for enzyme-linked immunosorbent assay: oligonucleotide A, 5′-AAGCATATCCGCCATTCCTCGTGGTGCCC-3′; and oligonucleotide B, 5′-CCCATCTTACACTTCTCGTCCATCTTCA-3′ (Ets-1-binding sites are underlined). Synthesized oligonucleotides were 5′-end-labeled with biotin maleimide and T4 polynucleotide kinase according to the instructions for the 5′-EndTag nucleic acid labeling system (Vector Laboratories, Burlingame, CA), and the complementary oligonucleotides were then annealed to generate double-stranded oligonucleotides. A 96-well microtiter immunoplate (MaxiSorp, Nalge Nunc International, Rochester, NY) was coated with anti-Ets-1 antibody (Santa Cruz Biotechnology, Inc.) blocked with 0.5% bovine serum albumin and then incubated with 10 μg of nuclear extracts from SH-SY5Y cells, which show significant expression of Ets-1. After extensive washing of the wells with phosphate-buffered saline and 0.05% Tween 20, the biotinylated oligonucleotides (12.5 pmol) were incubated for 60 min at room temperature. To detect the DNA-protein complexes, the wells were incubated with streptavidin-conjugated horseradish peroxidase and then with 3,3′-diaminobenzidine and hydrogen peroxide for 30 min. For measurement of the background level, the same experiment was conducted except that the nuclear extracts were excluded. The absorbance of each well at 490 nm was determined using an automated microtiter plate spectrophotometer (ImmuonMini NJ-2300, Nalge Nunc International).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed according to the instructions of Upstate (Temecula, CA). In brief, 1 × 10⁶ SH-SY5Y cells were treated with 1% formaldehyde at 37°C for 10 min and then harvested. The collected cells were resuspended in 200 μl of SDS lysis buffer (50 mM Tris-HCl (pH 8.1) containing 1% SDS, 10 mM EDTA, and protease inhibitors) and incubated for 10 min on ice. The cell lysates were sonicated with four sets of 30-s pulses using a Model UCD-200 ultrasonic generator (Tosho Electronic Corp., Tokyo, Japan) on ice and then centrifuged. The supernatant of the cell lysates was diluted 10-fold in ChIP dilution buffer (16.7 mM Tris-HCl (pH 8.1) containing 0.01% SDS, 1% Triton X-100, 2 mM EDTA, 167 mM NaCl, and protease inhibitors). Chromatin solutions (1 ml) were incubated overnight at 4°C with 2.5 μg of anti-Ets-1 antibody. For negative controls, no antibody or rabbit IgG was utilized for immunoprecipitation. The immune complexes were collected with 30 μl of protein A-agarose/salmon sperm DNA (50% slurry) at 4°C for 1 h with rotation. The beads were pelleted by centrifugation and washed sequentially for 5 min by rotation with 500 μl of the following buffers: low salt wash buffer (20 mM Tris-HCl (pH 8.0) containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl), high salt wash buffer (20 mM Tris-HCl (pH 8.0) containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 500 mM NaCl), and LiCl wash buffer (10 mM Tris-HCl (pH 8.1) containing 0.25 M LiCl, 1% Igepal CA-630, 1% sodium deoxycholate, and 1 mM EDTA). Finally, the beads were washed twice with 500 μl of TE buffer (10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA). The immune complexes were eluted by addition of 125 μl of 1% SDS in 0.1 M NaHCO₃ to the pelleted beads, and then incubated at room temperature for 15 min. This process was repeated, and the eluates were combined. Then, 10 μl of 5 M NaCl was added, and the mixture was incubated at 65°C for 6 h. The remaining proteins were digested with proteinase K and incubated at 45°C for 1 h. The DNA was recovered by phenol/chloroform extraction and then ethanol precip-
The potential binding sites of Ets-1 are indicated by hatched ovals. The transcriptional start site is indicated by an arrow.

**TABLE 1**

| Consensus core sequence | β-1,4-GalT V sequence (positions) |
|--------------------------|-----------------------------------|
| (A/C) GGA (A/T)          | tgaTCCGgc (−1921 to −1912)        |
|                          | ccAGGAgagg (−1763 to −1754)       |
|                          | atcCCCTCGGC (−574 to −565)        |
|                          | gcCCCTCGGC (−76 to −67)           |
|                          | ccAGGAggcg (+118 to +127)          |

The nucleotide positions are indicated relative to the transcriptional start site of the gene. Uppercase letters within the β-1,4-GalT V sequences indicate the core sequences for Ets-1-binding sites.

