

# Control of core 2 $\beta$ 1,6 *N*-acetylglucosaminyltransferase-I transcription by Sp1 in lymphocytes and epithelial cells

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Received: 9 March 2007 / Revised: 13 April 2007 / Accepted: 23 April 2007 / Published online: 26 May 2007  
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**Abstract** Core 2  $\beta$ 1,6 *N*-acetylglucosaminyltransferase-I (C2GnT-I) catalyzes the synthesis of one of the major core structures in GalNAc  $\alpha$ -Ser/Thr O-linked oligosaccharides, the core 2 branch. The production of the core 2 branch is required for the synthesis of glycoforms that are important for the cellular functions of lymphocytes, mucin-producing epithelial cells and other cell types. Therefore, proper molecular control of C2GnT-I expression is very important for different types of cells. C2GnT-I is transcribed from 4 promoters, with promoter 2 being the major promoter. C2GnT-I promoter 2 lacks a TATA box and is very GC rich. In this study, the analysis of this promoter finds that the transcription factor Sp1 is essential for transcription of C2GnT-I in both mesodermally derived T-cells (Jurkat) and in endodermal mucin producing epithelial cells (NCI H292). In Jurkat cells, all nine of the Sp1 binding sites within the minimal promoter region contribute to transcription, and there is a linear relationship between the number of Sp1 sites and the transcriptional activity of the promoter. In NCI H292 cells, only three of these Sp1 binding sites are required for transcription from promoter 2. Chromatin immunoprecipitation confirms that Sp1 binds to promoter 2 in NCI H292 cells *in vivo*.

**Keywords** Glycosyltransferase · Transcription · Gene expression · Core 2 branch · Sp1

## Abbreviations

C2GnT	Core 2 $\beta$ 1,6 <i>N</i> -acetylglucosaminyltransferase
sLeX	Sialyl Lewis X antigen
Sp1	Specificity protein 1
nCARE	Negative calcium response element
ATF	Activating transcription factor
GAPDH	Glyceraldehyde phosphate dehydrogenase
DHFR	Dihydrofolate reductase
ChIP	Chromatin immunoprecipitation
KLF	Kruppel-like factor
PP2A	Protein phosphatase 2A
ST6Gal I	$\beta$ -galactoside $\alpha$ 2,6 sialyltransferase I
hTERT	Human telomerase reverse transcriptase
VEGF	Vascular endothelial growth factor
Muc5AC	Mucin 5 subtype AC

## Introduction

A tremendous variety of GalNAc- $\alpha$ -Ser/Thr O-linked oligosaccharide structures can be synthesized on mammalian glycoproteins. Since the individual sugars are added in specific configurations by different glycosyltransferases, the genetic control of the glycoforms expressed by an individual cell is at the level of regulating the specific glycosyltransferases expressed in that cell. O-linked structures are synthesized by first constructing a “core” oligosaccharide that is further modified by the addition of sugars to produce the final glycoform product. One of the major core structures, core 2 [Gal $\beta$ 1 $\rightarrow$ 3(GlcNAc $\beta$ 1 $\rightarrow$ 6)GalNAc], is formed by the addition of an *N*-acetylglucosamine in a  $\beta$ 1-6 linkage to the *N*-acetylgalactosamine of the core 1 (Gal $\beta$ 1 $\rightarrow$

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3GalNAc- $\alpha$ 1-O-Ser/Thr) structure [1, 2]. This core 2 branch provides the preferred substrate for synthesis of several biologically significant terminal structures such as poly-*N*-acetylglucosamines and the Lewis antigens [3, 4]. While glycosylation is often thought to be carried out by constitutive, unregulated housekeeping genes, core 2 branched glycoforms are differentially expressed during development and in different adult cell types [5, 6] and have particular importance in T- and B-cells and in mucin producing epithelial cells.

There are three genes encoding enzymes that catalyze the synthesis of the core 2 branch, C2GnT-I, C2GnT-II and C2GnT-III. C2GnT-I, originally called C2GnT-Leucocyte or L form, [7] is the most widely expressed while C2GnT-II, also called C2GnT-M or mucin form [8] and C2GnT-III, or C2GnT-T or thymus form, are more restricted. C2GnT-II is principally found in mucus producing tissues and C2GnT-III is found predominantly in the thymus. C2GnT-I expression is very important for the function of cells of the immune system. C2GnT-I expression is induced upon activation of mature naïve T-cells and B-cells [9–11] to permit the synthesis of the sialyl Lewis X (sLe<sup>X</sup>) antigen [sialyl $\alpha$ 2-3Gal $\beta$ 1-4(Fuc- $\alpha$ 1-4)GlcNAc $\beta$ 1-3Gal-R]. The Lewis antigens on core 2 branched glycoforms are ligands for selectins that mediate trafficking of lymphocytes to sites of inflammation [12] and lymph nodes [13, 14]. C2GnT-I is also expressed by mucin-producing epithelial cells, where it participates in the glycosylation of mucin apoproteins. Most of the mass of mucin glycoproteins is made up of O-linked carbohydrates, many containing core 2 branches. These glycoconjugates contribute to mucin biochemical and biophysical properties to provide lubrication and a protective barrier from environmental insults for epithelial cells along the airway and gastro-intestinal tracts [15].

