

# Promoters of Human *Cosmc* and *T-synthase* Genes Are Similar in Structure, Yet Different in Epigenetic Regulation\*

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**Background:** Transcriptional regulation of human *T-synthase* and *Cosmc* is unknown.

**Results:** Minimal promoters for *Cosmc* and *T-synthase* are located in CpG islands, and the promoter of *Cosmc*, but not *T-synthase*, was hypermethylated in Tn4 B cells.

**Conclusion:** Promoters of *Cosmc* and *T-synthase* are similarly structured, yet different in epigenetic regulation.

**Significance:** These data aid in understanding the role of these genes in biology and diseases.

The T-synthase (core 1  $\beta$ 3-galactosyltransferase) and its molecular chaperone *Cosmc* regulate the biosynthesis of mucin type O-glycans on glycoproteins, and evidence suggests that both *T-synthase* and *Cosmc* are transcriptionally suppressed in several human diseases, although the transcriptional regulation of these two genes is not understood. Here, we characterized the promoters essential for human *Cosmc* and *T-synthase* transcription. The upstream regions of the genes lack a conventional TATA box but contain CpG islands, cCpG-I and cCpG-II for *Cosmc* and tCpG for *T-synthase*. Using luciferase reporter assays, site-directed mutagenesis, ChIP assays, and mithramycin A treatment, we identified the core promoters within cCpG-II and tCpG, which contain two binding sites for Krüppel-like transcription factors, including SP1/SP3, respectively. Methylome analysis of Tn4 B cells, which harbor a silenced *Cosmc*, confirmed the hypermethylation of the *Cosmc* core promoter but not for *T-synthase*. These results demonstrate that *Cosmc* and *T-synthase* are transcriptionally regulated at a basal level by the specificity protein/Krüppel-like transcription factor family of members, which explains their ubiquitous and coordinated expression, and also indicate that they are differentially epigenetically regulated beyond X chromosome imprinting. These results are important in understanding the regulation of these genes that have roles in human diseases, such as IgA nephropathy and cancer.

Mucin type O-glycosylation is one of the most abundant post-translational modifications and is initiated and com-

pleted by a set of glycosyltransferases in the Golgi apparatus. Among them is the core 1  $\beta$ 3-galactosyltransferase or C1GalT1 (T-synthase),<sup>5</sup> which is the key enzyme that transfers galactose from UDP-galactose to GalNAc $\alpha$ 1-Ser/Thr (Tn antigen) in glycoproteins to form the core 1 disaccharide Gal $\beta$ 1–3GalNAc $\alpha$ 1-Ser/Thr. Core 1 serves as a precursor for a variety of complex O-glycans (1). These core 1-based O-glycans play wide ranging roles in many pathways, including immunity (2–4), cell-cell interactions (3–7), vascular biology (8), angiogenesis (9), and lymphangiogenesis (10). Interestingly, the endoplasmic reticulum-localized molecular chaperone *Cosmc* (core 1  $\beta$ 3-galactosyltransferase-specific molecular chaperone or C1GalT1C1) is required for active T-synthase formation *in vivo* by preventing its aggregation and subsequent proteasomal degradation (11, 12). Disruption of either *T-synthase* or *Cosmc* is embryonic lethal due to hemorrhaging, suggesting the important role of O-glycosylation in vascular biology (9, 13).

The structures of O-glycans change during cell differentiation and animal development, indicating that expression of glycosyltransferases is both temporally and spatially regulated. Yet the details on the mechanisms of regulation of these enzymes, especially at the transcriptional level, are poorly understood. Unusual expression of Tn and its sialylated version, sialyl-Tn (STn), due at least in part to compromised expression of either *T-synthase* and/or *Cosmc*, are found in several human diseases, such as Tn syndrome (14), IgA nephropathy (IgAN) (15), and human tumors (16). For example, somatic mutations in *Cosmc* were identified in the blood cells from patients with Tn syndrome (17, 18). Tn/STn antigens, which are highly expressed in tumor cell lines and human primary cervical cancer tissue, also arise from point mutations in the *Cosmc* coding region or the deletion of *Cosmc* (19, 20). Furthermore, the Tn4 cells derived

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<sup>5</sup> The abbreviations used are: T-synthase, core 1  $\beta$ 3-galactosyltransferase (UDP-Gal:N-acetylgalactosaminyl- $\alpha$ 1-Ser/Thr  $\beta$ 3-galactosyltransferase); *Cosmc*, core 1  $\beta$ 3-galactosyltransferase specific molecular chaperone; TF, transcription factor; TSS, transcription start site; Tn antigen, GalNAc $\alpha$ 1-Ser/Thr; KLF, Krüppel-like transcription factor; SP, specificity protein; OGT, O-linked N-acetylglucosaminyltransferase; IP, immunoprecipitation; GT, glycosyltransferase; IgAN, IgA nephropathy; STn, sialyl-Tn; MeDIP, methylated DNA immunoprecipitation.

TABLE 1

Primers used for amplifying 5'-upstream region of human *Cosmc* and *T-synthase* and oligonucleotides for site-directed mutagenesis

Name	Sequence
C-P-F1	5'-CCCAGGTTCAAGCAATTC-3'
C-P-R1	5'-CTTCTGATGGTGATCCTAC-3'
C-550F	5'-ATAGGTACCGAATTCGAGGGAAGTGGGCACACGCGA-3'
C-400F	5'-ATAGGTACCGAATTCGTTGGCTTGGTCTCA-3'
C-260F	5'-ATAGGTACCGAATTCATTTCCATGCCAGGAAGGTG-3'
C-160F	5'-ATAGGTACC GAATTCAGGCCATAGCAAGTCTCTG-3'
C-100F	5'-ATAGGTACCGAATTCGCGCAGCGGGCGCCGCGCC-3'
C-60F	5'-ATAGGTACCGAATTCGGGCAAACGGACGGCTCCGCC-3'
C-10R	5'-GTAAAGCTTGGATCCAGTGGGGCGGGGTAAAGGAGC-3'
C-60R	5'-GTA AAGCTTGGATCCCGCCCGCAGCGCTCGAAG-3'
C+90R	5'-GTAAAGCTTGGATCCTACGCTCCTGACCAGGCTGTT-3'
C-150R	5'-GTAAAGCTTGGATCCTATAGGCCTGCGTGACCCGGACGC-3'
C-F9	5'-TTCTCCATAGAGGAGTTGTG-3'
C-R10	5'-TGTGGTTATACCAGTGCCACC-3'
C-42-45-SP1-F	5'-CGGCTCCTTTACCCCTAACCACTACCCCGCCT-3'
C-42-45-SP1-R	5'-AGGCGGGGTAGTGGTTAGGGGTAAAGGAGCCG-3'
C-17-15-SP1-F	5'-CAAACGGACGGCTCCTAACAGCCGCGGCTCCT-3'
C-17-15-SP1-R	5'-AGGAGCCGGCGGCTGTAGGAGCCGTCCGTTTG-3'
T-P-F5	5'-GCTCTCCGGCCATTGCCCC-3'
T-P-R5	5'-GCACCTCTGGAAGGAGCCCG-3'
T-334F	5'-CTTTCGTCCCGCTCAAGGTC-3'
T-169F	5'-ATAGGTACCGAATTCAGCCAATCCCGCTTCTCTC-3'
T-89F	5'-ATAGGTACCGAATTCGGCGGAGACGTCAGCGCCAG-3'
T-82R	5'-ATAGGTACCGAATTCGCTGCCGCCAGATTATTC-3'
T-38-36SP1-F	5'-GGGTGGCGGCTGCGCCATAGGATTGCCACGGCCTGGACGCGCTGC-3'
T-38-36SP1-R	5'-CGCGCTCCCGCCCGCTCGCTATTCCTCCCGCAGCGCTCCA-3'
T-7-5-SP1-F	5'-TGGACGCGCTGCGGGGAGGGGAATAGCGAGCGGGCGGAGCGC GC-3'
T-7-5-SP1-R	5'-CGCGCTCCCGCCCGCTCGCTATTCCTCCCGCAGCGCTCCA-3'

from an individual whose leukocytes carry Tn antigen harbor a silenced *Cosmc* but an active *T-synthase* due to hypermethylation of the putative promoter region of *Cosmc* (21). Galactose-deficient O-glycans or Tn/STn antigens are commonly found in the hinge region of immunoglobulin A1 (IgA1) molecules from sera of patients with IgAN, and they also occur in serum IgA1 from normal individuals in our study (22). Although the mechanism underlying a fraction of IgA1 from both healthy controls and IgAN patients carrying Tn/STn antigens is not fully understood, several studies have shown that under-galactosylated IgA1 is associated with a lower transcription level of *Cosmc* and *T-synthase* in peripheral B cells or immortalized B cells from patients with IgAN (23–26). Hypermethylation of *Cosmc* occurs in many human pancreatic cancer samples and is associated with Tn antigen expression and malignant behavior of the tumor cells (27). Therefore, a major mechanism for the expression of Tn antigen on cells is the loss of functional *Cosmc*, including both genetic mutations and epigenetic dysregulation of *Cosmc* (19, 21). Knock-out of either *T-synthase* or *Cosmc* in mice causes embryonic lethality and Tn antigen expression (9, 13), and specific deletion of *T-synthase* in endothelial and hematopoietic cells in mice causes defective lymphatic vessel formation (10) and loss of high endothelial venule integrity (28); specific disruption of either *Cosmc* (29) or *T-synthase* (30) in hematopoietic cells confers megathrombocytopenia and bleeding in mice.

