Regulation of expression of the human β -1,2-*N*-acetylglucosaminyltransferase II gene (*MGAT2*) by Ets transcription factors

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Oncogenic transformation of fibroblasts by the *src* oncogene has long been known to cause an increase in the size of cell-surface protein-bound oligosaccharides, owing primarily to increased N-glycan branching mediated by increased β -1,6-*N*-acetylglucosaminyltransferase V (GnT V) activity. The *src*-responsive element of the GnT V promoter was localized to Ets-binding sites and the promoter was transcriptionally stimulated by both *ets-1* and *ets-2* expression [Buckhaults, Chen, Fregien and Pierce (1997) J. Biol. Chem. **272**, 19575–19581; Kang, Saito, Ihara, Miyoshi, Koyama, Sheng and Taniguchi (1996) J. Biol. Chem. **271**, 26706–26712]. Because GnT V action requires the prior action of β -1,2-*N*-acetylglucosaminyltransferase II (GnT II) and the human GnT II promoter contains four putative Ets-binding sites [Chen, Zhou, Tan and Schachter (1998) Glycoconj. J. **15**, 301–308], GnT II might also be under oncogenic control via Ets

transcription factors. We now report that co-transfection into HepG2 or COS-1 cells of either *ets-1* or *ets-2* expression plasmids together with chimaeric GnT II promoter–chloramphenicol acetyltransferase plasmids results in a 2–4-fold stimulation of promoter activity. Mobility-shift assays and South-Western blots localized the functional Ets-binding site to one of the four putative sites on the GnT II promoter. The GnT II promoter, unlike the GnT V promoter, is not activated by either *src* or *neu*. Therefore although both promoters are stimulated by a member of the Ets family of transcription factors, the functional role of this Ets transcriptional control seems to be different for the two genes.

Key words: N-acetylglucosaminyltransferase II promoter, cancer, gene expression, N-glycans, oncogenes.

INTRODUCTION

One of the most common alterations in transformed or metastatic malignant cells is the presence of large highly branched N-glycans associated with an increased activity of UDP-GlcNAc:R₁Man α 1-6R₂ [GlcNAc to Man α 1-6] β -1,6-*N*-acetylglucosaminyltransferase V (GnT V; EC 2.4.1.155), which adds a GlcNAc residue in β 1-6 linkage to the Man α 1-6 residue of GlcNAc β 1-2Man α 1-6Man β 1-R (where R is the remainder of the N-glycan structure) [1–7]. Cells transformed with polyoma virus, Rous sarcoma virus or T24 H-*ras* [4,7–10] or treated with transforming growth factor β or phorbol ester [11] show significantly increased GnT V activity. GnT V transcription is stimulated by transfection of cells with *src* oncogene or with Ets-2 transcription factor [12,13], or with Ets-1 [14–16] or with *her-2/neu* oncogene [17].

UDP-GlcNAc: α -6-D-mannoside β -1,2-*N*-acetylglucosaminyltransferase II (GnT II; EC 2.4.1.143) is a medial Golgi enzyme that catalyses the incorporation of a GlcNAc residue in β -1,2 linkage to the Man- α -1,6 arm of the N-glycan core and is an essential step in the biosynthetic pathway leading from hybrid to complex N-glycans [18]. Because GnT II action is an essential prerequisite of GnT V action [19], and because the promoters of human GnT II [20] and GnT V [12,21] both have putative binding sites for the products of the *ets* and *c-myc* families of proto-oncogenes, we initiated the present study to determine whether the GnT II promoter, like the GnT V promoter, is activated by the products of the *src* and *ets* genes.

The Ets family of transcription factors is characterized by a conserved 84-amino-acid DNA-binding region termed the ‘ets

domain’ found in proteins from humans to *Drosophila* [22,23] and was first identified by sequence similarity to the *v-ets* oncogene in the genome of the E26 avian erythroblastosis retrovirus [22,24–26]. Ets proteins bind specifically to 10 bp DNA sequences that contain an invariant (C/A)GGA(A/T) motif. Ets activity requires the co-operation of other transcription factors, for example Ets-1 and Ets-2 require AP-1 (activator protein 1) transcription factors (Fos/Jun) for activation of the oncogene-responsive unit of the polyoma virus enhancer [22] and Ets-1 interacts co-operatively with SP1 in the human T-cell lymphotropic virus long terminal repeat [23,27]. Various growth modulators affect Ets activity at the levels of gene transcription, post-translational modification and protein stability [22]. Like many other oncoproteins, Ets family members seem to be involved in the development of *Drosophila* and other species [22]. Both Ets-1 and Ets-2 can be activated by the Ras–Raf–MEK [MAP (mitogen-activated protein) kinase/ERK kinase]–ERK (extracellular signal-regulated protein kinase) pathway [28].