**RESULTS**

**Analysis of Putative Binding Sites of Ets-1 in the Promoter Region of the Human β-1,4-GalT V Gene**—We cloned the 5′-flanking region of the human β-1,4-GalT V gene from a human genomic library (GenBank™ accession number AB067772) (13). To identify the putative binding sites of transcription factors, including Ets-1, a 2.3-kb 5′-flanking region of the human β-1,4-GalT V gene was analyzed using the MatInspector program (26). Five Ets-1-binding sites in the 5′-flanking region of the human β-1,4-GalT V gene (−1921 to −1912, −1763 to −1754, −574 to −565, −76 to −67, and +118 to +127 relative to the transcriptional start site of the gene) were identified (Fig. 1 and Table 1).

**Effect of Ets-1 or DN-Ets-1 on the Expression of the β-1,4-GalT V Gene**—The cDNAs encoding Ets-1 and DN-Ets-1, which contains a DNA-binding domain but lacks a transcriptional activation domain (Fig. 2), were amplified by PCR, and the expression constructs pcDNA/ets-1 and pcDNA/DN-ets-1 were obtained. Because A549 cells have been shown to produce a small amount of Ets-1 among several cancer cell lines (19), the cells were used to examine the effect of Ets-1 on the gene expression of β-1,4-GalT V. A549 cells were transiently transfected with pcDNA/ets-1 or pcDNA3.1 as a control and cultured for 48 h. The total RNA preparations (20 μg each) from the mock- and ets-1 cDNA-transfected cells were subjected to electrophoresis on a 2.2% agarose gel, stained with EtBr, and visualized using a UV light lamp. The results showed no significant differences in the amounts of 18 S and 28 S rRNAs for either total RNA preparation (Fig. 3A, lower panel). The total RNAs were blotted onto a Hybond N+ membrane by capillary diffusion, and Northern blot analysis was conducted. The results showed significant expression of the ets-1 gene with a molecular size of 1.3 kb in the ets-1 cDNA-transfected cells, whereas no expression of the endogenous ets-1 gene with a molecular size of 6.8 kb was detected in the mock- and ets-1 cDNA-transfected cells (Fig. 3A, upper panel). Under the same conditions, the expression of the β-1,4-GalT V gene increased by 4–6-fold in the ets-1 cDNA-transfected cells compared with that in the mock-transfected cells (Fig. 3A, middle panel).

DN-Ets-1 was shown to suppress the expression of the Ets-1-regulated genes encoding GlcNAcT V (19) and urokinase-type plasminogen activator (27). No significant differences in the amounts of 18 S and 28 S rRNAs were observed for either total RNA preparation (Fig. 3B, lower panel). When Northern blot analysis was performed using total RNA preparations (10 μg each) from mock- and DN-ets-1 cDNA-transfected HepG2 cells, which contain a large amount of endogenous Ets-1 among several cancer cell lines (19), the gene expression of β-1,4-GalT V decreased significantly compared with the mock-transfected cells (Fig. 3B, middle panel). Under the same conditions, a significant level of DN-ets-1 gene expression was detected (Fig. 3B, upper panel). These results indicate that the expression of the β-1,4-GalT V gene is regulated by Ets-1.

**Effect of Ets-1 or DN-Ets-1 on the Promoter Activity of the β-1,4-GalT V Gene**—To examine the effect of Ets-1 on the promoter activity of the β-1,4-GalT V gene, a construct containing the full-length promoter (pGL(−2099/+170)) was constructed as described previously (13). Cotransfection of pGL(−2099/+170) with pcDNA/ets-1 into A549 cells resulted in a 2-fold stimulation of promoter activity (Fig. 4A). In contrast, when SH-SY5Y and HepG2 cells were cotransfected with pGL(−2099/+170) and pcDNA/DN-ets-1, the promoter activities decreased dramatically to 5–10% of the control (Fig. 4B). These results indicate that the promoter activation of the human β-1,4-GalT V gene is regulated by Ets-1.
Activation of β-1,4-GalT V Transcription by Ets-1