Human *T-synthase* is located on 7p21 (earlier was designated as 7p14-p13) with three exons (31). By contrast, human *Cosmc* is encoded by a single exon gene located on Xq24 (11). Interestingly, human *Cosmc* and *T-synthase* are ubiquitously and coordinately expressed, yet their expression level varies substantially from tissue to tissue or cell type to cell type (12, 31). Here, we report the identification and characterization of the promoters for human *Cosmc* and *T-synthase*. Our results show that

the CpG islands at 5'-flanking regions of human *Cosmc* and *T-synthase* each containing two SP1/3-binding sites are the gene promoters. ChIP assays and site-directed mutagenesis analyses of either SP1/3 site confirmed the essential role of the SP1/3 sequence in the regulation of these two genes. Importantly, the methylome analysis of the Tn4 B cells harboring a silenced *Cosmc* revealed that the promoter, including the minimum promoter of *Cosmc* but not *T-synthase*, was hypermethylated.

### Experimental Procedures

**Reagents**—All routine chemical reagents and mithramycin A were purchased from Sigma, Fisher, or VWR (Suwanee, GA). Deoxyoligonucleotides were ordered from Integrated DNA Technologies, Inc. (Coralville, IA).

**Cell Culture and Mithramycin A Treatment**—Two human cell lines, FEMX-I (kindly provided by the group of Dr. Oystein Fodstad, Norwegian Radium Hospital Research Foundation, Norway) and HEK293T cells (ATCC 293tsA1609neo), which have high T-synthase activities, were chosen for the promoter characterizations and grown in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), penicillin (100 IU/ml), and streptomycin (100 IU/ml) at 37 °C, 5% CO<sub>2</sub>. Mithramycin A was dissolved in sterile Milli-Q water to make a 1 mM stock solution. For mithramycin A treatment, FEMX-I cells and 293T cells were seeded with 5 × 10<sup>6</sup> cells per T75 flask with the indicated concentration of mithramycin A for the desired duration of treatment. After drug exposure, cells were collected by centrifugation.

**Western Blot and Quantification**—Cells were lysed in an appropriate volume of 20 mM Tris-HCl buffer (pH 7.2) containing 300 mM NaCl, 0.5% Triton X-100, and proteinase inhibitor mixture (Roche Applied Science) for 30 min on ice. Lysates were then centrifuged at 15,000 rpm for 15 min at 4 °C. Protein

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concentration in supernatants was determined by bicinchoninic acid (BCA) protein assay (Pierce). For each lysate, 10  $\mu\text{g}$  of total reduced proteins were separated on Mini-PROTEAN TGX gels (Bio-Rad) and transferred electrophoretically onto a nitrocellulose membrane and immunoblotted with the indicated antibodies. The signals of immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce).

Pictures were inverted and subtracted from background value, and band densitometric intensities were then measured by FluorChem and expressed as a percentage relative to  $\beta$ -actin in the same sample, and the integrated density value of *T-synthase*/ $\beta$ -actin at day 0 was set as 100%. The values were averaged from three different gels from three experiments.

**Cloning and Characterization of the 5'-Flanking Region**—A list of all oligonucleotide primers used in this study is provided in Table 1. To generate pGlu-*CosmcP*(-2066/537) (transcription starting site (TSS) as +1), the *Cosmc* 5'-flanking region was amplified by PCR Phusion high fidelity polymerase (New England Biolabs) using genomic DNA from normal human leukocytes as template and the oligonucleotides C-P-F1 and C-P-R1 covering -2161 to +687 bp as primers. The PCR product was cloned into PCR-TOPO vector (Invitrogen), and then partially digested with EcoRI and subcloned into the multiple cloning sites of the pBluescript SK(+) vector. The insert was finally subcloned to pGlu-Basic vector (New England Biolabs) using the engineered restriction sites for HindIII and BamHI. The nucleotide sequences of the plasmid were confirmed by sequencing.

Other deletion constructs were generated by PCR with pGlu-*CosmcP*(-2066/537) as template, namely pGlu-*CosmcP*(-550/90), pGlu-*CosmcP*(-400/90), pGlu-*CosmcP*(-260/90), pGlu-*CosmcP*(-160/90), pGlu-*CosmcP*(-100/90), and pGlu-*CosmcP*(-60/90). The primers used for the above constructs were the reverse primer C+90R, combined with one of the forward primers C-550F, C-400F, C-260F, C-160F, C-100F, and C-60F, respectively. pGlu-*CosmcP*-sp1(-42/-45) and pGlu-*CosmcP*-sp1(-17-15) were constructed by introducing three point mutations into pGlu-*CosmcP*(-260/90) with the QuikChange kit (Stratagene), respectively. The primers used for generating the mutations were C-42-45-SP1-F/R and C-17-15-SP1-F/R (Table 1). The PCR product was purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified PCR product and plasmid pGlu-Basic were digested with NcoI and BamHI, purified with QIAquick gel extraction kit, and ligated according to the manufacturer's instructions. The ligation mixture was transformed into *Escherichia coli* strain Top10 competent cells. The sequences of the putative promoter region in the resulting plasmid were confirmed by DNA sequencing. The CpG island for *T-synthase* (tCpG) was cloned by PCR using primers T-P-F5/T-P-R5 covering -666 to +747 bp and human genomic DNA as the template. Similar methods were used for *T-synthase* promoter construction, and the primers for constructing plasmids for *T-synthase* promoter activity analysis are also listed in Table 1.

**Transient Transfection and Luciferase Assays**—FEMX-I and 293T cells were transfected 1 day after seeding in 6-well plates with up to  $1 \times 10^6$  cells per well using FuGENE 6 and 9 (Roche Applied Science). For each construct, triplicates were per-

formed, and 48 h after transfection, supernatants were collected, and luciferase activity was measured using the Gaussia luciferase assay kit (New England Biolabs). Briefly, luciferase activity was immediately measured by addition of 50  $\mu\text{l}$  of assay reagents mixed with 20  $\mu\text{l}$  of medium. The photon produced was measured by a Top Count NXT microplate scintillation and luminescence counter. Luciferase activities were calculated using fold difference as the activity of the control constructs pGlu-cCpGI-IIR (pGlu-*CosmcP*(-2066/537)-R) for *Cosmc* promoter constructs or pGlu-666/+747-R (pGlu-*TsynF5R5*-R) for *T-synthase* promoter constructs. The data were normalized with luciferase gene containing vector copy number. Unpaired Student's *t* tests were performed to determine statistical significance with \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , and \*\*\*,  $p < 0.001$ .

**Chromatin Immunoprecipitation (ChIP) and PCR Analysis**—Chromatin was isolated from formaldehyde-treated FEMX-I cells and was further treated with sonication. Antibodies used for immunoprecipitation were anti-SP1 and normal rabbit IgG (Millipore Upstate, Temecula, CA). PCR was carried out with the C-60F and C+90R primer sets to amplify SP1-binding sites containing regions within the *Cosmc* promoter. F-89 and R+63 primer sets were used for *T-synthase* ChIP analysis. To search for possible known cis-regulatory elements, the programs MacVector and Promoter Scan (PROSCAN, www-bimas.cit.nih.gov) were used.

**DNA Methylation Analysis**—DNA methylation profiling was performed as described (32). Briefly, genomic DNA of Tn4 and control C12 cells were isolated using a DNeasy Blood and Tissue kit (Qiagen). Methylated DNA immunoprecipitation (MeDIP) using 5mC-specific antibody was performed. Immunoprecipitated DNA along with input was used for library preparation with NEBNext ChIP-Seq Library Prep Reagent Set for Illumina, and 38-cycle single-end sequencing was performed using Version 4 Cluster Generation and Sequencing kits (parts 15005236 and 15002739) and Version 7.0 recipes. We use the standard Illumina Pipeline to process image and extract sequence.