The human [29], rat [30], pig [31] and *Caenorhabditis elegans* (J. Tan and H. Schachter, unpublished work) GnT II genes have been cloned. The human GnT II gene (*MGAT2*) is on chromosome 14q21 [29] and is on a single exon. The gene has a GC-rich 5′ untranslated region, consensus sequences suggestive of multiple binding sites for Sp1, multiple transcription initiation sites and a CCAAT box, but lacks a TATA box [20]. These properties and the wide tissue distribution of the gene [29] indicate that it is a constitutively expressed housekeeping gene. Ablation of the GnT II gene in mice results in the birth of stunted mice that die shortly after birth [32]. Inactivating mutations of the GnT II gene in humans cause carbohydrate-deficient glycoprotein syndrome

Abbreviations used: CAT, chloramphenicol acetyltransferase; GnT II, UDP-GlcNAc: α -6-D-mannoside β -1,2-*N*-acetylglucosaminyltransferase II; GnT V, UDP-GlcNAc:R₁Man α 1-6R₂ [GlcNAc to Man α 1-6] β -1,6-*N*-acetylglucosaminyltransferase V.

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type II, a disease involving serious malformation of the nervous and other systems [33]. An analysis of deletions of the GnT II promoter showed that the main promoter region lies in an 84 bp region 553 bp upstream of the ATG start codon [20]. This region contains four putative Ets-binding sites. Here we report co-transfection, gel mobility-shift and South-Western blotting experiments showing that one of these four sites is a functional Ets-binding site. However, unlike the GnT V promoter, the GnT II promoter is not activated by either the *src* or the *neu* oncogenes. The functional role of Ets transcriptional control therefore seems to be different for the two genes. Although human GnT II is a typical housekeeping gene, our findings indicate that in some circumstances the gene might be under the control of Ets transcription factors. A preliminary report of this work has appeared [34].

MATERIALS AND METHODS

Cultured cell lines

All cultured cell lines were grown in a humidified incubator at 37 °C in air/CO₂ (19:1). HepG2 (a human hepatocellular carcinoma cell line) and COS-1 (an African green monkey kidney cell line transformed with simian virus 40) were grown in α -minimum essential medium supplemented with 10% (v/v) fetal bovine serum.

Molecular biology procedures and plasmids

Unless otherwise stated, all molecular biology procedures were performed with standard techniques [35,36]. PCR was used to synthesize chimaeric constructs containing sections of the 5' untranslated region of the gene for GnT II fused to a chloramphenicol acetyltransferase (CAT) reporter gene [20]. Plasmids p-1000CAT and p-680CAT contained respectively GnT II promoter fragments from -1000 to -276 bp and from -680 to -276 bp relative to the ATG start codon at +1, subcloned into the pCAT-Basic vector (Promega). Competent *Escherichia coli* DH5 α cells (Gibco/BRL) transformed with plasmid were inoculated into Luria-Bertani medium containing 0.050 mg/ml ampicillin and were grown overnight at 37 °C. Plasmid DNA was purified with the QIAprep Spin Plasmid Kit (Qiagen) by following the manufacturer's protocol. The purity of the DNA was determined by the ratio of A_{260} to A_{280} ; a ratio of 1.8 was considered pure. A chimaeric construct pGL2-TV3, containing a section of the GnT V gene promoter fused to a luciferase reporter gene, was synthesized as described previously [12]. Plasmids pRK5-Ets2, pRK5 Δ 1-328Ets2 and pRK5 were kindly provided by Dr K. E. Boulukos (University of Nice, Nice, France) [37]; pRK5-Ets2 and pRK5 Δ 1-328Ets2 are eukaryote cell expression plasmids encoding respectively Ets-2 or a dominant-negative deletion mutant of Ets-2 (Δ 1-328Ets-2) subcloned into pRK5. Plasmids pEVRF-ets-1, pEVRF0 and pET-ets-1 were gifts from Dr B. J. Graves (University of Utah School of Medicine, Salt Lake City, UT, U.S.A.), pEVRF-ets-1 is an eukaryote cell expression plasmid encoding Ets-1 subcloned into pEVRF0, and pET-ets-1 is a bacterial expression plasmid encoding Ets-1. The pSV-Src and pcDNA3/*neu* eukaryote cell expression plasmids were gifts from Dr Tony Hunter (Salk Institute for Biological Studies, La Jolla, CA, U.S.A.) and Dr Lin Chen (University of Georgia, Athens, GA, U.S.A.) respectively. Purified FliB and SapB (Ets family proteins) were kindly supplied by Dr Cheryl H. Arrowsmith (Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada).