Aberrant C2GnT-I expression is a factor in the pathology of many diseases. In the immune system, C2GnT-I overexpression has been observed in immunodeficiency disorders including Wiskott–Aldrich syndrome and AIDS [16, 17]. Expression of C2GnT-I is also a factor in cancer pathology. Increased C2GnT-I transcription is correlated with the increased malignancy of many different cancers, including colon carcinomas [18], pulmonary adenocarcinomas [19], oral cavity carcinomas [20], and pancreatic cancers. [21]. C2GnT I is also increased in leukemia [22]. However, C2GnT is reduced in breast cancer [23, 24]. Malignant cells may take advantage of C2GnT-I expression by forming the core 2-type sLe<sup>X</sup> selectin ligand and using the structures in place for normal immune cell extravasation to metastasize. Notably, core 2 branching in activated T-cells has been shown to induce a transient localization of these cells to the lungs and liver, two primary sites for metastasis [10]. Changes in glycosylation consistent with increased core 2 branching have been characterized in airway diseases. For

example, mucin glycoproteins from cystic fibrosis patients show increases in the amounts of sialylation, sulfation and Lewis antigen expression in addition to increased total glycosylation [25–27]. Furthermore, the expression of these glycoforms may contribute to the pathogenesis of cystic fibrosis by promoting inflammation and *Pseudomonas aeruginosa* binding [28]. Therefore, regulation of C2GnT-I expression is very important in both lymphocytes and in mucin-producing epithelial cells.

In humans, the C2GnT-I gene spans over 60 kilobases and its transcription is very complex. There are four promoters that synthesize at least 5 different mRNAs, each of which varies in the 5' untranslated region [29]. Since the expression of this gene is regulated in two distinct cell types, T-cells and mucin producing epithelial cells that derive from distinct developmental lineages, mesoderm and endoderm, respectively; we sought to compare the factors that are required for transcription of C2GnT-I from promoter 2 both types of cells, a T-cell lymphoma line, Jurkat, and a mucin-producing, airway epithelial cell line. Both of these cell lines have been used to investigate cell-type specific expression in many studies, such as FoxP3 and IL-2 in Jurkat cells and mucin genes, including MUC5AC and Muc2, in NCI H292 cells [30–32]. These experiments show that the transcription factor Sp1 is important for C2GnT-I expression in both types of cells.

## Experimental procedures

**Cell culture** Human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was cultured in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), Hepes (25 mM), 2 mM L-glutamine (Invitrogen) and sodium pyruvate (1 mM, Invitrogen). Jurkat T lymphocytes were maintained in IMDM medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine (Invitrogen), and 2 mM Hepes (pH 7.4). Both cell lines were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

**Mithramycin A treatment** Mithramycin A (Sigma-Aldrich, St. Louis, MO) is an antibiotic, chemotherapeutic compound that binds to GC boxes in DNA and inhibits the binding of Sp1. NCI-H292 cells were treated with 100 nM mithramycin at confluence for 24 h. Jurkat cells were seeded at a density of 10<sup>6</sup> cells/ml and allowed to recover for about 2 h prior to treatment with mithramycin A for 24 h at concentrations ranging from 0–3  $\mu$ M.

**RT-PCR** Total RNA was prepared from NCI H292 and Jurkat cells using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol and used for PCR with Jumpstart RedTaq Ready Mix (Sigma-Aldrich, St. Louis, MO) for PCR and the following primer pairs and annealing temperatures:

GAPDH: 5'ATGACCACAGTCCATGCCAT3' and 5'GCCTGCTTACCACCTTCTT3'; 62°C. All C2GnT-I transcripts: 5'AATGGGGCAGGATGTCACC3' and 5'AAGTCTCCTTCGCAGCAACG3'; 60°C C2GnT-I Promoter 2 transcripts: 5'TGGGCATCCTCCTGAGACT3' and 5'CTTGAAGTTGTCAGTTTGC3'; 62°C Muc2: 5'CAAGAGGTGCAGCCAGAAGC3' and 5'TGGAGACGGACGAGATGAGC3'; 60°C Muc5AC: 5'ACTTGGGCACTGGTGCTG3' and 5'TCCGGCCTCATCTTCTCC3'; 60°C

**DNA subcloning and deletion mutants** A BAC clone (B57F11) containing genomic sequence from chromosome 9q22 was obtained from The Wellcome Trust Sanger Institute human genome sequencing project (Hinxton, Cambridge, CB10 1SA, UK) and used as template to amplify 2,526 bp of genomic sequence corresponding to promoter 2 of C2GnT-I using primers 5'AGACTGAGTGGGTGGATCG3' and 5'GCTCCTGCTCCACCAAGCT3'; 56°C. The amplicon was cloned into the pGEM T-easy vector (Promega, Madison, WI) for sequencing. The fragment cloned in the reverse orientation in the pGEM T-easy vector, was removed using NcoI and MluI restriction enzymes, and ligated upstream of the luciferase gene in the pGL3 vector (Promega, Madison, WI) cut with NcoI and MluI. This clone was used to make each of the other constructs by digesting the full-length construct with the indicated enzymes (NEB Ipswich, MA) using the provided buffers and recommended temperatures. Enzymes used as follows:  $\Delta$ -620 bp, BglII and MluI;  $\Delta$ -146 bp, SacI;  $\Delta$ -70 bp, SmaI and MluI;  $\Delta$ -45 bp, PstI. An additional deletion ( $\Delta$ -346 bp) was made using KpnI and XhoI to digest the -620 bp construct. Internal deletions were made from the -620 bp and -346 bp constructs using the following enzymes: to remove bases between -146 and -45, SacI and PstI;  $\Delta$ -146 to -70, SacI and SmaI;  $\Delta$ -210 to -114, BmgB1;  $\Delta$ -210 to +20, BmgB1 and EcoRI;  $\Delta$ -210 to -45, BmgB1 and SmaI;  $\Delta$ -210 to -40, BmgB1 and PstI. Ligations were transformed into JM109 cells (Promega, Madison, WI) for colony selection and screening.