Identification of the Ets-1-responsive Promoter Region in the β-1,4-GalT V Gene—To identify the Ets-1-responsive promoter region, various deletion constructs (pGL(-2099/+170), pGL(-1121/+170), pGL(-531/+170), pGL(-116/+170), and pGL(+23/+170)), as shown in Fig. 5A (left panel), together with pcDNA/ets-1, were transiently transfected into A549 cells, and promoter activation by Ets-1 was examined. The results showed that significant promoter activities and those Ets-1-responses with 2-fold activation were associated with pGL(-2099/+170), pGL(-1121/+170), pGL(-531/+170), and pGL(-116/+170), whereas no significant activity and activation were observed for pGL(+23/+170) (Fig. 5A, right panel), indicating that as long as the reporter construct contains region −116 to +22, the promoter is activated by Ets-1. Furthermore, pGL(-2099/+170), pGL(-1121/+170), pGL(-531/+170), pGL(-116/+170), and pGL(+23/+170), together with pcDNA/DN-ets-1, were transiently transfected into HepG2 cells, and the promoter suppression by DN-Ets-1 was examined. Significant promoter activities were detected in pGL(-2099/+170), pGL(-1121/+170), pGL(-531/+170), and pGL(-116/+170), and these promoter activities decreased to ~20% of the control upon DN-ets-1 transfection; but no significant activity was observed for pGL(+23/+170) (Fig. 5B). Similar results were obtained using SH-SY5Y cells (data not shown). These results indicate that the region between nucleotides −116 and +22, which contains one putative Ets-1-binding site, is responsible for the Ets-1-induced transcriptional activation of the β-1,4-GalT V gene in cancer cells.

Identification of Transcription Factors Bound to the Ets-1-responsive Promoter Region of the β-1,4-GalT V Gene—In the Ets-1-responsive promoter region, one Ets-1-binding site at nucleotides −76 to −67 was found to overlap with the Sp1-binding site at nucleotides −81 to −69 (Fig. 6A). To examine whether or not Ets-1 binds to promoter region −83 to −58 of the β-1,4-GalT V gene, an EMSA was conducted using oligonucleotide probe −83/−58, which contains one Ets-1-binding site and one Sp1-binding site (Fig. 6A). Because the β-1,4-GalT V gene promoter is highly activated in SH-SY5Y cells among the cancer cell lines examined (13) and because almost equal amounts of Ets-1 and Sp1 are contained in SH-SY5Y cells (data not shown), SH-SY5Y cells were used for the EMSA. Probe −83/−58 formed DNA-protein complexes with a nuclear extract of SH-SY5Y cells (Fig. 6B, lanes 1 and 6). The mobility of
Activation of β-1,4-GalT V Transcription by Ets-1

The DNA-protein complexes with Ets-1 or Sp1 probe was examined using the biotinylated Ets-1 or Sp1 consensus oligonucleotide as a probe. The results showed that a major DNA-protein complex with the Ets-1 probe moved faster than the complex with the Sp1 probe (Fig. 6B, lanes 2 and 3). On the basis of these results, we considered the mobility of a major DNA-protein complex with probe −83/−58 to be the same as that of a major DNA-protein complex with the Sp1 probe, indicating that the complex is formed with probe −83/−58 and Sp1. When competition analyses were performed with a 100-fold molar excess of either unlabeled Sp1 or Ets-1 consensus oligonucleotides, the formation of the complex was markedly reduced only by incubation with the Sp1 consensus oligonucleotides (Fig. 6B, lanes 4 and 5). Moreover, a major complex was detected as a supershifted band by incubation with anti-Sp1 antibody (Fig. 6B, lane 7). However, no supershifted band was detected by incubation with anti-Ets-1 antibody (data not shown). These results show that probe −83/−58 binds to Sp1, but not to Ets-1, indicating that Sp1 binds to the Ets-1-responsive promoter region of the β-1,4-GalT V gene.