Analysis of transcriptional regulation by using the CAT reporter system

Cells [(5–7) $\times 10^5$] were seeded in six-well culture plates or 60 mm tissue culture plates. Adherent cells were transfected 24 h after seeding at 80–90% confluence. Reporter constructs (p-680CAT or p-1000CAT) (0.5 μ g) were co-transfected with either pSV40- β -galactosidase plasmid (Promega) (0.5 μ g) or the luciferase expression plasmid pGL3-Control Vector (Promega) (0.5 μ g) to determine transfection efficiency, in both the absence and the presence of various effector plasmids and their controls (pRK5-Ets2, pRK5 Δ 1-328Ets2, pRK5, pEVRF-ets-1, pEVRF0, pSV-Src and pcDNA3/*neu*) (0.5–1.5 μ g) [20]. The various DNA mixtures were precipitated with ethanol and dissolved in TE buffer (10 mM Tris/HCl containing 1 mM EDTA) at pH 7.4. pCAT-Basic vector (Promega) was added to the transfection mixtures to ensure the same total DNA content in each transfection.

For transfection into COS-1 cells, growth medium containing neither serum nor antibiotics was added to the DNA mixture (5–10 μ g of total DNA) to a final volume of 0.1–0.15 ml. SuperFect Transfection Reagent (Qiagen) was added (5–6 μ l per μ g of DNA) and the mixture was incubated at room temperature for 10 min to allow complex to form. Growth medium containing serum and antibiotics (1 ml) was added to the DNA-SuperFect complex and the mixture was added to COS-1 cells that had previously been washed once with PBS. After incubation for 3 h in a humidified incubator at 37 °C in air/CO₂ (19:1), the cells were washed once with PBS, new growth medium containing serum and antibiotics was added and incubation was continued for a further 48 h.

The calcium phosphate method was used for transfection into HepG2 cells. Growth medium was changed 4 h before transfection. Deionized distilled water was added to the DNA mixture (5–10 μ g of total DNA) to a final volume of 0.36 ml; 0.040 ml of 2.5 M CaCl₂ was added and the mixture was added dropwise with a Pasteur pipette to 0.4 ml of aerated 2 $\times$ HBSP [1.5 mM Na₂HPO₄/10 mM KCl/0.28 M NaCl/12 mM glucose/50 mM Hepes (pH 7.05), filtered through a 0.22 μ m filter]. The solution was immediately vortex-mixed for 5 s and left at room temperature for 20 min. The resulting precipitate was spread evenly over the cells and mixed gently into the growth medium. After incubation for 4 h in a humidified incubator at 37 °C in air/CO₂ (19:1), the cells were collected and shocked for 2 min with 15% (v/v) glycerol. The glycerol was removed and the cells were washed twice with PBS and once with medium followed by the addition of 4 ml of fresh medium and incubation for a further 48 h.

Transfections were also performed with pCAT-Basic as negative controls. Three to six independent transfections, in duplicate wells, were performed for each experiment. After transfection, medium was removed and the cells were washed three times with PBS, followed by the addition of 1 ml of TEN buffer [40 mM Tris/HCl (pH 7.5)/1 mM EDTA/150 mM NaCl] and incubation for 5 min at room temperature. Cells were collected by scraping, pelleted in a microcentrifuge and resuspended in 50 μ l of 0.25 M Tris/HCl, pH 8.0. Cells were extracted by using the Tris buffer freeze-thaw protocol as described in the Promega technical bulletin. Cell lysates were assayed for CAT, luciferase or β -galactosidase.