**Transfections** Plasmid constructions containing a firefly luciferase reporter gene under the control of C2GnT-I promoter 2 (full length or deletions as above) were co-transfected with a CMV-renilla luciferase construct used for normalizing transfection efficiency at a ratio of 10:1 (firefly: renilla) in NCI H292 cells and at a ratio of 5:1 (firefly: renilla) in Jurkat cells. Transfections were done using the

Superfect reagent (Qiagen). Cells were incubated for 16 h then fed with complete media and incubated an additional 16–24 h before being harvested. For mithramycin A treatment, cells were transfected 4 h and allowed to recover for 1 h before being treated with mithramycin A for 24 h before being harvested and assayed.

**Chromatin immunoprecipitation (ChIP) assay** Experiments were carried out using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. Briefly, cells were fixed with formaldehyde for 10 min at 37°C then harvested by scraping and lysed in the presence of protease inhibitors. DNA was fragmented to an average size of 500 bp using sonication and the sample was split in half for immunoprecipitation with or without an antibody to amino acids 528–546 of Sp1 (sc-59; Santa Cruz Biotechnology, Santa Cruz, CA). Crosslinks were reversed and recovered DNA was used in PCR reactions to amplify a 119 bp region of C2GnT-I promoter 2 (Accession numbers AY196294 and AY196295) using the following primers: 5'CTAGAAAGCGGGGCCTCT3' and 5'TAGGAGACTCTGAGGCC3'; and annealing 60°C. The negative control for this assay is C2GnT-I promoter 3 (Accession number AY196296). A 110 bp region of promoter 3 was amplified with the following primers: 5'CACGGGAAGGAAGAACTCA3' and 5'AAGAATGAAGCTGCGGACC3'; and annealing at 60°C. As a positive control, a 68 bp region of the dihydrofolate reductase (DHFR) gene (Accession number NM\_000791) promoter was amplified with the same reagents and the following primers: 5'TCGCCTGCACAAATAGGGAC3' and 5'AGAACGCGCGGTCAA GTTT3'; and annealing at 57.4°C [33]. DNA recovered from chromatin samples corresponding to 10% of chromatin used for each immunoprecipitation reaction were collected before immunoprecipitation and used as loading controls (% input).

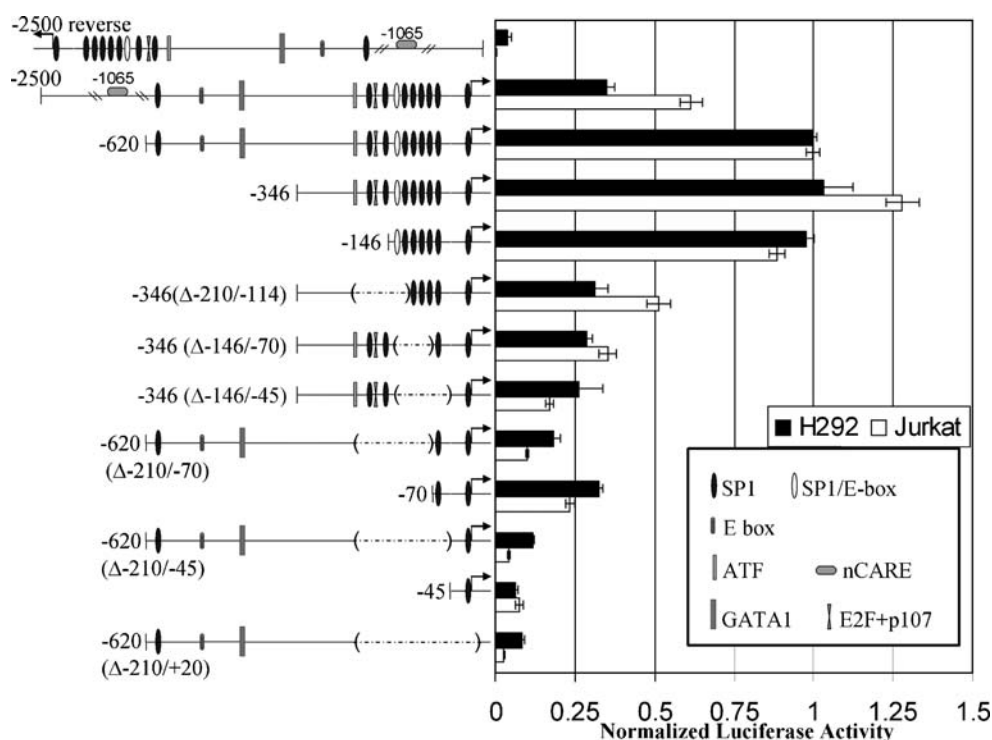
## Results

Previous experiments had identified multiple transcription initiation sites for the C2GnT I gene [29] suggesting the presence of multiple promoters for this gene. In addition, the majority of C2GnT I mRNAs were initiated from the second promoter (from 5' to 3'). To confirm that region of DNA contained a promoter sequence, a 2,500 bp fragment from this region was amplified by PCR from a bacterial artificial chromosome clone that was obtained from The Wellcome Trust Sanger Institute (Cambridge, UK) and cloned into the pGL3 luciferase reporter vector in both the forward and reverse orientations. As shown in Fig. 1, this

fragment showed significant promoter activity in the forward but not the reverse orientation after transfection into both Jurkat and NCI H292 cells.