FASTQ sequence files were aligned to the reference genome using Bowtie, keeping only unique nonduplicate genomic matches with no more than two mismatches within the first 25 bp. Unique and nonduplicate reads from nonenriched input genomic DNA and 5mC-enriched sequence set will be counted in 100-, 1000-, and 10,000-bp bins genome-wide and subsequently normalized to the total number of nonduplicate reads in millions. Input-normalized values will then be subtracted from 5mC-enriched values per bin to generate normalized 5mC signals. To determine genomic regions that present differential 5mC profiles, we identified true 5mC-enriched regions or "peaks" using the model-based analysis of ChIP-Seq (MACS) algorithm.

**Quantitative RT-PCR**—Total RNA of drug-treated 293T and FEMX-I cells was extracted with RNeasy mini kit (Qiagen). RT-PCR was carried out with 1  $\mu\text{g}$  of mRNA as template followed by SuperScript First-strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. The quantitative real time PCR was performed in triplicate in a 20- $\mu\text{l}$  reaction mixture containing gene-specific primers and



Power SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY) using the 7500 Standard Real Time PCR systems (Applied Biosystems). Primers for human *Cosmc*, *T-synthase* transcripts, and internal control 18S rRNA were designed using Primer Express 3.0 software (Applied Biosystems) and are listed in Table 2. The relative quantifications were calculated by  $\Delta\Delta C_t$  method and normalized to the control samples.

## Results

**CpG Island-II at 5'-Flanking Region of Human *Cosmc* Has Gene Promoter Activity**—Human *Cosmc* is a single exon gene originally designated within a 20-kb DNA fragment on Xq24, although it is now annotated as 4.47 kb in the GRCh38 Primary Assembly ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Analysis of the 5'-flanking region of *Cosmc* with the on-line program CpG Island Searcher identified two GC-rich regions or CpG islands (CpG-I)

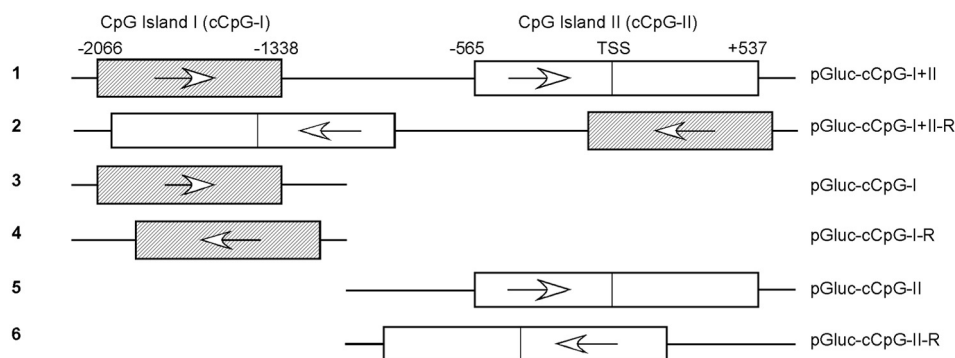
upstream of the *Cosmc* exon, designated as cCpG-I and cCpG-II. cCpG-I localizes between  $-4980'$  and  $-4237'$  bp (designated the translation initiation site as  $+1'$  bp) with a size of 744 bp, and cCpG-II is between  $-3478'$  and  $-2377'$  bp with 1102 bp. Alignment between the 5'-untranslated region (5'-UTR) of the *Cosmc* cDNA revealed that the TSS falls in cCpG-II, suggesting that CpG-II is the putative promoter of human *Cosmc*. However, this promoter region does not have a typical TATA box, consistent with early studies that CpG island promoters may often lack TATA boxes (33).

To experimentally confirm the promoter activity of the cCpG-II for *Cosmc*, we performed luciferase reporter assays using Gaussia luciferase-containing vector, pGluc (New England Biolabs). The constructs pGluc-cCpG-I-II, containing both CpG islands I and II, and pGluc-cCpG-I and pGluc-cCpG-II, containing cCpG island I and cCpG island II, respectively, as well as the reverse-oriented cCpG-I-II (pGluc-cCpG-I-II-R) and cCpG-II (pGluc-cCpG-II-R) are listed in Fig. 1A. All of the constructs were transfected into a human melanoma cell line, FEMX-I, and at 48 h post-transfection the luciferase activity in the cell culture media was measured. Compared with the activity of luciferase from pGluc-cCpG-I-II-R and the basic vector pGluc-Basic,  $\sim 48$ -, 1-, and  $\sim 562$ -fold increases of the luciferase activity were detected in the media of cells transfected with pGluc-cCpG-I-II, pGluc-cCpG-I, and pGluc-cCpG-II, respectively (Fig. 1B), indicating that human *Cosmc* CpG-II contains

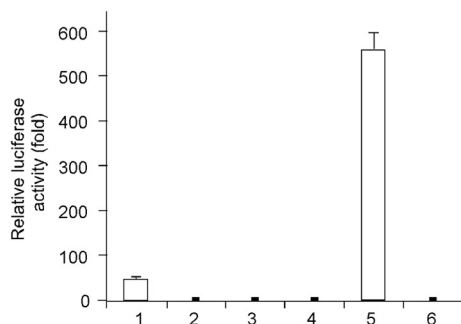
**TABLE 2**  
Primers used for quantitative RT-PCR of human *Cosmc* and *T-synthase*

Gene name	Sequence
<i>Cosmc</i>	Forward, 5'-CCTTGTAACCAAGATGTGAGT-3' Reverse, 5'-TGTCACAGTGTGGTCCAAGTC-3'
<i>T-synthase</i>	Forward, 5'-GGACAGAATCATCTAGAAGGACAAATG-3' Reverse, 5'-CAATGTCTGTCTCATCTTTATGTTG-3'
18S rRNA	Forward, 5'-CGGCTACCACATCCAAGGAA-3' Reverse, 5'-CCTGTATTGTTATTTTCGTCACCT-3'

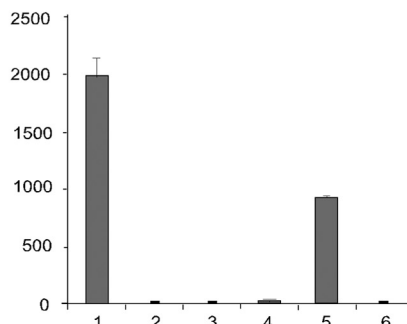
### A. Constructs of Reporter Assay for *Cosmc* Promoter



### B. Luciferase Activity of Constructs for *Cosmc* Promoter in FEMX-I Cells



### C. Luciferase Activity of Constructs for *Cosmc* Promoter in 293T Cells



**FIGURE 1. CpG islands at 5'-flanking regions of human *Cosmc* have gene promoter activity.** A, schematic structure of human *Cosmc* promoter showing putative CpG islands at 5'-flanking regions predicted by bioinformatic tools. The name of each construct was assigned according to the numbers of CpG islands (shaded box, cCpG-I; open box, cCpG-II) located upstream of the initiation codon ATG. B and C, luciferase activity of FEMX-I (B) and 293T (C) cells transfected with human *Cosmc* promoter constructs containing CpG islands in pGluc-basic reporter plasmid. The activity of each construct was normalized to the copy number of the promoter region on the chromosome or plasmid with semi-quantitative PCR. Error bars represent mean  $\pm$  1 S.D. of three biological replicates. The luciferase activity of each construct is calculated as fold-change relative to the background activity of pGluc-Cosmc-CpG-I-II-R vector.

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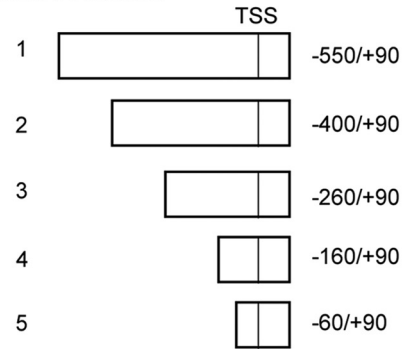
strong promoter activity. Importantly, pGluc-cCpG-II-R had no detectable activity, which is consistent with the importance of the promoter orientation. Unexpectedly, the activity of pGluc-cCpG-I was close to that of background, indicating that the CpG-I island itself does not contain a *Cosmc* promoter. Interestingly, the DNA fragment containing both cCpG-I and -II, pGluc-Cosmc-CpG-I-II, had much lower activity than pGluc-cCpG-II, 48- versus 562-fold, suggesting that CpG-I island may contain regulatory elements that could suppress the transcription of *Cosmc* in certain cell types.