Regulation of Sp1 Gene Expression by Ets-1—Our previous study showed that the expression of the β-1,4-GalT V gene is regulated by Sp1 and that Sp1 plays an essential role in the promoter activation of the β-1,4-GalT V gene in cancer cells (13). The above results suggest that the activation of the β-1,4-GalT V gene by Ets-1 is mediated by Sp1. Therefore, we examined whether or not the expression of the Sp1 gene is regulated by Ets-1. When blots containing total RNA preparations from mock- and ets-1 cDNA-transfected A549 cells were rehybridized with the Sp1 cDNA as a probe after removing the β-1,4-GalT V cDNA probe, a significant increase in the expression of the Sp1 gene with a molecular size of 8.2 kb was observed in the ets-1 cDNA-transfected cells compared with the mock-transfected cells (Fig. 7A, upper panel). In accordance with the increased expression of the Sp1 gene, the expression of the Sp1 protein with a molecular size of 95 kDa was increased in the ets-1 cDNA-transfected cells (Fig. 7D, upper panel). Therefore, we concluded that the increased expression of the Sp1 gene results in the increased expression of the β-1,4-GalT V gene in A549 cells. However, when blots containing total RNA preparations from mock- and DN-ets-1 cDNA-transfected HepG2 cells were rehybridized with the Sp1 cDNA as a probe after removing the β-1,4-GalT V cDNA probe, a faint signal with a molecular size of 8.2 kb was detected in both gene-transfected cells, but the signals could not be quantified. Therefore, the gene expression of Sp1 was analyzed by RT-PCR using oligonucleotide primers specific to the Sp1 gene. The results showed a marked decrease in Sp1 gene expression in the DN-ets-1-transfected cells compared with the mock-transfected cells (Fig. 7C, upper panel), whereas the expression levels of the glyceraldehyde-3-phosphate dehydrogenase gene in the cells remained constant (lower panel). The linearity of the amounts of RT-PCR products was confirmed by conducting the analysis with three different cycles. In accordance with the decreased expression of the Sp1 gene, the expression of the Sp1 protein with a molecular size of 95 kDa decreased in the DN-ets-1 cDNA-transfected cells (Fig. 7D, upper panel). Therefore, we concluded that the decreased expression of the Sp1 gene results in the decreased expression...
Activation of β-1,4-GalT V Transcription by Ets-1

A 295-bp fragment of the β-1,4-GalT V gene, that of the β-1,4-GalT V gene in HepG2 cells. These results indicate that the expression of the Sp1 gene is regulated by Ets-1.

Regulation of the Sp1 Gene Promoter by Ets-1—Although the nucleotide sequence of the 5′-flanking region of the human Sp1 gene has been reported (23), no Ets-1-binding site has yet been identified. To identify the putative binding sites of transcription factors, including Ets-1, a 1.6-kb 5′-flanking region of the Sp1 gene was analyzed using the MatInspector program (26). Two Ets-1-binding sites in the promoter region of the Sp1 gene (−413 to −404 and −291 to −282 relative to the transcriptional start site of the gene) were identified (Fig. 8A and Table 2). To examine whether or not the promoter of the Sp1 gene is activated by Ets-1, the construct pGL-Sp1(−480/−2), which contains two Ets-1-binding sites, was transiently cotransfected with pcDNA/ets-1 into A549 cells. After transfection, the cells were cultured for 48 h, and luciferase activity was assayed using cell extracts. The results showed that the ectopic coexpression of Ets-1 stimulated the promoter activation of the Sp1 gene by 3-fold (Fig. 8B). On the other hand, when pGL-Sp1(−480/−2) was transiently cotransfected with pcDNA/DN-ets-1 into HepG2 cells, the ectopic coexpression of DN-Ets-1 decreased the promoter activation of the Sp1 gene to 67% of the control (Fig. 8C). The promoter activity of the β-1,4-GalT V gene was decreased dramatically by DN-Ets-1. In contrast to the promoter activity of the β-1,4-GalT V gene, that of the Sp1 gene was decreased by 67% of the control by DN-Ets-1. Because the promoter activity of the Sp1 gene is regulated mainly by Sp1 (23), partial suppression of the Sp1 gene promoter by DN-Ets-1 might be observed. This indicates that the contribution of Ets-1 to the promoter activation of the Sp1 gene is <40%. Similar results were obtained using several other cancer cell lines (data not shown). These results suggest that the promoter of the Sp1 gene is regulated not only by Sp1, but also by Ets-1.