This 2,500 bp sequence was examined for putative transcription factor binding sites using the SiteSeer program and the Transfac v.6.0 database [34]. This analysis found a large number of putative transcription factor-binding sites some of which are indicated in Figs. 1 and 2. To determine which of the transcription factor binding sites within this 2.5 kb fragment were important for the transcription of C2GnT-I, 5' deletions were constructed in the promoter fragment to identify the smallest proximal fragment with maximal promoter activity. The first deletion called -620, was made at a BglII site, 620 bp upstream of the transcription initiation site. This deletes 1,880 bp from the 5' end and shows increases in promoter activity of 38% in Jurkat cells and 65% in H292 cells (Fig. 1), suggesting that a repressor is present in the region from -620 bp to -2,500 bp. Sequence analysis identified a putative repressor in this region, a negative calcium response element (nCARE) found at -1,065 bp.

The nucleotide sequence of the region between -620 bp and the transcription start site (+1) with identified transcription factor consensus binding sequences is shown in Fig. 2. There are no proximal TATA or CAAT boxes, but it is very GC rich, containing a CpG island, and 10 binding sites for transcription factors of the Sp1 family (numbered 1 to 10 from 3' to 5'). One of these Sp1 binding sites, designated Sp1-7, overlaps with an E-box binding site. There are also putative binding sites for factors such as ATF, GATA-1, and E2F (Fig. 1). To determine whether any of these factors are important for transcription from promoter 2, additional 5' deletions were constructed and tested for promoter activity as shown in Fig. 1. In Jurkat cells, deletion from -620 to -346 bp removing Sp1-10, GATA-1, and a distal E-box shows an additional increase of 28% suggesting a repressor is also present in this region. Deleting the region from -346 to -146 bp, removing binding sites for ATF and E2F+p107 as well as Sp1 sites 8 and 9, decreases promoter activity by 40% suggesting a role for one or more of these elements. Deletion from -146 to -70 bp results in a further decrease in promoter activity



**Fig. 1** Deletion analysis of C2GnT-I promoter 2. The various promoter fragments and deletions are diagrammed on the Y-axis with their relative luciferase activities plotted as bars along the X-axis. The longest (2,500 bp) fragment shown at the top is not drawn to scale and was tested in both the reverse and forward orientations. The symbols in the diagram represent the positions of transcription factor binding consensus sequences and are defined in the box attached to the figure. The fragments are numbered with the base position of the 5' end and

the bases removed from internal deletions (indicated in parenthesis around a dashed line) with the transcription initiation site as +1. The bars represent the mean value (and standard error) of the ratio of firefly to renilla luciferase activities obtained from Jurkat cells (white bars) and NCI H292 cells (black bars) normalized to the -620 bp construct. The data represent at least two independent transfections, each done in triplicate

**Fig. 2** Nucleotide sequence of human C2GnT-I promoter 2. The nucleotide sequence of C2GnT-I promoter 2 is shown with nucleotides numbered with the transcription initiation site as +1. The transcribed sequence is *bold and italicized*. Consensus transcription factor binding sites in the forward orientation are indicated by *boxes*, and in the inverse orientation are *underlined and italicized*. Individual Sp1 binding sites are numbered 1–10 from 3' to 5'. The restriction endonuclease cleavage sites used to construct the deletion mutants are shown with *small vertical arrows*. The beginning of the 654 bp CpG island is indicated by a *large arrow* at –250 bp