We also tested the promoter activity of the constructs in another human cell line, HEK293T (human embryonic kidney 293T, 293T). We chose 293T and human melanoma FEMX-I for the reporter assay because they are developmentally distant cell types, yet they all have robust activity of T-synthase. The trend of reporter luciferase activity of constructs 2–5 in 293T cells was similar to that in FEMX-I cells confirming that cCpG-II is the promoter for human *Cosmc* (Fig. 1C). Interestingly, construct 1, having both cCpG-I and cCpG-II (pGluc-cCpG-I-II), had a similar activity of pGluc-cCpG-II in 293T cells (Fig. 1C). Combining this result with the result from FEMX-I cells, the data strongly suggest that there is TF regulating the activity of cCpG-I as a suppressor, and furthermore, this TF either does not exist in 293T cells or the pathway causing this TF to be released is not activated in 293T cells, in comparison with FEMX-I cells.

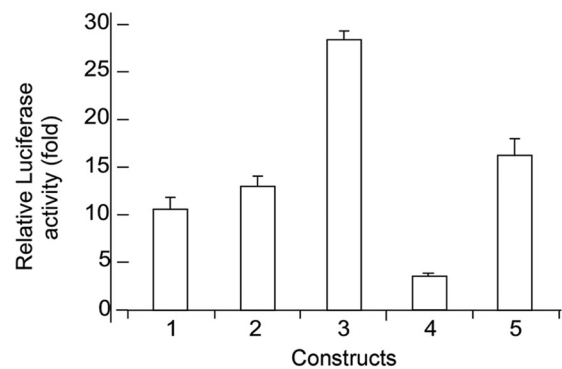
To fairly compare the promoter activities between the constructs, the relative activity or fold-change for each construct in each experiment was normalized by the apparent activity of luciferase divided by the copy number of the plasmid. The copy number of the plasmids in the transfected cells was analyzed by semi-quantitative PCR using the primers listed in Table 1 for amplifying the vector-insert chimeras and genomic DNA isolated from the cells at the time when the media were harvested. The copy number of the plasmids was amplified in a linear range, and the DNA intensity was carefully measured using a densitometer. The copy number of the *Cosmc* coding region amplified by C-F9/C-R10 was used as an internal control. In all experiments with luciferase reporter assays, the activity was normalized and represented the same way.

**Identification of the Minimal Promoter of *Cosmc***—To better understand the transcriptional regulation of *Cosmc*, the information on the minimal DNA region required for promoter activity was investigated. To identify the minimal promoter for *Cosmc*, DNA fragments with serial truncations at the 5'-end of the *Cosmc* promoter within the region  $-550$  to  $+90$  bp (TSS as  $+1$ ) were generated by PCR and subcloned into a pGluc-Basic vector (Fig. 2A). These constructs were transfected into FEMX-I, and the medium was collected at 48 h post-transfection to measure luciferase activity. As shown in Fig. 2B, all of the constructs had some promoter activity, although variations between different constructs were observed. The smallest DNA fragment  $-60/+90$  (pGluc-cCpG-II-60 + 90) had substantial activity, indicating this 150-bp DNA in the cCpG-II is the minimal promoter for human *Cosmc*. Interestingly, the fragment of  $-260/+90$  (pGluc-cCpG-II-260 + 90) had the highest promoter activity, in contrast to the fragments of  $-160/+90$  (pGluc-CpG-II-160 + 90) with the lowest activity. These data

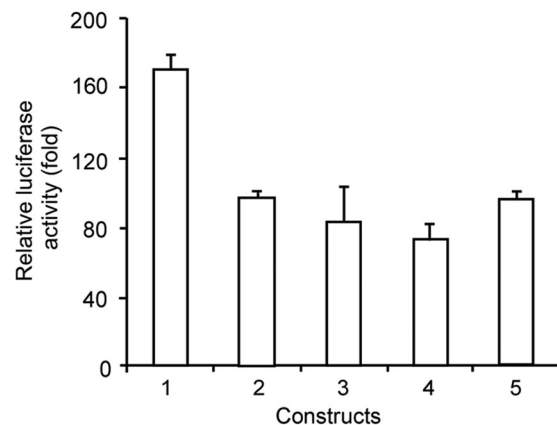
### A. Constructs with Truncations at 5'-End of *Cosmc* Promoter



### B. Luciferase Activity of Constructs in FEMX-I Cells



### C. Luciferase Activity of Constructs in 293T Cells



**FIGURE 2. Identification of *Cosmc* gene core promoter.** A, schematic structure of *Cosmc* promoter deletion/truncation constructs. B and C, luciferase activity of FEMX-I (B) and 293T (C) cells transfected with human *Cosmc* promoter deletion constructs. The activity of each construct was normalized to the copy number of the promoter region on the chromosome or plasmid with semi-quantitative PCR. Error bars represent mean  $\pm$  1 S.D. of three biological replicates. The luciferase activity of each construct is calculated fold-change relative to the background activity of pGluc-CpG-I-II-R vector.

suggested that the 70 bp within  $-260/-190$  bp of *Cosmc* might have an enhancer element for FEMX-I cells. In 293T cells (Fig. 2C), a different pattern of luciferase activity from different constructs was observed: construct 1 ( $-550/+90$ ) had the highest activity, and construct 3 ( $-260/+90$ ) had relatively low activity in contrast to that in FEMX-I cells, indicating that the TF profiles of FEMX-I and HEK293 cells are very different. Neverthe-

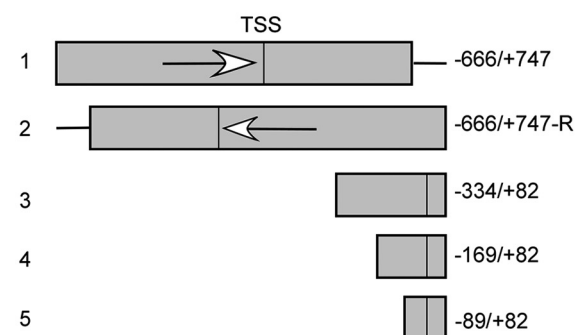
less, the DNA fragment of  $-60/+90$  in the cCpG-II in both cells gave a similar robust luciferase activity, suggesting the 150-bp DNA fragment ( $-60/+90$ ) in cCpG-II is the minimal promoter for human *Cosmc*.

**CpG Island at 5'-Flanking Region of Human *T-synthase* Has Gene Promoter Activity**—Early studies showed that human *Cosmc* and *T-synthase* are coordinately expressed (12). To understand this phenomenon, we also analyzed the promoter for human *T-synthase*. Human *T-synthase* is composed of three exons on 7p14–13 stretching over 115 kb, which is much larger than *Cosmc*. Using a similar strategy for identification of the gene promoter for *Cosmc*, one GC-rich DNA fragment (CpG island) with 1.4 kb at the 5'-flanking region was identified, and the 5'-UTR of *T-synthase* cDNA fell in this region. Similar to the human *Cosmc* gene promoter, no typical TATA box was found in this putative promoter region for *T-synthase*. With a similar strategy to the promoter reporter assay for *Cosmc*, the 1.4-kb DNA fragment containing the CpG island (tCpG) of human *T-synthase* was cloned into a pGluc-Basic vector, designated as pGluc-tCpG (TSS as +1) (Fig. 3A), and its promoter activity was measured at 48 h post-transfection into both FEMX-I cells and 293T cells. As shown in Fig. 3, B and C, the luciferase activity of pGluc-tCpG (construct 1  $-666/+747$ ) was low but consistently greater than that of the reverse-oriented tCpG vector, pGluc-tCpG-R (construct 2  $-666/+747$ -R), indicating that the tCpG has the promoter activity for human *T-synthase*.

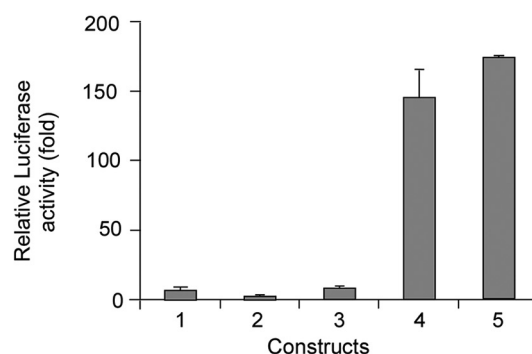
To identify the minimal DNA fragment with its *T-synthase* promoter activity, serial constructs with truncations at the 5'-end of tCpG within  $-334$  to  $+82$  bp (Fig. 3A, constructs 3–5) were also analyzed for their promoter activity in both FEMX-I and 293T cells. In FEMX-I cells, the promoter activity increased along with the 5'-truncations of the tCpG (Fig. 3B), with construct 4 representing  $-169/+82$  bp and construct 5 representing  $-89/+82$  bp, having similar activity to each other, suggesting that 171 bp from  $-89/+82$  bp was the minimal promoter for human *T-synthase*. These data also suggested that the 497-bp fragment from  $-666$  to  $-169$  bp contains a negative regulatory element, because the construct containing this region had little activity. In 293T cells (Fig. 3C), constructs 4 and 5 had the highest activity as seen in FEMX-I cells, although construct 3 showed much higher activity than that from FEMX-I cells. The differences in the activity observed in different cell lines might also arise from the differences in TF profiles between these two different types of cells. Nevertheless, these results from two different cell lines indicated that the minimal promoter for human *T-synthase* is the  $-89/+82$ -bp DNA fragment.