Identification of the Ets-1-binding Site for Promoter Activation of the Sp1 Gene by Ets-1—To analyze the binding of Ets-1 to Ets-1-binding sites −413 to −404 and −291 to −282 in the Sp1 gene promoter, an enzyme-linked immunosorbent assay using anti-Ets-1 antibody and biotinylated oligonucleotides containing Ets-1-binding sites was performed. The biotinylated oligonucleotides were incubated with streptavidin-conjugated horseradish peroxidase and then with o-phenylenediamine and hydrogen peroxide. The absorbance of each well at 490 nm was measured in a microtiter plate spectrophotometer. The results showed that higher absorbance was associated with oligonucleotides A and B compared with the Ets-1 consensus oligonucleotides used as a control (Fig. 9A), indicating that Ets-1 can bind to both Ets-1-binding sites in the Sp1 gene promoter. To demonstrate that Ets-1 binds to the promoter region of the Sp1 gene in vivo, ChIP assay was performed. Chromatin fragments from SH-SYSY cells were immunoprecipitated with anti-Ets-1 antibody, and DNA from the immunoprecipitates was isolated. A 295-bp fragment of the Sp1 promoter region was amplified by PCR using the DNA as a template (Fig. 9B), and the results showed that Ets-1 bound to the promoter region of the Sp1
Gene in vivo. To identify which Ets-1-binding site is involved in the Ets-1 activation of the Sp1 gene, three constructs with mutations in the Ets-1-binding sites (pGL-Sp1mET1, pGLSp1mET2, and pGL-Sp1mET3), as shown in Fig. 10 (left panel), together with pcDNA/ets-1, were transiently transfected into A549 cells, and the promoter activation by Ets-1 was examined. The results showed significant promoter activity associated with pGL-Sp1(−480/−2) that was activated 3-fold by Ets-1 (Fig. 10, right panel). When the mutation was introduced in Ets-1-binding site −413 to −404, the promoter activity and its activation by Ets-1 were nullified completely (Fig. 10, right panel). This indicates that Ets-1-binding site −413 to −404 is essential for the promoter activation of the Sp1 gene. Following ets-1 transfection, a 2-fold increase in promoter activity was observed for pGL-Sp1mET2-transfected cells (Fig. 10, right panel), indicating that Ets-1-binding site −291 to −282 is not involved in the promoter activation of the Sp1 gene by Ets-1.

These results indicate that only Ets-1-binding site −413 is −404 in the promoter region of the Sp1 gene is involved in the activation of the gene by Ets-1.

When the mutation is introduced into the Sp1-binding site of the β-1,4-GalT V gene promoter, no Sp1 binds to the Sp1-binding site (13). To show the importance of the binding of Sp1 to the Sp1-binding site for the promoter activation of the β-1,4-GalT V gene by Ets-1, the pGL(−116/+170)-Sp1 mutation was transiently transfected with pcDNA/ets-1 into A549 cells. The results showed that a significant activation by Ets-1 was observed for pGL(−116/+170)-transfected cells, whereas no activation by Ets-1 was observed for pGL(−116/+170)-Sp1 mutation-transfected cells (Fig. 11), indicating that the binding of Sp1 to the Sp1-binding site in the β-1,4-GalT V gene promoter is essential for promoter activation by Ets-1. In conclusion, this study has demonstrated that Ets-1 enhances the expression of the β-1,4-GalT V gene through activation of the Sp1 gene in cancer cells.
Activation of β-1,4-GalT V Transcription by Ets-1