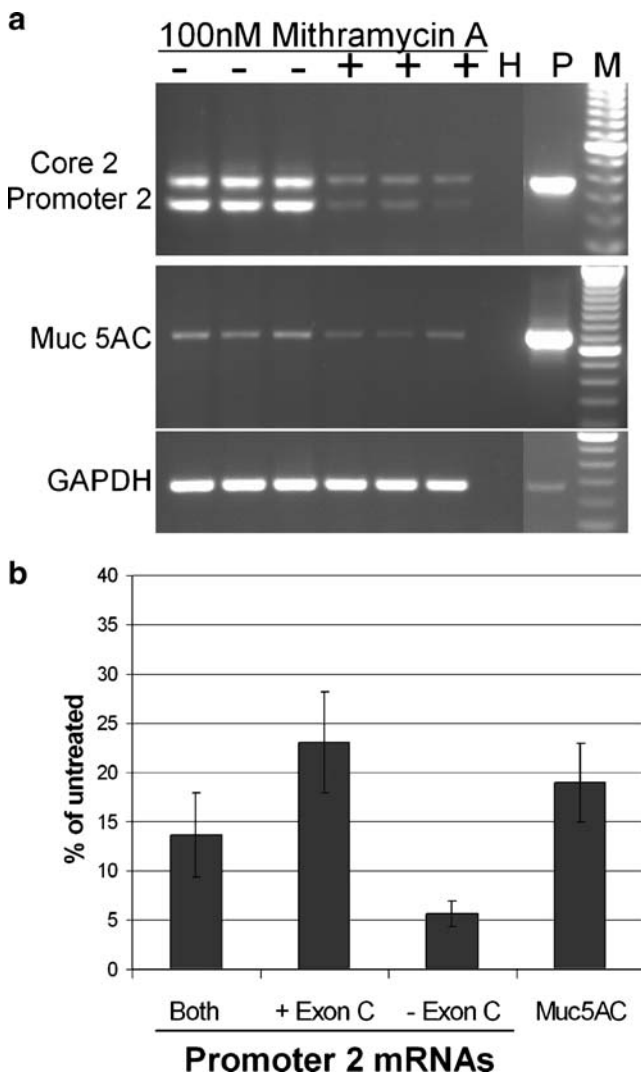


to 35% of the –620 bp construct, and deleting to –45 bp decreases expression to near background levels. Adding back the region from –620 to –210 (Fig. 1; –620(Δ–210/–45)) to these deletions produced a further reduction in activity again suggesting an inhibitory element in this segment. Deleting all of these proximal sequences from –210 to +20 (Fig. 1; –620(Δ–210/+20)) reduces promoter activity to background levels showing the importance of these sequences for transcription. These data indicate that sequences beginning at –346, including the GC rich region, contain most of the promoter activity for Jurkat cell transcription. The results obtained from NCI H292 cells follow a similar pattern with a couple of differences. The 5' deletions at –620, –346 and –146 all show similar activities, indicating that unlike the Jurkat cells, transcription in NCI H292 cells is not affected by this region and the sequences beginning at –146 are sufficient for complete promoter activity.

In order to refine the location of the regulatory sequences, several additional internal deletions were constructed and tested for promoter activity. Deleting the region from –210 to –114, containing Sp1 sites 6 and 7 (–346(Δ210/–114)), reduced transcriptional activity by 49% in Jurkat and by 69% in NCI H292 cells, indicating the importance of these sites to both cell types. Removal of Sp1 sites 3 and 4 by deleting the region from –146 to –70 (–346(Δ–146/–70)), and Sp1-2 (–70) results in further decreases in transcriptional activity in Jurkat cells that is linear to the number of sites (Fig. 1), suggesting that each site contributes to transcriptional activity in this cell line. The NCI H292 cells do not show a linear response to number of Sp1 sites, but decrease

transcription when sites 6 and 7 are removed, and then again with the removal of Sp1-2, suggesting that these specific sites have a greater impact on transcription in NCI H292 cells.

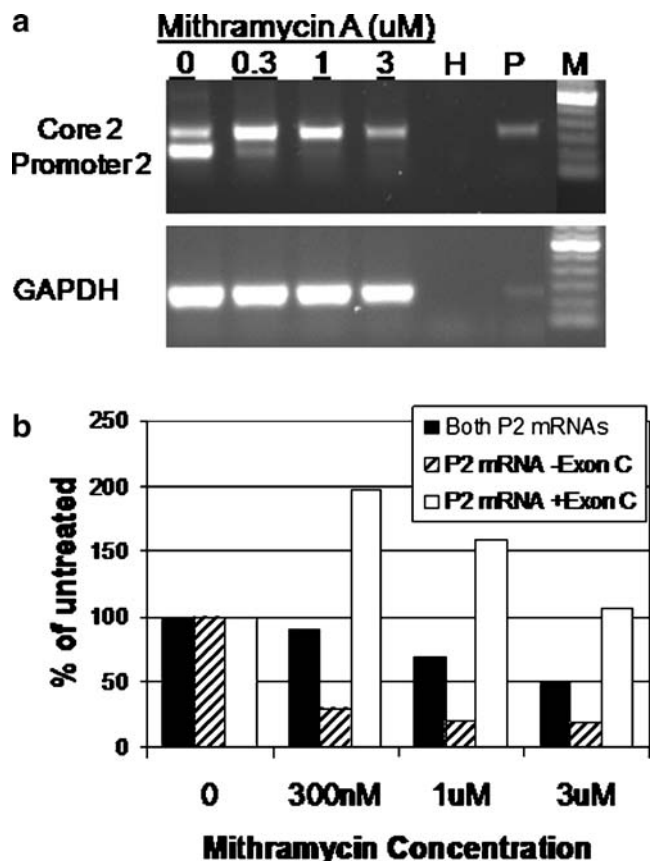
The deletion analysis experiments suggested that GC rich, putative Sp1 binding regions proximal to promoter 2 of C2GnT-I are very important for transcription. Mithramycin A is an anti-neoplastic compound used in chemotherapy that binds to the GC box in DNA, inhibiting the binding of Sp1 to its binding sites [35] and had previously been shown to inhibit Sp1 mediated, Muc5AC gene transcription in NCI H292 cells [30]. Therefore, if Sp1 binding is important for C2GnT-I transcription, mithramycin A treatment should block transcription from this promoter. To test this hypothesis, NCI H292 cells and Jurkat cells were treated with mithramycin A and the C2GnT-I transcripts were measured by RT-PCR using two sets of primers. One set of primers was specific to the two transcripts that are initiated from promoter 2. The promoter 2 primers give 2 amplification products due to alternative splicing of an exon, exon C [29] in the 5' untranslated region of mRNA transcribed from promoter 2. An additional set of primers was designed from a sequence that is common to all C2GnT-I transcripts, transcribed from all four of the promoters and therefore provides a measure of all C2GnT-I mRNA. The second set of C2GnT-I primers are specific to the two transcripts that initiate from promoter 2. Muc5AC was used as a control for Mithramycin A activity in NCI H292 cells. The results of this experiment shown in Fig. 3 (a and b) indicate that the transcripts from C2GnT-I promoter 2 are reduced by almost 90% in NCI H292 cells treated with 100 nM mithramycin A for 24 h. Interestingly, the smaller splice variant that omits exon C (small P2 transcripts)