**Two Binding Sites for SP1/3 in the 150-bp Minimal Promoter of *Cosmc* and 171-bp Minimal Promoter of *T-synthase* Are Essential for Basal Promoter Activity**—Knowing the minimal DNA sequences that were required for the promoter activity of *Cosmc* and *T-synthase*, we investigated the potential TFs for transactivating the expression of these two genes. We used two programs, Promoter Scan program ([www.bimas.cit.nih.gov](http://www.bimas.cit.nih.gov)) and MacVector Nucleotide Sequence Analysis-Mammalian TF, to search the possible TFs that bind to the *Cosmc* promoter cCpG-II and *T-synthase* promoter tCpG. No TATA or CCAAT

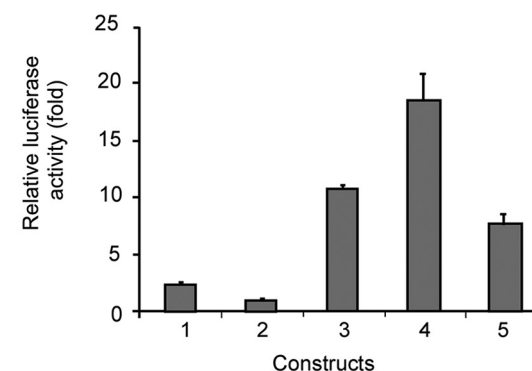
### A. Constructs with Truncations at 5'-End of *T-synthase* Promoter



### B. Luciferase Activity of Constructs in FEMX-I cells



### C. Luciferase Activity of Constructs in 293T cells



**FIGURE 3. Identification of *T-synthase* gene core promoter.** A, schematic structure of human *T-synthase* promoter deletion constructs. B and C, luciferase activity of FEMX-I (B) and 293T (C) cells transfected with human *T-synthase* promoter constructs with truncations at the 5'-end. The activity of each construct was normalized to the copy number of the promoter region on the chromosome or plasmid with semi-quantitative PCR. Error bars represent mean  $\pm$  1 S.D. of three biological replicates. The luciferase activity of each construct is calculated as fold-change relative to the background activity of pGluc-666/+747-R vector.

box was found in either gene (Figs. 4 and 5); however, many putative TF-binding sequences were predicted in those promoters. Significantly, close to the TSS of mRNA for *Cosmc* and *T-synthase*, two putative binding sites for SP1/3, 5'-ACCCCG-CCCC-3' and 5'-GCTCCGCCCA-3', were located on the minus strand in the cCpG-II and two SP1/3-binding sequences, 5'-ATAGCGGGCCA-3' and 5'-GGGGCGGAGC-3', were also found in tCpG. Because their binding sites are located upstream proximal to the TSS, SP1/3 are general TFs and *Cosmc* and *T-synthase* are ubiquitously expressed, we considered these



## Promoters of Human *Cosmc* and *T-synthase*



FIGURE 4. **Promoter sequences of human *Cosmc*.** The full sequence and potential transcription factor-binding sites predicted by MacVector 12 of promoter (cCpG-II) for human *Cosmc* is shown. Putative transcription factor consensus binding sites are indicated by *bold*, *italics*, *underlines*, or *strikethroughs*. Transcription start sites are indicated. The TSS for human *Cosmc* predicted by DBTSS (chromosome X:120625226–120631269) is –64 bp upstream. The binding sites and sequences by two critical TFs, SP1/3, upstream and close to the TSS are in *bold* and highlighted in *yellow*.

SP1/3-binding sequences in *Cosmc* and *T-synthase* good candidates for regulating the minimal promoter activity.

To examine whether the promoter activity is controlled by these KLF/SP TF SP1/3-binding sequences within the *Cosmc* minimal promoter region, each of the SP1/3-binding sequences in the constructs was mutated individually, designated as pGluc-*Cosmc*-M1 and -M2 or doubly designated as pGluc-*Cosmc*-M1/2 (*starred* constructs in Fig. 6A). These constructs were transfected into FEMX-I cells, and the media were collected at 48 h post-transfection and measured for luciferase activities. Either mutation of the two SP1/3 sequences or double mutant abolished the promoter activity (Fig. 6B), demonstrating that the basal promoter activity for *Cosmc* is controlled by these two SP1/3-binding sites. Interestingly, in 293T cells, mutations in the SP1/3-binding sequences in the *hCosmc* min-

imal promoter only caused a modest decrease in promoter activity (Fig. 6C). These results are not totally surprising, because the profile of TFs in melanoma FEMX-I cells is likely very different from that in embryonic kidney 293T cells; this also indicates that the mutated sequences could be transactivated by some TFs existing in 293T cells that are expressed at low levels in FEMX-I cells.

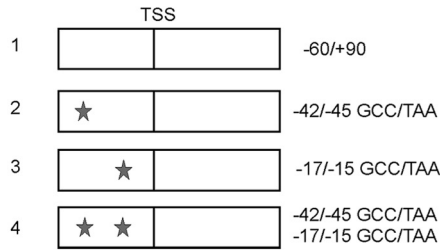
Similar to the *Cosmc* promoter, the *T-synthase* minimal promoter region (–89/+82 bp) also contains two SP1/3 sites. Mutations were introduced into either SP1 sites, pGluc-Tsyn-M1 and pGluc-Tsyn-M2, or both sites, pGluc-Tsyn-M1/2 (*starred* constructs in Fig. 6D), which were then transfected into FEMX-I cells and 293T cells. As shown in Fig. 6E in FEMX-I cells, the luciferase activity in either one or both SP1/3 mutations was abrogated, demonstrating that these two SP1-



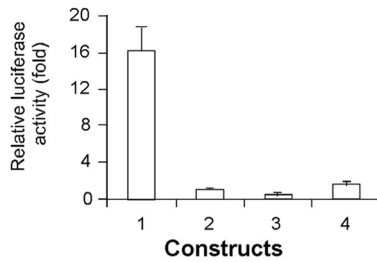


## Promoters of Human *Cosmc* and *T-synthase*

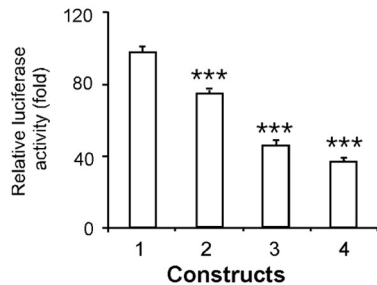
### A. Constructs of *Cosmc* Core Promoter



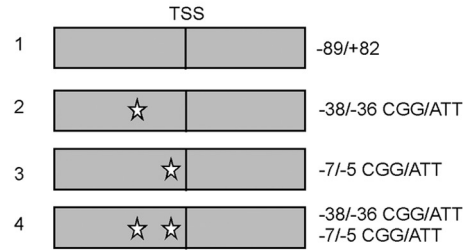
### B. Luciferase Activity of *Cosmc* Core Promoter Constructs in FEMX-I cells



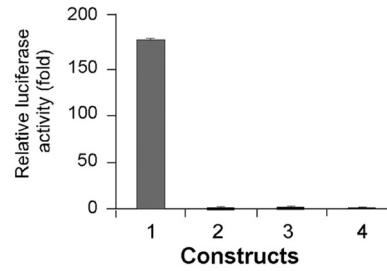
### C. Luciferase Activity of *Cosmc* Core Promoter Constructs in 293T Cells



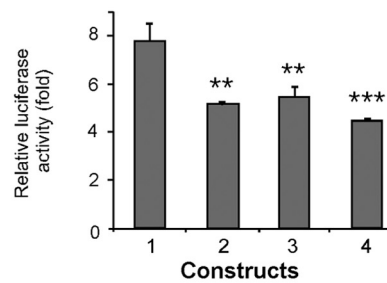
### D. Constructs of *T-synthase* Core Promoter