**DISCUSSION**

The Galβ1→4GlcNAcβ1→6Man structure in highly branched N-linked oligosaccharides, which is formed by the sequential action of GlcNAcT V and β-1,4-GalT V, is associated with the malignant phenotypes of cancer cells (4–6). To elucidate the effective biosynthesis of such oligosaccharides, it is necessary to determine the regulatory mechanisms of the gene expression of the transferases at transcriptional levels. The expression of the GlcNAcT V gene has been shown to be regulated by Ets-1 (17–19). Our previous study showed that the GlcNAcT V gene expression of the Fas ligand (28) and platelet-derived growth factor A (29) when both of the Sp1- and Ets-1-binding sites are present independently in the promoter regions and bound to Sp1 and Ets-1, respectively. In the case of the β-1,4-GalT V gene, however, one Ets-1-binding site in the Ets-1-responsive promoter region was found to overlap the Sp1-binding site, and EMSA clearly showed that Sp1, but not Ets-1, can bind to the region. We hypothesized that the activation of Sp1 by Ets-1 enhances β-1,4-GalT V gene expression and examined whether or not the expression of the Sp1 gene is regulated by Ets-1. Our results clearly show that the gene expression and promoter activity of Sp1 are regulated by Ets-1. Therefore, we concluded that Ets-1 enhances the expression of the β-1,4-GalT V gene through activation of the Sp1 gene. On the basis of our results and previous reports, we propose that the expression of the Galβ1→4GlcNAcβ1→6Man structure in highly branched N-linked oligosaccharides is regulated by Ets-1 through activation of the GlcNAcT V and β-1,4-GalT V genes.

Besides the β-1,4-GalT V and GlcNAcT V genes, the gene expression of human β-1,2-GlcNAcT II, which transfers GlcNAc to Manα1→6Manβ1→R (where R is the remainder of the N-linked oligosaccharide), and the human α-1,3-fucosyltransferase IV gene, which is responsible for the synthesis of the Lewis X structure (Galβ1→4(Fuco1→3)GlcNAcβ1→), have been shown to be regulated by Ets-1 and Ets-2 (30) and by Elk-1 (31), which is a member of the Ets family of transcription factors, respectively. The expression of the β-1,4-GalT I gene during mouse spermatogenesis has been shown to be regulated by TASS-1, which can bind to GGA1/A/T sequences like Ets-1 (32). Furthermore, the expression of the mouse UDP-Gal:Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→Manα1→6Manβ1→R (where R is the remainder of the N-linked oligosaccharide) glycoside family of genes has also been shown to be activated by Ets-1 (33).

The *ets-1* gene was originally identified as a cellular counterpart of the *v-ets* retroviral gene, one of two oncogenes in the E26 avian erythroblastosis virus (34), and is known to be expressed preferentially in lymphocytes (35). The targeted disruption of the *ets-1* gene in mouse results in reduced numbers of T cells, a defect upon T cell receptor stimulation, and an increased proportion of IgM+ cells (36, 37), indicat-
ing that Ets-1 is essential for maintaining the normal pool of resting T and B lineage cells.

The malignant behaviors, invasion, and metastasis of cancer cells involve a large number of molecules such as angiogenic factors, growth factors and their receptors, adhesion molecules, proteinases, intracellular signaling molecules, and transcription factors. Because Ets-1 has been shown to activate the transcription of a number of angiogenesis-, invasion-, and metastasis-associated genes (reviewed in Refs. 20 and 21), Ets-1 is considered to play critical roles in cancer progression. The expression of abnormally activated Ets-1 has been observed in human gastric carcinoma (38), hepatocellular carcinoma (39), lung carcinoma (40), and ovarian carcinoma (41). Elevated Ets-1 expression has also been shown to correlate with malignancy and reduced survival of patients with ovarian carcinoma (41). In human gastric, hepatocellular, and lung carcinomas, the expression of the β-1,4-GalT V gene increases compared with normal counterparts.4 The elevated expression of the β-1,4-GalT V gene could be due to the expression of abnormally activated Ets-1 in these carcinomas.

Transfection of mouse NIH3T3 cells with the ets-1 cDNA results in the formation of colonies in soft agar and the induction of tumors in nude mice (42). Moreover, transfection of the ets-1 cDNA into Bel-7402 hepatocellular carcinoma cells results in marked increases in the expression levels of the c-met, matrix metalloproteinase-1, matrix metalloproteinase-9, and urokinase-type plasminogen activator genes in response to the increased invasive activity of the cells (43). These results indicate that an abnormal expression of Ets-1 is associated with the malignant phenotypes of cancer cells. In contrast, the expression levels of the c-met, matrix metalloprotease-1, matrix metalloprotease-9, and urokinase-type plasminogen activator genes in Bel-7402 cells decrease, and the invasive activity of the cells is suppressed when the cells are transfected with the antisense Ets-1 oligonucleotide (43). When the DN-ets-1 cDNA is transfected into U251 human glioma cells, the expression levels of the urokinase-type plasminogen activator, integrin α5, and integrin β3 genes are markedly reduced, and the experimental metastasis of the cells into the livers of chick embryos is suppressed dramatically (27). However, the N-glycosylation of Bel-7402 and U251 cells has not yet been studied. It will be of interest to investigate whether or not the biosynthesis of the Galβ1 →4GlcNAcβ1 →6Man structure in N-linked oligosaccharides characteristic of cancer cells is inhibited by the antisense Ets-1 oligonucleotide and DN-Ets-1 and whether or not such a change in N-glycosylation results in the suppression of invasion and metastasis.