Extracts to be used for CAT assays were heated at 60 °C for 10 min before assay to inactivate endogenous deacetylase activity. CAT was assayed with [¹⁴C]chloramphenicol (60 mCi/mmol, 50 μ Ci/ml) (New England Nuclear Life Science Products) and n-butyryl-CoA (5 mg/ml) (Promega) as substrates; the incubation

Figure 1 Presence of four putative Ets-binding sites in the GnT II promoter region at positions —616, —623, —636 and —642 nt upstream of the translation start codon (underlined) [20]

Double-stranded oligodeoxynucleotide probes with GGA → GTA mutations in these Ets-binding sites were synthesized and the sequences are indicated. We also synthesized an Ets-binding site consensus sequence 5'-CGGCAACCAAGTGTGTGC-3' as a positive control.

time was 30 min and the mixed-xylenes (Aldrich) phase-separation assay (Liquid Scintillation Counting assay) was used in accordance with the Promega protocol. CAT activity was determined from a linear standard curve with a CAT standard (Promega; 1 unit of enzyme activity corresponds to 1 nmol of acetate transferred to chloramphenicol in 1 min at 37 °C). Luciferase was assayed with the Luciferase Assay System with Reporter Lysis Buffer (Promega; activity was expressed in relative units based on fluorescence values). β -Galactosidase was assayed with *o*-nitrophenyl β -galactoside (Sigma; 1 unit of enzyme activity corresponds to the hydrolysis of 1 nmol of *o*-nitrophenyl β -galactoside in 1 min at 28 °C, with an ϵ_{420} of 4500 M⁻¹·cm⁻¹ for *o*-nitrophenol). Expression of promoter activity as determined by CAT activity was normalized to transfection efficiency as determined by either the luciferase activity or the β -galactosidase activity.

Synthesis of double-stranded oligodeoxynucleotide probes

There are four putative Ets-binding sites in the GnT II promoter region (Figure 1). Six sets of complementary oligodeoxynucleotides (Figure 1) were synthesized and annealed for the analysis of Ets binding to these four sites. The same probes were used in both gel mobility-shift assays and South-Western blots (see below). Either one Ets-binding site (probes E642, E636, E623 and E616) or two (probes EMu-1 and EMu-2) were mutated from GGA to GTA. Probe EB (Ets-binding consensus sequence) 5'-CGGCAACCAAGTGTGTGC-3' was synthesized as a positive Ets-binding control [22,38]. Oligodeoxynucleotides were synthesized on a Pharmacia DNA synthesizer and purified by the cartridge method (Hospital for Sick Children–Pharmacia Biotechnology Centre, Toronto, Ontario, Canada). 5'-End-labelling of the oligonucleotide probes was performed with T4 polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP (6000 Ci/mmol, 10 mCi/ml; Amersham Life Science). The specific radioactivities of the probes were approx. 5 × 10⁸ c.p.m./ μ g.

Gel mobility-shift assays

Nuclear extracts [39] from HepG2 and COS-1 cells transfected with the pRK5-Ets2 expression plasmid, or recombinant Ets-1 protein prepared from *E. coli* transfected with pET-Ets-1 plasmid (see South-Western blot protocol, below), or purified FltB or SapB were used as sources of Ets protein. Gel mobility-shift assays were performed as described in Current Protocols in Molecular Biology [35]. Nuclear extracts (2–3 μ g) or purified Ets family protein (3 μ g) were mixed with double-stranded radio-labelled DNA probe (10000 to 20000 c.p.m.) in a total volume of 20 μ l with buffer comprising 25 mM KCl, 50 mM MgCl₂, 25 mM

Hepes, pH 8.0, 3 μ g/ μ l BSA and 20% (v/v) glycerol. For competition analysis, proteins were incubated in the presence of a 200-fold excess of unlabelled oligodeoxynucleotides. Salmon sperm DNA (0.5 μ g) was used as a non-specific competitor. Samples were incubated at 4 °C for 30 min followed by electrophoretic analysis on 4% (w/v) non-denaturing polyacrylamide gels (acrylamide-to-bisacrylamide ratio 60:1) with 1 × Tris/glycine electrophoresis buffer [0.25 M Tris/1.9 M glycine/10 mM EDTA (pH 8.3)]. The gels were pre-run at 4 °C at 20 mA for 1 h, samples were added and the gels were run at 4 °C at 40 mA for 2–3 h. After being dried under vacuum with heat, the gels were exposed to X-ray film (Kodak BioMax Film) with an intensifying screen at –70 °C overnight.