**Fig. 3** The effect of mithramycin A treatment on promoter 2 transcripts in NCI H292 cells. **a** The ethidium bromide stained agarose gels of RT-PCR amplification products from equal amounts of RNA from triplicate samples of NCI H292 cells were treated for 24 h with mithramycin (+) or without mithramycin (-), 100 nM as indicated at the top. The primers used were specific for the mRNAs indicated at the left of each gel. Muc5AC is included as a positive control. The two bands in the Core 2 Promoter 2 gel represent splice variants containing exon C (+exon C; upper band) or omitting exon C (-exon C; lower band). Lanes H are the no template PCR negative controls, Lanes P are plasmid template, PCR positive controls and Lanes M are 100 bp DNA size markers. **b** Graphic representation of the Chemidoc (BioRad) fluorometric quantification of the bands from the gels in (a). Bars represent the average intensity ratio of amplicon bands (%mithramycin A treated/untreated) normalized to GAPDH

is reduced more than the larger variant that contains exon C (large P2 transcripts), suggesting a difference in RNA stability or a mithramycin effect on splicing. Jurkat cells were resistant to 100 nM mithramycin A treatment but at higher concentrations, C2GnT-I promoter 2 transcripts were reduced relative to the glyceraldehyde phosphate dehydrogenase (GAPDH) message. Again, the smaller mRNA appeared to be more sensitive to mithramycin A than the larger transcript. (Fig. 4)

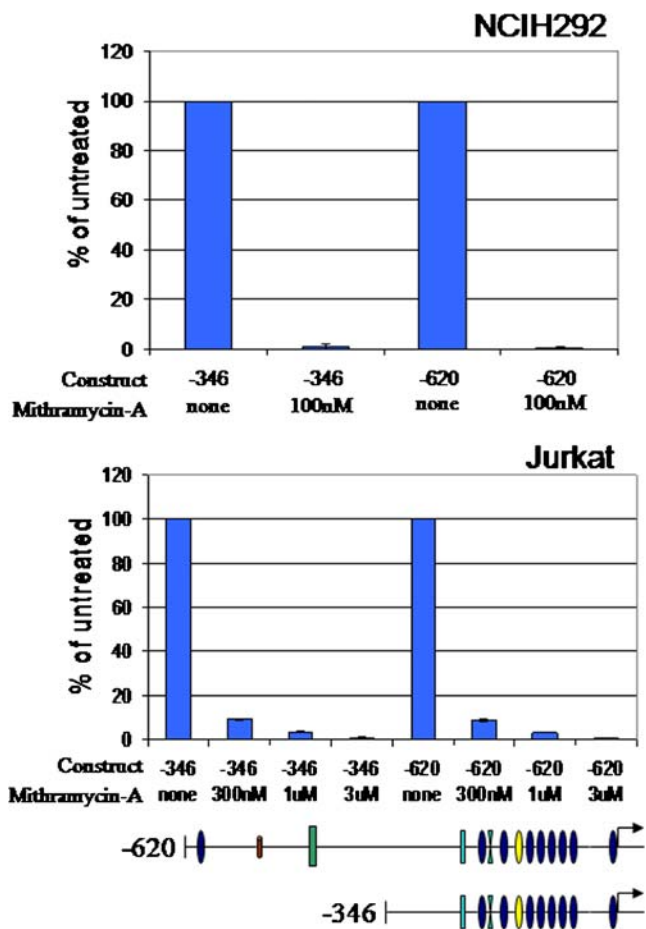
Since RT-PCR analysis measures steady state mRNA levels, the previous experiment is not a direct assay for promoter activity but may reflect a difference in mRNA stability. Therefore, an *in vivo* assay using transfected reporter constructs was also performed. The firefly luciferase reporter constructs containing either -346 or -620 bp of C2GnT-I promoter 2 sequence were tested for expression in the presence or absence of mithramycin A. Transfected cells were treated with mithramycin A for 24 h. In both cell lines mithramycin A treatment dramatically suppressed transcriptional activity of the -346 bp and the -620 bp reporter constructs by 99% (Fig. 5). These results clearly show that transcription of C2GnT-I from promoter 2 in Jurkat and NCI H292 cells is inhibited by mithramycin A



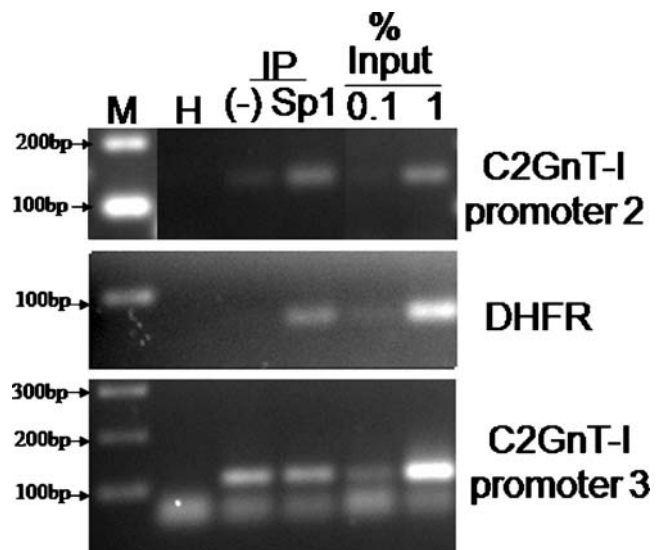
**Fig. 4** Effect of mithramycin A treatment on promoter 2 transcripts in Jurkat cells. **a** The ethidium bromide stained agarose gel analysis of RT-PCR amplification products using equal amounts of template RNAs from Jurkat cells treated for 24 h with the concentration mithramycin A indicated at the top the lane. The primers specific for the C2GnT-I and GAPDH mRNAs are indicated at the left of each gel. The C2GnT-I primers amplify two mRNAs, one containing exon C (+exon C) produces the larger amplicon and the other, omitting exon C produces the smaller amplicon. Lanes H are the no template, negative PCR control; Lanes P are plasmid template, PCR positive controls and Lanes M are 100 bp DNA size markers. **b** Graphic representation of the Chemidoc (Biorad) fluorometric quantification of the bands from the gels in (a). Bars represent the relative intensity, normalized to GAPDH of the upper band (+exon C; white), lower band (-exon C; grey) or the sum of both bands (black)