In summary, this study has shown, for the first time, that the gene expression of Sp1 is regulated by Ets-1 in cancer cells. Because the downstream target molecules of Ets-1 and Sp1 have been shown to be involved in tumor growth, invasion, and metastasis (43–46), the malignant phenotypes of cancer cells can be suppressed by regulating the expression level of the ets-1 gene in cancer cells. Our study has focused on the development of an effective system to control the expression of the ets-1 gene in cancer cells for eventual application to cancer therapy.

---

4 T. Sato and K. Furukawa, unpublished data.

REFERENCES

1. Kobata, A. (1984) in Biology of Carbohydrates (Ginsburg, V., and Robbins, P. W., eds) Vol. 2, pp. 87–161, John Wiley & Sons, Inc., New York
2. Yamashita, K., Okhura, T., Tachibana, Y., Takasaki, S., and Kobata, A. (1998) J. Biol. Chem. 273, 10834–10840
3. Pierce, M., and Arango, I. (1986) J. Biol. Chem. 261, 10772–10777
4. Dennis, J. W., Laferte, S., Wagborne, C., Brettman, M. L., and Kebel, R. S. (1987) Science 236, 582–585
5. Dennis, J. W., and Laferte, S. (1989) Cancer Res. 49, 945–950
6. Asada, M., Furukawa, K., Segawa, K., Endo, T., and Kobata, A. (1997) Cancer Res. 57, 1073–1080
7. Demetriou, M., Nabi, I. R., Coppolino, M., Dedhar, S., and Dennis, J. W. (1995) J. Cell Biol. 130, 383–392
8. Granovsky, M., Fata, J., Pawling, J., Muller, W. J., Khokha, R., and Dennis, J. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 95, 472–477
9. Amado, M., Almeida, R., Schwientek, T., and Clausen, H. (1999) Biochim. Biophys. Acta 1473, 35–53
10. Furukawa, K., and Sato, T. (1999) Biochim. Biophys. Acta 1473, 54–66
11. Sato, T., Furukawa, K., Bakker, H., Van den Eijnden, D. H., and Van Die, I. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 472–477
12. Guo, S., Sato, T., Shirane, K., and Furukawa, K. (2001) Glycobiology 11, 813–820
13. Sato, T., and Furukawa, K. (2004) J. Biol. Chem. 279, 39574–39583
14. Shirane, K., Sato, T., Segawa, K., and Furukawa, K. (1999) Biochem. Biophys. Res. Commun. 265, 434–438
15. Sato, T., Shirane, K., Kido, M., and Furukawa, K. (2000) Biochem. Biophys. Res. Commun. 276, 1019–1023
16. Jiang, J., Chen, X., Shen, J., Wei, Y., Wu, T., Yang, Y., Wang, H., Zong, H., Yang, J., Zhang, S., Xie, J., Kong, X., Liu, W., and Gu, J. (2006) J. Biol. Chem. 281, 9482–9489
17. Kang, R., Saito, H., Ibara, Y., Miyoshi, E., Koyama, N., Sheng, Y., and Taniguchi, N. (1996) J. Biol. Chem. 271, 26706–26712
18. Buckhout, P., Chen, L., Fregien, N., and Pierce, M. (1997) J. Biol. Chem. 272, 19575–19581
19. Ko, J. H., Miyoshi, E., Noda, K., Ekuni, A., Kang, R., Ikeda, Y., and Taniguchi, N. (1999) J. Biol. Chem. 274, 22941–22948
20. Dittmer, J., and Nordheim, A. (1998) Biochim. Biophys. Acta 1377, F1–F11
21. Sementchenko, V. I., and Watson, D. K. (2000) Oncogene 19, 6533–6548