South-Western blots

The pET-ets-1 plasmid contains 886 bp of mouse Ets-1 sequence subcloned into the pET3a vector [40]. Recombinant Ets-1 protein was partly purified from pET-ets-1 plasmid-transfected *E. coli* strain BL21(DE3)pLysS [40]. Total cell lysate of non-transfected *E. coli* cells was used as a negative control. *E. coli* cell lysate (20 μ g) or partly purified recombinant Ets-1 (10 μ g) was subjected to SDS/PAGE [41] and proteins were electroblotted to a nitrocellulose/cellulose acetate membrane (Millipore Immobilon-NC) [42]. The membranes were dried in air for 15 min and washed in succession with 100 ml of binding buffer [25 mM NaCl/5 mM MgCl₂/0.5 mM dithiothreitol/25 mM Hepes (pH 7.9)] for 15 min, 100 ml of binding buffer supplemented with 5% (w/v) Carnation non-fat dried milk for 30 min and 100 ml of binding buffer supplemented with 2.5% (w/v) Carnation non-fat dried milk for 30 min. The washed membranes were then incubated with gentle shaking for 2 h at 4 °C in 10 ml of binding buffer supplemented with 2.5% (w/v) Carnation non-fat dried milk containing ³²P-labelled double-stranded oligodeoxynucleotide probes (10⁶ c.p.m./ml; see above). The membranes were washed three times with 100 ml of binding buffer containing 2.5% (w/v) Carnation non-fat dried milk for 15 min, blotted briefly on filter paper, wrapped in Saran wrap ('cling film') and exposed to X-ray film (Kodak BioMax Film) with an intensifying screen at –70 °C overnight. A Western blot was also performed with N-276 antibody against Ets-1 (2 mg/ml; catalogue number sc-111; Santa Cruz Biotechnology).

RESULTS

Transcriptional regulation by Ets-1 and Ets-2 with the use of the CAT reporter system

Transient co-transfection experiments were performed in HepG2 and COS-1 cells by using either p-680CAT or p-1000CAT as reporter plasmids and a variety of effector plasmids and controls. We found that there was consistent activation (2–4-fold) of GnT II promoter activity on co-transfection of either *ets-1* or *ets-2* with plasmid p-680CAT into HepG2 cells (Figure 2) and COS-1 cells (Figure 3) and with plasmid p-1000CAT into HepG2 cells (results not shown) and COS-1 cells (Figure 4). A dominant-negative deletion mutant of Ets-2 (Δ 1–328Ets-2) caused the inhibition of *ets-2*-mediated stimulation of GnT II promoter, as determined by p-680CAT expression in HepG2 cells (Figure 2) (this effect was not always observed) and by both p-680CAT (Figure 3) and p-1000CAT (Figure 4) expression in COS-1 cells.

Because GnT V promoter expression had previously been shown to be stimulated by *src* [12], we tested our pSV-Src plasmid on GnT V promoter expression in our system and

Figure 2 Expression of p-680CAT in HepG2 cells

A DNA fragment from -276 bp to -680 bp, relative to the ATG start codon at $+1$, from the 5' untranslated region of the GnT II gene, was subcloned into a CAT reporter plasmid to produce the p-680CAT plasmid. p-680CAT was transfected into HepG2 cells either without or with plasmids pRK5-Ets2 (Ets2), pRK5, pRK5 $\Delta 1-328$ Ets2 (Δ Ets2), pEVRF-ets-1 (Ets1), pEVRF0 or pSV-Src. pRK5-Ets2 and pSV-Src were both added to the transfection mixtures at three different concentrations (0.5, 1.0 and 2.0 μ g). β -Galactosidase expression was used to determine transfection efficiency. The ratio of CAT activity to β -galactosidase activity is given as a percentage of the value for p-680CAT. pCAT-Basic-transfected cells, mock-transfected cells and cells transfected with plasmids pRK5 or pEVRF0 served as negative controls. The two bars in each category represent independent transfections. Various parts of the experiment were repeated three to six times with similar results. It is observed that Ets1 and Ets2 stimulated CAT activity. Expression of $\Delta 1-328$ Ets2 inhibited the Ets2-mediated stimulation of CAT activity in this experiment but this effect was not observed in all experiments. Expression of $\Delta 1-328$ Ets2 and *src* inhibited CAT activity.