### E. Luciferase Activity of *T-synthase* Core Promoter Constructs in FEMX-I Cells



### F. Luciferase Activity of *T-synthase* Core Promoter Constructs in 293T Cells



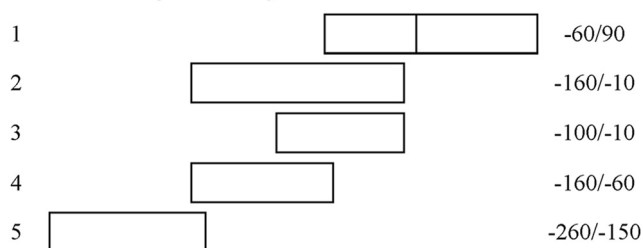
**FIGURE 6. GC-rich sequences, such as SP1-binding sites, are important for maintaining minimal promoter activity of human *Cosmc* and *T-synthase* gene promoter.** *A*, schematic structure of human *Cosmc* promoter mutant constructs. Stars indicate mutation sites. *B* and *C*, mutation of consensus nucleotides of putative SP1- or SP3-binding sites result in reduced human *Cosmc* core promoter-driven luciferase activity in FEMX-I (*B*) and 293T (*C*) cells. The activity of each construct was normalized to the copy number of the promoter region on the chromosome or plasmid with semi-quantitative PCR. Error bars represent mean  $\pm$  1 S.D. of three biological replicates. The luciferase activity of each construct is calculated as fold-change relative to the background activity of pGluc-*Cosmc*-CpG-III-R vector. *D*, schematic structure of human *T-synthase* promoter mutant constructs. *E* and *F*, mutation of consensus nucleotides of putative SP1- or SP3-binding sites result in reduced human *T-synthase* core promoter-driven luciferase activity in FEMX-I (*E*) and 293T (*F*) cells. The activity of each construct was normalized to the copy number of the promoter region on the chromosome or plasmid with semi-quantitative PCR. Error bars represent mean  $\pm$  1 S.D. of three biological replicates. The luciferase activity of each construct is calculated as fold-change relative to the background activity of pGluc-Tsyn-F5R5-R vector. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

with the expected size of 150 bp was generated from the PCR after IP with anti-SP1 and anti-SP3 antibodies, while no detectable product was seen in the IP with the isotype control antibody and with blank material (Fig. 8A). These results demonstrate that SP1 and SP3 are associated with the *Cosmc* minimal promoter. Similarly, ChIP assay for the human *T-synthase* promoter was also performed using primers covering -89 to +82 bp, including the two critical SP1 and SP3 sites in the minimal promoter. An expected product with a size of 171 bp corresponding to the minimal promoter for *T-synthase* was amplified (Fig. 8B) in the specific anti-SP1 and anti-SP3 antibody pulldown materials but not in the isotype control antibody and blank material experiment, confirming that SP1/3 sequences of human *T-synthase* minimal promoter were bound by SP1 and SP3 TFs.

Through these combined approaches, the promoters of human *Cosmc* and *T-synthase* are identified and characterized, and the full sequences are shown in Fig. 4 for *Cosmc* and Fig. 5

for *T-synthase*. It is noteworthy that both *Cosmc* and *T-synthase* are coordinately and ubiquitously expressed, and the identification of the important KLFs, SP1/SP3, clearly explains this phenomenon. Furthermore, these two genes are also differentially expressed in different tissues or cell types (12, 31), suggesting that the tissue-specific TFs are also involved in their transcriptional regulation by playing the enhancer or suppressor role. Indeed, there are many consensus sequences being recognized by putative TFs, such as AP2,  $\gamma$ -IRE, and other KLFs for both *Cosmc* and *T-synthase*. Taken together, our results suggest that the basal levels of human *Cosmc* and *T-synthase* are similarly transcriptionally regulated by KLF SP1/3 TFs and also enhanced or suppressed by some cell- or tissue-specific TFs.

*Treatment with Mithramycin A Caused Reduction of *Cosmc* and *T-synthase* mRNAs and *T-synthase* Protein and Activity*—The above results indicated that KLFs, including SP1/3, are the important TFs regulating *Cosmc* and *T-synthase* transcription.

A. Constructs of Reporter Assay for *Cosmc* Promoter

## B. Luciferase Activity of Constructs in FEMX-I Cells

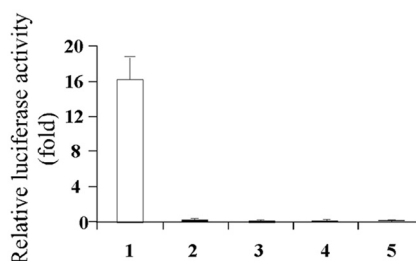


FIGURE 7. **Characterization of *Cosmc* 3'-truncated promoter constructs.** *A*, schematic illustration of *Cosmc* promoter with 3'-deletion constructs. *B*, luciferase activity. FEMX-I cells were transfected with *Cosmc* promoter 3'-truncation constructs. The activity of each construct was normalized to the copy number of the promoter region on the chromosome or plasmid with semi-quantitative PCR. Error bars represent mean  $\pm$  1 S.D. of three biological replicates. The luciferase activity of each construct is calculated by fold-change relative to the background activity of pGluc-*Cosmc*-CpG-II-R construct.

Some studies (34, 35) reported that mithramycin A generally binds to GC-rich DNA sequences and consequently prevents the binding of TFs leading to compromised transcription. Thus, we explored whether mithramycin A could affect the binding of KLFs to the DNA sequence leading to alteration of *Cosmc* and *T-synthase* transcription.

To determine the concentration of mithramycin A for treatment, we cultured 293T and FEMX-I cells in the presence of a range of concentrations of mithramycin A for 8 days. Mithramycin A did not significantly alter the viability of 293T cells at concentrations  $\leq$ 100 nM and FEMX-I cells at concentrations  $\leq$ 10 nM. Therefore, 293T and FEMX-I cells were treated with two different concentrations (50 and 100 nM for 293T cells and 5 and 10 nM for FEMX-I cells) of mithramycin A for different periods of time (0–8 days). To examine whether mithramycin A affected the transcription of *Cosmc* and *T-synthase*, quantitative RT-PCR to measure their mRNA levels was performed. As shown in Fig. 9A, the transcriptional levels of *Cosmc* and *T-synthase* were decreased in a dose-dependent manner in both cell lines tested. As expected, Western blots demonstrated that the protein levels of *Cosmc* and *T-synthase* were reduced in the mithramycin A-treated cells in a similar manner (Fig. 9B). To examine whether the mithramycin A treatment effect is relatively specific on the genes, including *Cosmc* and *T-synthase* who have the GC-rich promoters, a control glycosyltransferase,  $\beta$ -O-acetylglucosaminyltransferase (OGT), which catalyzes the addition of O-GlcNAc to cytoplasmic and nuclear proteins, was also analyzed by Western blotting. OGT was not significantly affected in cells by treatment with mithramycin A (Fig. 9B). Consistent with the decline of the protein levels, the activity of *T-synthase* in 293T and FEMX-I cells also decreased in a dose- and time-dependent manner upon treatment of mithramycin A

as follows: moderate decrease at a low concentration (50 and 5 nM, respectively), and a significant decrease 80 and 60% at a high concentration (100 and 10 nM) for 293T cells and FEMX-I cells, respectively, after 8 days of treatment (Fig. 9C). Taken together, these results confirm the important role of the GC-rich sequence of CpG islands in the regulation of transcription and expression of *Cosmc* and *T-synthase*.

