Identification and characterization of transcriptional control region of the human beta 1,4-mannosyltransferase gene

Tetsuo Takahashi · Takashi Nedachi · Takuya Etoh ·
Hiroyuki Tachikawa · Xiao-Dong Gao

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Abstract All asparagine-linked glycans (*N*-glycans) on the eukaryotic glycoproteins are primarily derived from dolichol-linked oligosaccharides (DLO), synthesized on the rough endoplasmic reticulum membrane. We have previously reported cloning and identification of the human gene, *HMT-1*, which encodes chitobiosyldiphosphodolichol beta-mannosyltransferase (β 1,4-MT) involved in the early assembly of DLO. Considering that *N*-glycosylation is one of the most ubiquitous post-translational modifications for many eukaryotic proteins, the *HMT-1* could be postulated as one of the housekeeping genes, but its transcriptional regulation remains to be investigated. Here we screened a 1 kb region upstream from *HMT-1* open reading frame (ORF) for transcriptionally

regulatory sequences by using chloramphenicol acetyl transferase (CAT) assay, and found that the region from −33 to −1 positions might act in *HMT-1* transcription at basal level and that the region from −200 to −42 should regulate its transcription either positively or negatively. In addition, results with CAT assays suggested the possibility that two GATA-1 motifs and an Sp1 motif within a 200 bp region upstream from *HMT-1* ORF might significantly upregulate *HMT-1* transcription. On the contrary, the observations obtained from site-directed mutational analyses revealed that an NF-1/AP-2 overlapping motif located at −148 to −134 positions should serve as a strong silencer. The control of the *HMT-1* transcription by these motifs resided within the 200 bp region could partially explain the variation of expression level among various human tissues, suggesting availability and importance of this region for regulatory role in *HMT-1* expression.

Keywords Transcription · Gene expression · *N*-glycosylation · Dolichol-linked oligosaccharide · Mannosyltransferase

T. Takahashi (✉) · T. Nedachi · T. Etoh
Laboratory of Glycobiology and Glycotechnology,
Department of Applied Biochemistry, School of
Engineering, Tokai University, 4-1-1 Kitakaname,
Hiratsuka City, Kanagawa 259-1292, Japan
e-mail: tetsuo@keyaki.cc.u-tokai.ac.jp

H. Tachikawa
Laboratory of Biological Chemistry, Graduate School of
Agricultural and Life Sciences, The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

X.-D. Gao
Laboratory of Carbohydrate Chemistry and
Biotechnology, Ministry of Education School of
Biotechnology, Jiangnan University,
Wuxi 214122, Jiangsu Province, China

Abbreviations

DLO	Dolichol-linked oligosaccharide
MT	Mannosyltransferase
rER	Rough endoplasmic reticulum
β 1,4-MT	Beta 1,4-mannosyltransferase
HMT-1	Human mannosyltransferase I

kb	Kilo base pair
ORF	Open reading frame
CAT	Chloramphenicol acetyl transferase
GATA-1	GATA-binding factor 1
Sp1	Specificity protein 1
bp	Base pair
NF-1	Nuclear factor 1
AP-2	Activating protein 2
Man	Mannose
Dol	Dolichol
GlcNAc	<i>N</i> -Acetylglucosamine
UDP	Uridine diphosphate
GDP	Guanosine diphosphate
Glc	Glucose
Dol-P	Dolichyl phosphate
DPAGT1	Dolichyl-phosphate (UDP- <i>N</i> -acetylglucosamine) <i>N</i> -acetylglucosaminephosphotransferase 1
GlcNAc-1-P	<i>N</i> -Acetylglucosamine-1-phosphate
ALG1	Asparagine-linked glycosylation 1
CDG-I	Congenital disorder of glycosylation type I
α 1,3/ α 1,6-MT	Alpha 1,3/alpha 1,6-mannosyltransferase
α 1,2-MT	Alpha 1,2-mannosyltransferase
DMEM	Dulbecco's modified Eagle medium
RT-PCR	Reverse transcription-polymerase chain reaction
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
DEPC	Diethylpyrocarbonate
PBS	Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
OD	Optical density
ONPG	Ortho-nitrophenyl-beta-D-galactopyranoside
TLC	Thin layer chromatography
SE	Standard error
USF	Upstream stimulatory factor
AP-1	Activating protein 1
AP-4	Activating protein 4
Dex	Dexamethasone
RSV	Rous sarcoma virus
MMTV	Mouse mammary tumor virus
LTR	Long terminal repeat
Ets-1	E-twenty-six-1
NF-kB	Nuclear factor-kappa B