Figure 3 Expression of p-680CAT in COS-1 cells

p-680CAT was co-transfected into COS-1 cells either without or with plasmids pRK5-Ets2 (Ets2), pRK5, pRK5 $\Delta 1-328$ Ets2 (Δ Ets2), pEVRF-ets-1 (Ets1), pEVRF0, pSV-Src, pcDNA3/*neu* or pcDNA3. β -Galactosidase expression was used to determine transfection efficiency. The ratio of CAT activity to β -galactosidase activity is given as a percentage of the value for p-680CAT. Cells transfected with pCAT-Basic and control plasmids pRK5, pEVRF0 or pcDNA3 served as negative controls. The two bars in each category represent independent transfections. Various parts of the experiment were repeated three to six times with similar results. It is observed that both Ets-1 and Ets-2 stimulated CAT activity, whereas $\Delta 1-328$ Ets2 inhibited the Ets-2 mediated stimulation of promoter activity. Expression of *src* inhibited promoter expression, whereas the effect of *neu* expression did not differ from control plasmid pcDNA3 alone.

showed a similar effect (Figure 5). However, we found consistent inhibition of GnT II promoter expression on co-transfection of the *src* plasmid with p-680CAT in HepG2 cells (Figure 2) and COS-1 cells (Figure 3) and p-1000CAT in HepG2 cells (results not shown) and COS-1 cells (Figure 4). Similarly, although GnT V expression is stimulated by the *her-2/neu* oncogene [17], co-transfection of a *neu* expression plasmid in our system had no

significant effect on GnT II promoter activation with either p-680CAT (Figure 3) or p-1000CAT (Figure 4) in COS-1 cells or p-1000CAT in HepG2 cells (results not shown). These results indicate that, although both the GnT II and GnT V promoters are stimulated by Ets-1 and Ets-2 transcriptional activators, the upstream control of this stimulation is different for the two promoters.

Figure 4 Expression of p-1000CAT in COS-1 cells

p-1000CAT was transfected into COS-1 cells either without or with plasmids pRK5-Ets2 (Ets2), pRK5Δ1–328Ets2 (ΔEts2), pEVRF-ets-1 (Ets1), pEVRF0, pSV-Src, pcDNA3/*neu* or pcDNA3. β -Galactosidase expression was used to determine transfection efficiency. The ratio of CAT activity to β -galactosidase activity is given as a percentage of the value for p-680CAT. Cells transfected with plasmids pCAT-Basic, pEVRF0 and pcDNA3 served as negative controls. The two bars in each category represent independent transfections. The experiment was repeated twice with similar results. It is observed that both Ets-1 and Ets-2 stimulated CAT activity, whereas *src* was inhibitory and pcDNA3/*neu* expression had no effect relative to plasmid pcDNA3 alone. Δ1–328Ets2 inhibited the activation caused by Ets-2.

Figure 5 Expression of *src* oncogene activates GnT V promoter expression

pGL2-TV3 was transfected into HepG2 cells either without or with plasmid pSV-Src. CAT expression was used to determine transfection efficiency. The ratio of luciferase activity to CAT activity is given in arbitrary units. The two bars in each category represent independent transfections.

Gel mobility-shift assays

Gel mobility-shift assays [16] were performed with radioactive oligodeoxynucleotide probes for the GnT II promoter (Figure 1) and nuclear extracts from COS-1 cells (results not shown) and HepG2 cells (Figure 6A) that had been transfected with pRK5-Ets2. With both nuclear extracts, a specific band shift was seen only with probe E623 containing the GGA Ets-binding sequence at a site on the GnT II gene 623 nt upstream of the ATG translation initiation site (at position +1). The band shift with the E623 probe (Figure 6A, lane 2) was considered specific

because it was prevented by the addition of competing non-radioactive native probe (lane 3), but not by non-radioactive mutated probe (lane 4) nor by a non-specific probe (lane 5). Band shifts in Figure 6(A) that were inhibited by competitor probe, mutant probe and non-specific probe were considered non-specific. Probes lacking a GGA sequence at the –623 position but with a GGA sequence at other sites or with a GTA sequence at the –623 site resulted in either no detectable shift or relatively minor specific band shifts.