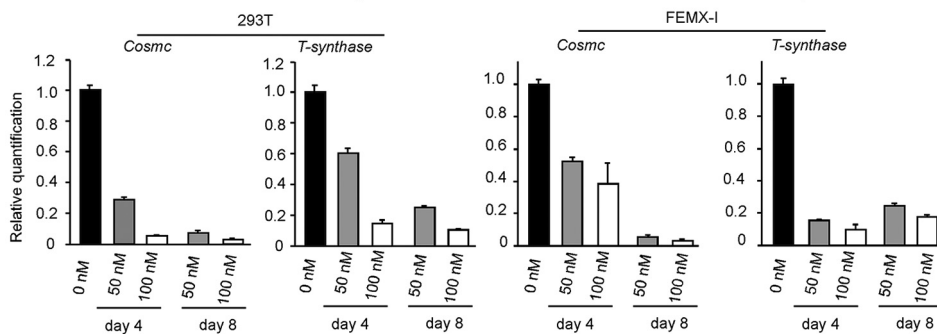
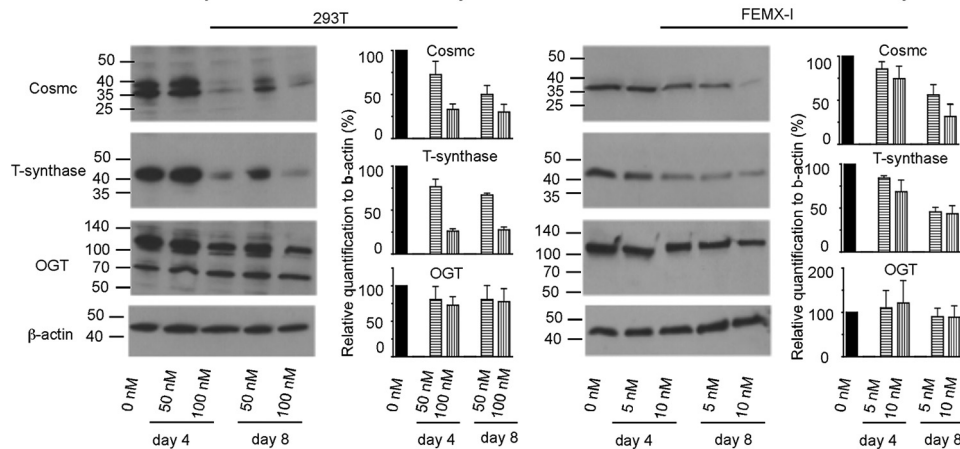
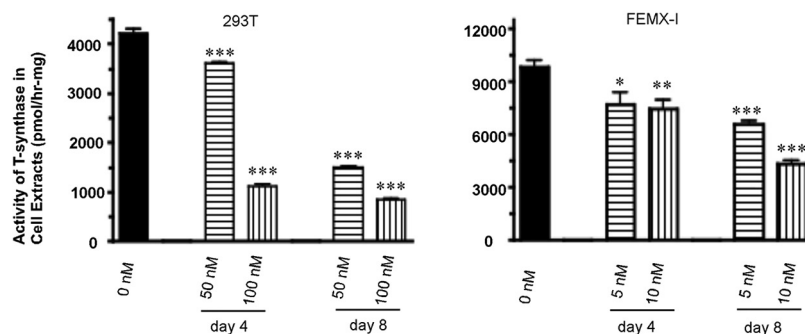
**Methylome Analysis Revealed Hyper-methylation of *Cosmc* Promoter in Tn4 B Cells**—DNA hypermethylation is generally found in CpG islands in gene promoter regions, and the degree of methylation reversibly correlates with the gene transcription level. Normal DNA methylation is essential for cell development and differentiation through regulation of gene transcription, chromatin structure, and accessibility (36). Dysregulation of DNA methylation is found to be associated with human diseases, such as carcinogenesis (37). Our previous study (21) using bisulfite sequencing showed that Tn4 B cells originally derived from a male individual with Tn-positive leukocytes have a silenced *Cosmc* due to core promoter hypermethylation but have a normally expressed *T-synthase*. To fully assess the DNA methylation in the Tn4 cells, we profiled the genome-wide DNA methylation by methylated DNA immunoprecipitation sequencing (MeDIP-seq). As shown in Fig. 10, *A* and *B*, in the control B cells (C12) immortalized from a healthy male individual both cCpG-II and tCpG were not methylated; the cCpG-II but not tCpG in Tn4 cells was hypermethylated. Examining the DNA sequences more closely, the hypermethylated region revealed by Methylome analysis falls in the central position of cCpG-II, including the minimum promoter,  $-60/+90$ -bp fragment (Fig. 2 and 5), further confirming the promoters of *Cosmc* and *T-synthase*, as well as verifying the experimental results (Figs. 2, 3, 6, and 8). Taken together, these results demonstrate that human *Cosmc* and *T-synthase* have similar promoter structures, yet they are differently regulated epigenetically beyond the X chromosome inactivation or imprinting of the X chromosome.

## Discussion

In this report, we have identified the promoters for both human *Cosmc* and *T-synthase*, two functionally related genes whose expression is essential for animal development and important for many biological processes through controlling the normal mucin type O-glycan biosynthesis. In addition, we have shown that these two genes are similarly regulated by GC-rich binding TFs, including the general TFs SP1 and SP3. Structurally, human *Cosmc* and *T-synthase* promoters have similarities as follows: CpG island with two GC-rich sequences proximal to the TSS, yet lacking a typical TATA box. Importantly, promoter activities of both *Cosmc* and *T-synthase* were reduced when SP1/3-binding sites were mutated, demonstrating that both genes are regulated by SP1/3 TFs. Furthermore, treatment with mithramycin A, a competitor for binding GC-rich DNA, results in a reduction in the endogenous transcription level of both *Cosmc* and *T-synthase* and a decrease in their activity levels, further confirming the essential role of SP1/3, and possibly other SP/KLF family members, in the regulation of the transcription and translation of these genes. Our data explain the nature of the coordinate expression of human





**A. Relative mRNA levels of *Cosmc* and *T-synthase* in 293T and FEMX-I cells treated with mithramycin A****B. Western Blots and quantification of *Cosmc* and *T-synthase* in 293T and FEMX-I cells treated with mithramycin A****C. *T-synthase* activity in 293T and FEMX-I cells treated with mithramycin A**

**FIGURE 9. Inhibition of SP1 differentially regulates human *Cosmc* and *T-synthase* expression.** *A*, mithramycin A treatment reduces the transcription levels of human *Cosmc* and *T-synthase* in 293T and FEMX-I cells at different concentrations as indicated. The levels of human *Cosmc* and *T-synthase* mRNA were normalized to that of the housekeeping gene 18S. Error bars represent mean  $\pm$  1 S.D. of three experimental replicates. *B*, Western blot analysis shows the protein expression levels of *Cosmc* and *T-synthase* after mithramycin A treatment at varying concentrations. Densitometry of relevant bands from Western blots was quantified by FluorChem and expressed as a percentage relative to  $\beta$ -actin in the same samples. Doublet in *Cosmc* gel represents both nonglycosylated and glycosylated forms, and both OGT band intensities were combined to determine the value. Data are means  $\pm$  1 S.E. of three independent assays. Anti- $\beta$ -actin antibodies were used as loading control. *C*, mithramycin A treatment caused a decline in *T-synthase* activity in 293T cells and FEMX-I cells. Error bars represent mean  $\pm$  1 S.E. of two biological replicates and three experimental replicates within each biological replicate. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

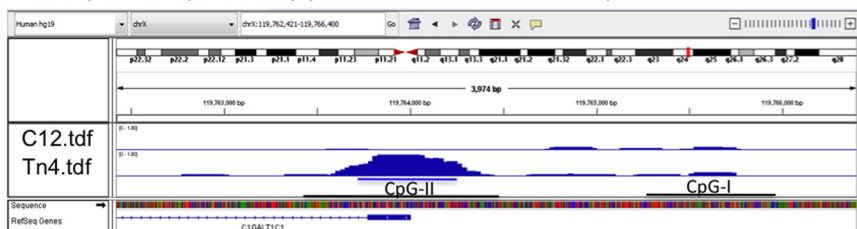
ment factors Sox5, Puralpha, and Olf1 (51). In the mucin type O-glycan biosynthetic pathway, the transcriptional regulation of a few GTs has been reported. The UDP-*N*-acetyl- $\alpha$ -D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-3 (ppGalNAc-T3) gene is restricted to epithelial glands and is regulated by the interaction of p300/CBP-associated factor with nuclear respiratory factor-1 (52). Core 2  $\beta$ 1, 6-*N*-acetylglucosaminyltransferase-I, which catalyzes the synthesis of core 2 structures in O-glycans, is a gene with a promoter that is putatively recognized by multiple tissue-specific

TFs (53) and is regulated by SP1 in lymphocytes and epithelial cells (54).

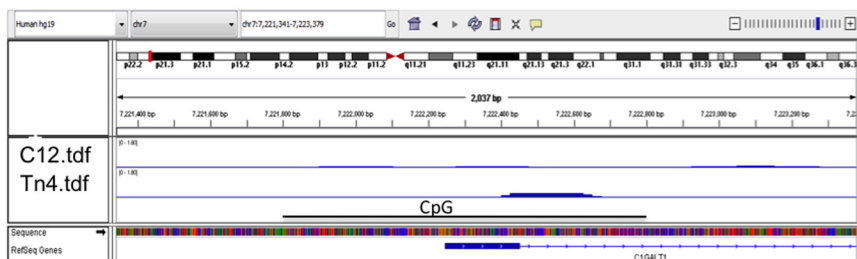
Most normal O-glycans are synthesized from the common precursor core 1 disaccharide, the product of *T-synthase*. Thus, it is not surprising that *T-synthase* is regulated by KLF, a family of general TFs, including SP1 and SP3. Naturally, its molecular chaperone *Cosmc* is also under control in a similar fashion. Our findings explain how both *Cosmc* and *T-synthase* are coordinately and ubiquitously expressed. Krüppel-like factors, including SP1/SP3, are a family of ubiquitously expressed C2H2-type

## Promoters of Human *Cosmc* and *T-synthase*

**A.** *Cosmc* promoter CpG islands (CpGI: 119,765,998-119,765,258; CpGI: 119,764,497-119,763,408)



**B.** *T-synthase* promoter CpG island (7,221,800-7,222,800)



**FIGURE 10. Methylation analysis.** DNA methylation in *Cosmc* (A) and *T-synthase* (B) genes in Tn4 and a control healthy B cell line C12 were analyzed by MeDIP. The methylation profile is shown in custom track in IGV2.3 (Integrative Genomics Viewer). Regions enriched for hypermethylated DNA in Tn4 cells are indicated by a *blue bar*, and the hypermethylation peaks that only existed in Tn4 cells are *underlined*. Predicted CpG islands of *T-synthase* and *Cosmc* were shown with a *line below*. The analyzed genes lie at the bottom of the panel, where *blue boxes* indicate exons. The *arrow* shows the transcriptional direction of the gene.