STAT5a	Signal transducer and activator of transcription 5a
TCF-1	Transcription factor 1
Alg7p	Asparagine-linked glycosylation 7 protein
Alg14p	Asparagine-linked glycosylation 14 protein

Introduction

Protein *N*-glycosylation is one of the most important events for maintenance of both structure and function of many glycoproteins in higher eukaryotes, especially those present in the extracellular and intracellular spaces (Varki 1993; Helenius and Aebi 2001), as well as yeast (Kukuruzinska et al. 1987). *N*-Glycans are divided into three classes: high mannose (Man), complex and hybrid types. These three species are derived from a common precursor, dolichol (Dol)-linked oligosaccharide (DLO), synthesized on the rough endoplasmic reticulum (rER) membrane. Assembly process of DLO is further divided into three stages: the first, early assembly of up to Man₅*N*-acetylglucosamine (GlcNAc)₂-PP-Dol on the cytoplasmic side by UDP-GlcNAc or GDP-Man dependent glycosyltransferases; the second, translocation of Man₅GlcNAc₂-PP-Dol into the luminal side by a putative flippase; and finally, late assembly of full-sized DLO [glucose (Glc)₃Man₆GlcNAc₂-PP-Dol] by Dol-P-Man or Dol-P-Glc dependent glycosyltransferases (Aebi 2013). These highly ordered steps are well conserved all through eukaryotes (Schwarz and Aebi 2011).

Of these three stages, early DLO assembly from dolichyl phosphate to Man₅GlcNAc₂-PP-Dol is fundamental and essential event for viability of eukaryotic cells, especially multicellular organisms. For example, using transgenic mice, it has been demonstrated that recessive deletion in the *DPAGT1* encoding *N*-acetylglucosaminyl-1-phosphate (GlcNAc-1-P) transferase, an enzyme that is first involved in early DLO assembly, causes embryonic lethality (Marek et al. 1999). In early DLO assembly, five steps of mannosylation occur, which involve three different GDP-Man dependent mannosyltransferases (MTs). Of these, the yeast *Saccharomyces cerevisiae* Alg1p encoded by the *ALG1* gene has been firstly

demonstrated to catalyze the first mannosylation in which the transfer of mannose from GDP-Man to chitobiosyldiphosphodolichol (GlcNAc₂-PP-Dol) occurs and beta-1,4 Man linkage is formed (EC 2.4.1.142; Couto et al. 1984; Albright and Robbins 1990). Previously we have cloned the open reading frame (ORF) encoding the human beta-1,4 mannosyltransferase (β 1,4-MT) gene from brain cDNA library as ortholog of the yeast *ALG1* gene and designated it as *HMT-1*. The *HMT-1* complemented the temperature-sensitive growth defect in yeast *alg1-1* mutant, indicating that it substantially encodes human β 1,4-MT (Takahashi et al. 2000).