and consistent with the model that factors are binding to GC rich, putative Sp1 binding sites.

In order to determine whether Sp1 does, in fact, bind to these sequences in the C2GnT-I promoter 2, we did chromatin immunoprecipitation or ChIP assays. We chose these assays because the putative Sp1 binding sequences present in the C2GnT-I promoter have all been previously shown to bind Sp1 using electrophoretic mobility shift assays (EMSA) and supershift assays, therefore, repeating these assays would not be helpful. However, the ChIP assay would show whether Sp1 is bound to these sites in the context of promoter 2 *in vivo*, and therefore be a better indication of Sp1 interacting with this promoter. DNA binding proteins were crosslinked to DNA, *in situ*, with formaldehyde, and chromatin was sheared and immunoprecipitated with anti-Sp1 antibodies as described in Experimental procedures. After uncrosslinking, C2GnT-I promoter 2 DNA in the immunoprecipitates was identified by PCR using primers



**Fig. 5** The effect of mithramycin A on transcription of transfected promoter deletions. NCI H292 and Jurkat cells were transfected with the C2GnT-I promoter/firefly luciferase constructs indicated on the x-axis, diagrams of promoter constructs are at the bottom of the figure. Mithramycin A (at the concentration indicated on the x-axis) was added 5 h after transfection. Cells were lysed 24 h after addition of mithramycin A and luciferase activity was measured. Bars represent the mean luciferase activities normalized to the untreated control



**Fig. 6** ChIP analysis of *in vivo* Sp1 binding to C2GnT-I promoter 2. NCI H292 cells were fixed with formaldehyde, immunoprecipitated using an antibody specific for Sp1, and genomic DNA was isolated as described in the Experimental procedures. Immunoprecipitated DNA was analyzed by PCR with primers spanning the transcription start site of C2GnT-I promoter 2. The DHFR promoter, and C2GnTI promoter 3 are assayed as positive and negative controls, respectively. Dilutions equivalent to 0.1% and 1% if the total input DNA (% input) were removed prior to immunoprecipitation and included as amplification controls. Lane M, 100 bp DNA markers and lane H, no template

from positions -100 to +18 bp of C2GnT-I promoter 2. PCR was also done using primers for the dihydrofolate reductase (DHFR) promoter that was previously shown by this technique to bind Sp1 *in vivo* [33]. As a negative control, primers from a region of C2GnT-I promoter 3 that has no detectable Sp1 consensus sequences in the vicinity were used. Chromatin immunoprecipitation analysis shows that Sp1 binds to promoter 2 in NCI H292 cells. (Fig. 6) C2GnT-I promoter 2 and the DHFR promoter are both enriched by ChIP with a Sp1-specific antibody. This shows that Sp1 binds to C2GnT-I promoter 2 *in vivo*. These results confirm the importance of Sp1 binding for transcriptional activity from C2GnT-I promoter 2.

### Discussion

These experiments show that the transcription factor, Sp1, is important for transcription from the core 2  $\beta$ 1,6 N-acetylglucosaminyltransferase I promoter 2 in two cell lines, NCI H292 lung cancer derived mucin-producing epithelial cells, and Jurkat, a T-cell lymphoma. This promoter has a negative calcium response element located about 1 kb upstream of the transcription initiation site. These nCARE elements are characterized by a T-rich stretch of about 20 bases followed by a 15 bp consensus that contains an inverted repeat. This inhibitory element

functions at large distances upstream of the human renin [36] and parathyroid genes [37], being located 1.7 kb and 3.5 kb upstream, respectively. Intracellular calcium leads to nuclear localization of the multifunctional Redox factor 1 (Ref-1) that binds to the nCARE sequence and represses transcription.

The proximal promoter is very GC rich with multiple potential Sp1 binding sites. The importance of Sp-1 binding in promoter 2 was confirmed by treatment with mithramycin A, and direct binding of Sp1, *in vivo*, was shown by chromatin immunoprecipitation. Although Sp1 is important for transcription in both cell lines, differences in the mechanisms for C2GnT-I expression by these cells might be expected because the cell lines in this study are derived from cells of two very different embryonic lineages. Accordingly, some differences in C2GnT-I transcription control were observed in these cell types. In Jurkat cells, 346 bp of sequence upstream of the transcription start site gives maximum transcriptional activity. This region is very GC rich, and contains nine Sp1 binding sites, or GC boxes, all of which are important for transcription, since the number of Sp1 sites present at the promoter is linearly correlated with the amount of transcriptional activity (Fig. 7). In H292 cells, on the other hand, maximum transcriptional activity can be conferred by