22. Watson, D. K., McWilliams, M. J., Lapis, P., Lautenberger, J. A., Schweinfest, C. W., and Papas, T. S. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7862–7866
23. Nicolas, M., Noe, V., Jensen, K. B., and Ciudad, C. J. (2001) J. Biol. Chem. 276, 22126–22132
24. Sato, T., Furukawa, K., Greenwalt, D. E., and Kobata, A. (1993) J. Biochem. (Tokyo) 114, 890–900
25. Sato, T., Takahashi, M., Kawada, T., Takayama, E., and Furukawa, K. (2005) Eur. J. Pharmac. Sci. 25, 221–227
26. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) Nucleic Acids Res. 23, 4878–4884
27. Kita, D., Takino, T., Nakada, M., Takahasi, T., Yamashita, J., and Sato, H. (2001) Cancer Res. 61, 7985–7991
28. Kavurma, M. M., Bobryshev, Y., and Khachigian, L. M. (2002) J. Biol. Chem. 277, 36244–36252
29. Santiago, F. S., and Khachigian, L. M. (2004) Circ. Res. 95, 479–487
30. Zhang, W., Revers, L., Pierce, M., and Schachter, H. (2000) Biochem. J. 347, 511–518
31. Withers, D. A., and Hakomori, S. (2000) J. Biol. Chem. 275, 40588–40593
32. Charron, M., Shaper, N. L., Rajput, B., and Shaper, J. H. (1999) Mol. Cell. Biol. 19, 5823–5832
33. Raimondi, L. P., Daniotti, I. L., and Maccioni, H. J. F. (2004) FEBS Lett. 576, 487–491
34. Leprince, D., Gégonne, A., Coll, L., de Taisne, C., Schneeberger, A., Lagrou, C., and Stenelin, D. (1983) Nature 306, 395–397
35. Bhat, N. K., Komschlies, K. L., Fujiiwara, S., Fisher, R. J., Mathieson, B. I., Gregorio, T. A., Young, H. A., Kasik, J. W., Ozato, K., and Papas, T. S. (1989) J. Immunol. 142, 672–678
36. Bories, J. C., Willerford, D. M., Grevin, D., Davidson, L., Camus, A., Martin, P., Stehelin, D., and Alt, F. W. (1995) *Nature* 377, 635–638
37. Muthusamy, N., Barton, K., and Leiden, J. M. (1995) *Nature* 377, 639–642
38. Nakayama, T., Ito, M., Ohtsuru, A., Naito, S., Nakashima, M., Fagin, J. A., Yamashita, S., and Sekine, I. (1996) *Am. J. Pathol.* 149, 1931–1939
39. Ito, Y., Miyoshi, E., Takeda, T., Sakon, M., Noda, K., Tsujimoto, M., Monden, M., Taniguchi, N., and Matsuura, N. (2000) *Am. J. Clin. Pathol.* 114, 719–725
40. Bolon, I., Gouyer, V., Devouassoux, M., Vandenbunder, B., Wernert, N., Moro, D., Brambilla, C., and Brambilla, E. (1995) *Am. J. Pathol.* 147, 1298–1310
41. Davidson, B., Reich, R., Goldberg, I., Gotlieb, W. H., Kopolovic, J., Berner, A., Ben-Baruch, G., Bryne, M., and Nesland, J. M. (2001) *Clin. Cancer Res.* 7, 551–557
42. Seth, A., and Papas, T. S. (1990) *Oncogene* 5, 1761–1767
43. Jiang, Y., Xu, W., Lu, J., He, F., and Yang, X. (2001) *Biochem. Biophys. Res. Commun.* 286, 1123–1130
44. Lietard, J., Musso, O., Theret, N., L’Helgoualch’, A., Campion, J. P., Yamada, Y., and Clement, B. (1997) *Am. J. Pathol.* 151, 1663–1672
45. Qin, H., Sun, Y., and Benveniste, E. N. (1999) *J. Biol. Chem.* 274, 29130–29137
46. Zhu, G. H., Lenzi, M., and Schwartz, E. L. (2002) *Oncogene* 21, 8477–8485
47. Sato, Ti, and Furukawa, K. (2005) *Glycoconj. J.* 22, 365