In most eukaryotes, orthologous genes of the *ALG1* have been identified and several motifs and domains in the encoded enzyme have been shown to be highly conserved among species (Takahashi et al. 2000). As many *N*-glycans on glycoproteins are known to play an important role in maintenance of cell viability, any defect of enzymes involved in biosynthesis of the precursor DLO could cause various lesions in multiple organ, mainly neuromuscular system, which is known as congenital disorder of glycosylation type I (CDG-I; Haeuptle and Hennet 2009; Jaeken 2010; Cantagrel and Lefeber 2011). Indeed, dolichol kinase (Kranz et al. 2007; Helander et al. 2013; Lieu et al. 2013) and several glycosyltransferases involved in early DLO assembly, including GlcNAc-1-P transferase (Wu et al. 2003; Adamowicz et al. 2011; Würde et al. 2012; Timal et al. 2012; Carrera et al. 2012; Basiri et al. 2013), β 1,4-MT (de Koning et al. 1998; Grubenmann et al. 2004; Kranz et al. 2004; Schwarz et al. 2004; Dupré et al. 2010; Snow et al. 2012; Morava et al. 2012; Rohlfing et al. 2014), α 1,3/1,6-MT (Thiel et al. 2003) and α 1,2-MT (Rind et al. 2010; Thiel et al. 2012; Regal et al. 2015), are critical, because these deficiencies lead to relatively severe underglycosylation of *N*-glycoproteins and consequently cellular dysfunction (Buczowska et al. 2015). Thus, the *HMT-1* is well postulated to be one of housekeeping genes. Although expression of the *ALG1* has been reported to be controlled during cell division cycle in yeast (Lennon et al. 1995), no other analysis for expression of its ortholog has been reported in other organisms including human. In this study, we present data on regulatory mechanism for the *HMT-1* transcription. This is the first demonstration about functionality on transcriptional regulation by upstream region of a mammalian gene encoding a glycosyltransferase involved in early DLO synthesis. Analyses with

the chloramphenicol acetyl transferase (CAT) assays using HeLa cells were conducted over the 1 kb region upstream from initiation codon for the *HMT-1*. Collectively, results obtained in our study suggested that the 200 bp region upstream from *HMT-1* open reading frame (ORF) is crucial for both positive and negative regulation of the *HMT-1* transcription.

Materials and methods

Cell and media

HeLa cells, their transfectants made in this study and KB cells were routinely cultured in DMEM medium (Nissui, Tokyo, Japan) supplemented with 10 % FCS (Nissui), 0.2 % NaHCO₃, streptomycin (Meiji Seika, Tokyo, Japan) (40 μ g/mL) and penicillin G (Meiji Seika) (40 U/mL). They were all grown at 37 °C in a humidified 5 % CO₂ atmosphere in CO₂ incubator. For visible cell counting, standard trypan blue exclusion was carried out.

RT-PCR

The Expand Long Template PCR System (Roche Diagnostics, Tokyo, Japan) and PTC-150 Minicycler (MJ Research, Tokyo, Japan) were used for all PCRs performed below, according to the instructions from suppliers. First-stranded cDNAs from Human MTC cDNA Panels I and II (Clontech, Mountain View, CA, USA) were subjected to two-step PCR with the primers, A1-10RTF and A1-13R (Table 1), according to the instructions from the supplier, under the following condition; 94 °C 30 s (2 min at only the initial cycle) and 68 °C 3 min (5 min at only the last cycle), varying the repeating numbers from 22 to 34 cycles in a total volume of 50 μ L. 10 μ L of each reaction was analyzed by 1 % agarose gel electrophoresis. Control PCR for glycerol-3-phosphate dehydrogenase (*G3PDH*) was also performed under the same condition except to use human *G3PDH* control primers (forward: 5'-TGAA GGTTCGAGTCAACGGATTGTTGGT-3'; reverse: 5'-CATGTGGGCCATGAGGTCCACCAC-3') supplied in the above kits.