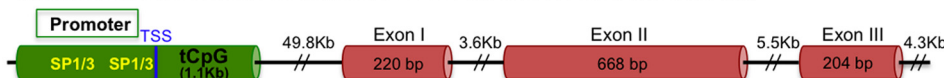
**A. Gene Structure of Human *Cosmc* (*C1GalT1C1*) (4.47 Kb) (Xq24)**

(Reference GRCh38 Primary Assembly: NC\_000023.11, Range: 120625674...120630150 bp)



**B. Gene Structure of Human *T-synthase* (*C1GalT1*) (66.1 Kb) (7p21.3)**

(Reference GRCh38 Primary Assembly: NC\_000007.14, Range: 7,182,547...7,248,651bp)



**FIGURE 11. Schematic representation of *Cosmc* and *T-synthase* gene structures.** A, human *Cosmc* gene on Xq24 comprises a promoter region in CpG-II and one single exon spanning 4473 bp based on the GRCh38 Primary Assembly, and it may contain an upstream suppressor element as the CpG-I was included in the older version of Primary Assembly GRCh37. B, human *T-synthase* gene spanning 66.1 kb on 7p21.3 (mapped on 7p14–13 earlier) comprises a promoter in the CpG island (tCpG) and three exons, with the number of nucleotides indicated. In both genes, upstream CpG(s) sequences and the exons are shown as *cylinders*, with the putative binding sites of SP1/3 indicated. The intronic sequences are shown as *lines with two italic bars* to indicate that the entire lengths are not included. The positions of *Cosmc* and *T-synthase* genes are shown in the GRCh38 Primary Assembly.

zinc finger-containing DNA-binding proteins (55, 56). This family of TFs regulates expression of numerous genes in the metabolic pathway of nucleic acids (e.g. thymidylate synthase, adenine deaminase, and DNA polymerase), cell cycle, and proliferation, such as cyclin D1, E2F, c-Fos, and TGF- $\alpha$  (57–60). Several GTs involved in O-glycan biosynthesis are also under control of SP1. In P19 cells, the ST6GalNAc-III gene is transcriptionally regulated by two of the three SP1-binding sites in its promoter, whereas in NIH3T3 cells, the ST6GalNAc-IV gene is transcriptionally under control of all three SP1-binding sites (61). Sato and Furukawa (62) found that the activation of the human  $\beta$ 1,4-galactosyltransferase-V gene is through the sequential action of Ets-1 and SP1, and altered expression was involved in abnormal glycosylation characteristic of cancer cells.

We found that the cis-elements of both *Cosmc* and *T-synthase* lack the TATA box but have the GC-rich region (CpG island) containing two independent yet nearby SP1/3-binding

sequences, which proved to be essential for their transcription. Generally the promoters of SP1-regulated genes lack a *bona fide* TATA-box that binds to the TATA-binding protein and nucleates the assembly of TATA-binding protein-associated factors (63). In a TATA-less element, SP1 facilitates a sequential recruitment of TFIID and RNA polymerase II; SP1 binds to TFIID via TAFII130 and TAFII55 subunits (64–66). Interestingly, the promoter activity of *Cosmc/T-synthase* requires both SP1 sites for their basal transcription, as seen in many GTs that are controlled by multiple TFs. Indeed, compelling evidence indicates that SP1-dependent activation and repression of target genes are modulated by its interactions with a repertoire of heterogeneous transcriptional factors, such as c-Myc, GATAs, p53, MEF2C, Smad2–4, Rb, NF- $\kappa$ B, NF-YA, VHL, MyoD, E2F, NFAT-1, YY1, and others (67). This might be the mechanism for the regulation of *Cosmc* and *T-synthase* in a tissue-specific manner, through the interaction of SP1 with the tissue or cell-specific transcription factor(s). These multiple TFs have to be

recruited by their corresponding DNA sequences in concert or sequential to the cis-regulatory element to form functional transcriptional complexes.

Mithramycin A is an antineoplastic antibiotic produced by *Streptomyces plicatus* because it has an inhibition activity on DNA/RNA polymerase. Many studies showed that it can interact with the GC-rich DNA, competing with TF SP1 binding, thereby inhibiting gene transcription (35). The promoters of human *Cosmc* and *T-synthase* are composed of GC-rich islands. Because mithramycin A inhibition of SP1 affects transcription of many genes, including some GTs (49), we tested whether mithramycin A could down-regulate their transcription. Both *Cosmc* and *T-synthase* transcripts, as well as *T-synthase* activity, declined upon long term treatment of mithramycin A. However, the protein level of another glycosyltransferase (OGT), which was determined as a control, was not significantly changed, suggesting that the overall translation machinery is not affected by mithramycin A. These data indicate that the decrease of functional transcripts of *Cosmc* and *T-synthase* was caused by the mithramycin A treatment.

The dysregulated expression of *Cosmc* and/or *T-synthase* may be important in IgAN. This is the most common primary glomerulonephritis (68–70) worldwide and is characterized by deposition of IgA1 in the mesangium. Studies suggest that a deficiency of galactose in the *O*-glycans and concomitant expression of the Tn antigen or STn in the hinge region of IgA1 might be responsible for initiating the pathogenesis (15, 23, 71–74) of IgAN in patients. However, we recently identified two distinct glycoforms as follows: the major fraction of IgA1 has normal *O*-glycans, and the minor fraction carries Tn/STn antigens in the plasma from both patients with IgAN and healthy controls (22), suggesting a more complex role of Tn/STn antigens on IgA1 in the disease. Several studies showed that the transcript levels of *Cosmc* and/or *T-synthase* are reduced in the B cells of patients with IgAN (24–26, 76–78). Suzuki *et al.* (75) reported that IgA1-secreting cell lines from patients with IgAN produce aberrantly glycosylated IgA1 due to lower transcripts of both *Cosmc* and *T-synthase* and up-regulated *ST6GalNAc-II*. Yet the mechanisms by which *Cosmc* and *T-synthase* are suppressed is unknown. Our results show that *Cosmc* and *T-synthase* are transcriptionally regulated by the general TF SP1 for their basal activity, and our data also suggest that other TFs may play enhancer or suppressor roles in overall control of gene transcription. It is also worth noting that the cCpG-I upstream of the promoter cCpG-II in *Cosmc* seems to play a suppressor role in the transcription of *Cosmc* in some types of cells. There are many potential transcription factor-binding sequences in cCpG-I, including NF- $\kappa$ B. It will be of great interest to define the role of these in plasma cells, which may be crucial to reveal the role of *Cosmc* and/or *T-synthase* in IgAN.

In summary, we have characterized the promoters for both human *Cosmc* and *T-synthase*, two functionally related genes that are similarly transcriptionally regulated by a general TF SP1/3 through two proximal SP1/3-binding sequences. Our data help to explain the observation that human *Cosmc* and *T-synthase* are ubiquitously expressed together, because their promoters are similar to each other. We also observed that the

expression levels of human *Cosmc* and *T-synthase* in different tissues varies substantially (12, 31), indicating that tissue-specific TFs may also play important roles in the regulation of these two genes. Indeed, as shown in our results, the upstream DNA sequences of the minimal promoters harbor many putative TF recognition sites (Figs. 4 and 5), which could also affect their activities, indicating that additional TFs other than SP1/3 could bind to the sequences in this region to enhance or repress the expression of *Cosmc* and *T-synthase* in a tissue- or cell-specific manner. To fully understand the transcriptional regulation of these two genes, the tissue-specific TFs, for example the human B cell-specific or plasma cell-specific TFs and the pathways associated with these TFs, also need to be further characterized. A better understanding of the regulation of *Cosmc* and *T-synthase* will certainly aid in elucidating the mechanisms for the expression of abnormal *O*-glycans, Tn and STn, in IgA1 from patients with IgAN as well as in transformed cells from patients with tumors, such as colorectal carcinoma and breast cancer.

**Author Contributions**—T. J. and R. D. C. conceived and coordinated the study and wrote the paper. J. Z., R. M., Y. W., and L. S. designed, performed, and analyzed most of the experiments and data. T. J., J. Z., L. L., Y. B., Y. L., and P. J. designed, performed, and analyzed the experiments shown in Fig. 10. I. V. D. and A. B. C. provided critical reagents and analyzed and interpreted data. All authors reviewed the results and approved the final version of the manuscript.

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