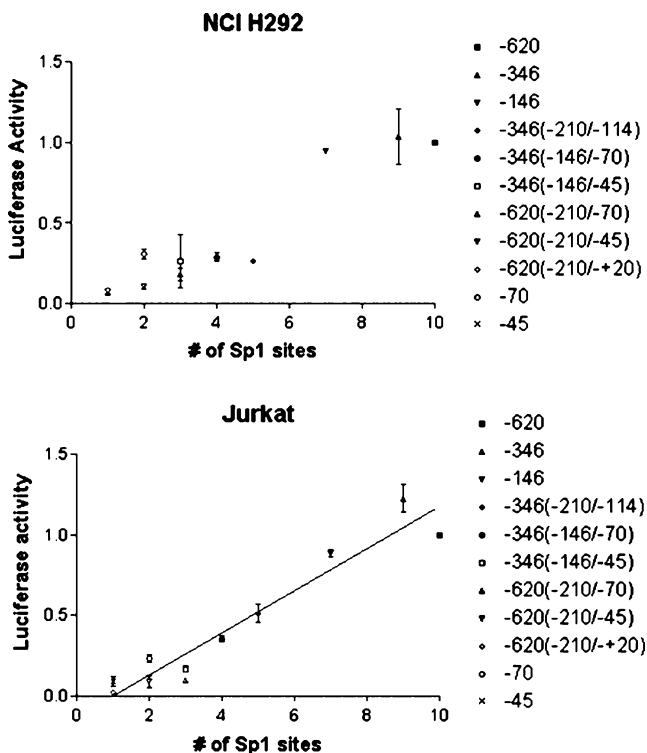
–146 bp of sequence, and only Sp1 binding sites 2, 6, and 7 are required (Figs. 1 and 2) resulting in a biphasic distribution when the promoter activity of each deletion is plotted relative to the number of Sp1 binding sites (Fig. 7). The different mechanisms used by these two cell types for transcription from promoter 2 may reflect differences in the expression level of Sp1, which varies dramatically depending upon tissue type, with the highest expression in hematopoietic cells > lung > intestine [38]. Furthermore, these differences in Sp1 sensitivity suggest that additional, cell type specific factors might be involved in initiation complex formation and/or RNA polymerase II activation.

There are a number of different transcription factors, including Sp1, Sp3, and members of the XKLF family, that bind to the same GC box recognition sequences. These proteins can act as repressors or activators of transcription depending upon the promoter context, the isoform expressed, the presence or absence of post-translational modifications, and/or the relative abundance of different family members [39]. For example, phosphorylation at any of a number of different sites that can lead to either activation or repression of transcription from different Sp1 responsive promoters. Dephosphorylation of Sp1 by protein phosphatase 2A (PP2A) increases the DNA binding affinity of Sp1 [40]. This process may be important for the transcription of C2GnT-I in activated T-cells. Activation of naïve T-cells stimulates cell division and expression of PP2A. This would lead to increased Sp1 binding to the C2GnT-I promoter 2, transcription of C2GnT-I, and the synthesis of the selectin ligands necessary for extravasation.

Other kinases and phosphatases have been shown to affect the phosphorylation of Sp1 including P42/44 MAPK, PPI, PKA, and PKC. In addition, O-GlcNAcylation of Sp1 may repress transcription by preventing the interaction of Sp1 with the TATA-binding-protein-associated factor dTAF (II)110 or by interfering with the access of kinases to Sp1 and preventing phosphorylation [41].

In mucin producing epithelial cells the expression of C2GnT-I and other glycosyltransferases is important for the proper biosynthesis and secretion of mucus. It has recently been shown that the expression of mucin apoprotein genes MUC1, MUC2 and MUC5AC and other glycosyltransferase genes, ST6Gal I, and  $\beta$ 1,4 galactosyltransferase are mediated by Sp1 [30, 42, 43]. This common requirement for Sp1 may be a regulatory mechanism for the coordinate regulation of both mucin and glycosyltransferase genes to ensure efficient mucus production.

Increased expression of C2GnT-I in transformed and malignant cells may be the result of elevated levels of Sp1 [44]. Many of the genes that are regulated by Sp1, like the hTERT oncogene, are involved in cellular proliferation [45] and can be repressed by Sp3 [46]. Increased expression of Sp1 leads directly to the expression of oncogenes [47] as



**Fig. 7** Diagram of C2GnT-I promoter 2 activity relative to the number of Sp1 binding sites present in each construct. The various promoter deletions are listed on the Y-axis. Symbols represent the ratio of firefly to renilla luciferase activity from each construct normalized to the activity of the –620 bp construct. The data represent two to seven independent transfections, each done in triplicate



well as other genes that promote angiogenesis and tumor growth, such as VEGF [48] and has been correlated with an overall increase in the malignancy of transformed cells in many cases [49, 50]. Our data indicate that C2GnT-I is another member of the family of genes that are over-expressed in malignant cells as a result of Sp1 expression. As with many of these genes, increased expression of C2GnT-I promotes tumor progression by boosting the synthesis of selectin ligands that provide the initial interaction required for extravasation.

In summary, the most often used of the four identified promoters of C2GnT-I, promoter 2, contains multiple consensus binding sites for the binding of Sp1. The presence of these GC rich regions has been historically believed to be indicative of constitutively expressed housekeeping genes. However, the data presented are consistent with the model that these sequences mediate regulated and tissue specific expression as well. Further work will be necessary to unravel the mechanism for these regulatory processes.

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