Primer extension assay

Total RNAs were isolated from 1×10^7 HeLa or KB cells using High Pure Total RNA Isolation Kit

Table 1 Oligonucleotides used in this study

Name	Nucleotide sequence	Purpose
A1-10RTF	5'-TGCGGGCCAGCCAAGATGGCGGCCTCATGC-3'	Forward primer for RT-PCR
A1-13R	5'-ATACGGGGGCTGCGGCCACGTCGCCCAGC-3'	Reverse primer for RT-PCR
A1-12RB	5'-biotin-GCACCACCGCTACTACATGCCGGG-3'	Primer extension
A1-G1F	5'-ccggATCCCTTCCCATGTGCTTTAAATC-3'	Forward primer for ET-8 construction
A1-13R	5'-ATACGGGGGCTGCGGCCACGTCGCCCAGC-3'	Reverse primer for ET-8 construction
PM-F	5'-CTGATACCGCTCGCCGAGCCG-3'	Primer for forward sequencing
PM-R	5'-GGTACATTGAGCAACTGACTGAAATG-3'	Primer for reverse sequencing
HMT1U-5F	5'-ggcatatgÄTTGTGTGGAAGGGACCCAGTGG-3'	Forward primer for NUP-1000 construction and sequencing
HMT1U-4F	5'-ggcatatgÄGTGAGGCCTTCCAGCCATGTG-3'	Forward primer for NUP-800 construction and sequencing
HMT1U-3F	5'-ggcatatgÄGAGGCTCACTGTGGAATTTCC-3'	Forward primer for NUP-600 construction and sequencing
HMT1U-2F	5'-ggcatatgGCAGAACCCACCAGCCCCCTAAC-3'	Forward primer for NUP-400 construction and sequencing
HMT1U-1F	5'-ggcatatgTAGGGAATCTGGTGTATGTGCG-3'	Forward primer for NUP-200 construction and sequencing
HMT1U-6F	5'-ggcatatgCTAATTCTTGGCCCGAGGGGC-3'	Forward primer for NUP-156 construction
HMT1U-7F	5'-ggcatatgCTGGGGGTCCCCGGGCCCTG-3'	Forward primer for NUP-132 construction
HMT1U-10F	5'-ggcatatgTTAGGGAACCGCGTCCACTTCT-3'	Forward primer for NUP-112 construction
HMT1U-8F	5'-ggcatatgCGGAGGCTCGGTCTGCGGCTGC-3'	Forward primer for NUP-70 construction
HMT1U-11F	5'-ggcatatgCGGCTGCGACAGCGCAGGAA-3'	Forward primer for NUP-57 construction
HMT1U-9F2	5'-cgcatatgCGAGGAAGCGCGGTCACGTGAC-3'	Forward primer for NUP-41 construction
HMT1U-9F	5'-ggcatatgGCGCGGTCACGTGACTGTGCG-3'	Forward primer for NUP-33 construction
HMT1U-1R	5'-ggcctagcÄTTGGCTGGCCCGAGCAGTCAC-3'	Reverse primer for all NUP series construction
HMT1U-6FN	5'-ggcatatgCTAATTCTTtCCCCGAGGGGC-3'	Forward primer for NUP-156/ <i>mutN</i> construction
HMT1U-6FA	5'-ggcatatgCTAATTCTTGGCCCGAAttGGC-3'	Forward primer for NUP-156/ <i>mutA</i> construction
HMT1U-7FK	5'-ggcatatgCTtttGGTTCCCCGGGCCCTG-3'	Forward primer for NUP-132/ <i>mutK</i> construction
HMT1U-7FN	5'-ggcatatgCTGGGGGTCCCCGttaCCCTG-3'	Forward primer for NUP-132/ <i>mutA</i> construction

In nucleotide sequences, the lowercase letters indicate artificial sequences, and restriction sites (*Bam* HI, *Nde* I or *Nhe* I) for cloning are underlined. All primers listed in above table were custom-made by Greiner Japan

(Roche Diagnostics, Japan). 10 µg of them were mixed with the 5'-biotinylated primer A1-12RB (Table 1) at final concentration of 20 µM in the 30 µL DEPC-treated water and incubated at 65 °C for 10 min and then chilled on ice for 2 min. Subsequently, reverse transcription was conducted at 42 °C for 30 min using Ready-To-Go RT-PCR beads (GE Healthcare, Tokyo, Japan). The reaction products were purified by phenol/chloroform extraction, concentrated by ethanol precipitation, and entirely subject to polyacrylamide gel electrophoresis, together with a sequencing reaction mix produced with another 5'-biotinylated primer supplied from Sequencing high Chemilumi kit (TOYOBO, Osaka, Japan) as ladder size marker. The procedures of electrophoresis followed by detection of signal bands have been previously described (Takahashi et al. 2000).