Recombinant Ets-1 prepared by the expression of plasmid pET-ets-1 in *E. coli* (Figure 6D) and Ets family proteins SapB

Ets-1 can also control GnT V expression [14–16]. Co-transfection into human bile-duct carcinoma HuCC-T1 cells of a chimaeric GnT V promoter–luciferase reporter gene construct with an *ets-1* expression plasmid showed enhanced luciferase activity [16]. Competitive gel mobility-shift assays [15,16] showed specific binding of Ets-1 to two Ets-binding sites on the GnT V promoter that were at different locations from the sites shown to bind Ets-2 [12]. In 16 cancer cell lines, the levels of GnT V mRNA were correlated closely with Ets-1 mRNA expression [15]. Similar experiments with NIH 3T3 and HepG2 cells showed that *her-2/neu* also stimulates transcription from the human GnT V promoter [17]; co-transfections with dominant-negative mutants of either Ets-2 or Raf kinase inhibited stimulation by *neu*, indicating that *neu* action, like that of the *src* oncogene, is mediated by the Ras–Raf–Ets signal transduction pathway.

We have shown previously that the human GnT II promoter [20], like the human GnT V promoter [12,21], has putative binding sites for the products of the *ets* and *c-myc* families of proto-oncogenes and that GnT II action is a prerequisite for GnT V action [19]. In the present study we used transient co-transfection experiments in HepG2 and COS-1 cells to demonstrate a consistent 2–4-fold activation of GnT II promoter activity by either *ets-1* or *ets-2*. Gel mobility-shift and South-Western blot analyses both showed that only one of the four putative Ets-binding sites on the GnT II promoter (the site 623 nt upstream of the ATG translation initiation site at position +1) was capable of binding various members of the Ets family (Ets-1, FliB and SapB). Consistent with these observations is the previous finding [20] that transfection of the chimaeric GnT II promoter–CAT plasmid p-636CAT (carrying the GnT II promoter region from –276 to –636 bp relative to the ATG start codon at +1) (Figure 1) resulted in the same CAT activation (75% of maximum) as transfection of plasmid p-680CAT; plasmid p-636CAT has only two intact Ets-binding sites, at positions –616 and –623 nt upstream of the translation start codon, whereas plasmid p-680CAT has four intact Ets-binding sites. Plasmid p-553CAT, lacking all four Ets-binding sites, shows only 10% of maximum CAT activity.

Whereas the GnT V promoter is activated by *src* and *neu*, neither of these oncogenes stimulated the GnT II promoter. Therefore, although both the GnT V and GnT II promoters are activated by Ets transcription factors, the upstream control of this stimulation is different for the two promoters.

It is of interest that transfection of cells with the *src* oncogene consistently inhibited GnT II promoter activity (Figures 2–4). However, there are two lines of evidence that indicate that there is sufficient GnT II activity after transformation with Rous sarcoma virus to allow adequate GnT V action. First, it has been shown that cells transformed with Rous sarcoma virus express higher levels of triantennary and tetra-antennary N-glycans than non-transformed cells [2,3,9,13]; secondly, a 50% decrease in GnT II activity, as occurs in the parents of children with carbohydrate-deficient glycoprotein syndrome type II, does not lead to any detectable phenotypic abnormality, indicating that GnT II activity is not rate-limiting [33,43].

Our findings that two members of the Ets family of transcription factors (Ets-1 and Ets-2) stimulate GnT II promoter activity in intact cells and that three Ets family members (Ets-1, FliB and SapB) bind to a specific site on the GnT II promoter in a cell-free system provide no information on which endogenous Ets factors actually control GnT II expression *in vivo*. The results in Figures 2–4 show that a dominant-negative deletion mutant of Ets-2 (Δ 1–328Ets-2) causes not only the expected inhibition of *ets-2*-mediated stimulation of GnT II promoter activity but also causes a small inhibition of basal GnT II promoter activity. The

latter observation suggests that endogenous Ets-2 might be involved in GnT II promoter activation, at least in HepG2 and COS-1 cells. It is possible that other Ets factors are similarly involved and that these factors might vary with the tissue and over time.

The constitutively expressed housekeeping glycosyltransferase, UDP-Gal:GlcNAc-R β -1,4-galactosyltransferase I (β -4-GalT I'), which normally transfers Gal to GlcNAc-R to make the Gal- β -1,4-GlcNAc linkage on N-glycans, has been recruited for other more specific biosynthetic functions [44–49], the production of lactose (Gal- β -1,4-Glc) localized to the lactating mammary gland, and expression specific to male germ cells. Like the GnT II promoter, the promoter region necessary for germ-cell-specific expression of β -1,4-galactosyltransferase I contains an Ets-binding motif [50]. It is therefore of interest that the GnT II gene, which is also a constitutively-expressed housekeeping enzyme glycosyltransferase might under some circumstances come under tissue- or time-dependent transcriptional control by an Ets-type transcriptional activator.

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