Cloning of an upstream region of the *HMT-1* gene

Genomic DNAs were prepared from 1×10^7 HeLa cells by standard method (Sambrook et al. 1989), and, as a template, 1 µg of them were subjected to PCR with final 10 µM primers A1-G1F and A1-13R (Table 1) under the following condition; 94 °C 30 s (2 min at only the initial cycle), 60 °C 30 s and 68 °C 6 min (7 min at only the last cycle) by 30 cycles in a total volume of 50 µL. The products were in part analyzed on 1 % agarose gel electrophoresis using λ DNA (Takara, Kusatsu, Shiga, Japan) digested with *Sly* I (Roche Diagnostics, Japan) as size marker. Finally they were digested with *Bam* HI and *Sma* I, purified and concentrated by High Pure PCR Product Purification Kit (Roche Diagnostics, Japan), and ligated into pUC118 vector (Vieira and Messing 1987), pre-digested with the same restriction enzymes, using DNA Ligation Kit Ver.2 (Takara, Japan). The ligation

mixture was used to transform *E. coli* JM109 strain by SEM method (Inoue et al. 1990). The resultant transformant, designated ET-8, was selected by the standard method (Sambrook et al. 1989).

For preparation of NUP construct series for CAT assays, the PCR with the primers HMT1U-5F and HMT1U-1R (Table 1) was first performed using ET-8 as template, under the following condition; 94 °C 30 s (1 min at only the initial cycle), 60 °C 30 s and 68 °C 1 min (2 min at only the last cycle) by 36 cycles in a total volume of 50 µL. The 1 kb amplified fragments were digested with *Nde* I and *Nhe* I (Roche Diagnostics, Japan), purified with High Pure PCR Product Purification Kit (Roche Diagnostics, Japan) and subsequently ligated into the vector pMAMneoCAT (Clontech, United States) treated with the same enzymes, using DNA Ligation Kit Ver.2 (Takara, Japan). The ligation mixture was used to transform *E. coli* JM109 strain by SEM method (Inoue et al. 1990) and the resultant subclone, designated as NUP-1000, was selected by the standard method (Sambrook et al. 1989). For nucleotide sequencing of NUP-1000, single-stranded DNAs were prepared and subjected to sequencing analyses by the same procedure as previously described (Takahashi et al. 2000), except usage of different sequencing primers listed in Table 1. Sequencing data were assembled and edited using GENETYX-MAC ver.12 software (GENETYX, Tokyo, Japan) on a personal computer.

Next, for construction of truncated subclones of NUP-1000, PCRs with corresponding forward primers listed in Table 1 and the reverse primer HMT1U-1R were conducted using NUP-1000 as template, under the same condition as case of NUP-1000 cloning. The products with corresponding lengths were subcloned into pMAMneoCAT by the same procedure as described above.

Detection of potential *cis*-acting motifs in a 1 kb region upstream from initiation codon of the *HMT-1*

For detection of potential motifs, which are located within the 1 kb region upstream from the initiation codon of the *HMT-1*, the region sequenced above was subjected to analysis using TF SEARCH software (<http://www.cbrc.jp/research/db/TFSEARCHJ.html>) and TRANSFAC 6.0 Public database (<http://www.gene-regulation.com/pub/databases.html>) on the web, or GENETYX-MAC ver.12 software (GENETYX, Japan) on a personal computer.

CAT assay

HeLa cells were co-transfected with 1.5 µg each NUP construct together with 1.0 µg control pMH lacZ6 vector (Roche diagnostics, Japan), which was used for normalization of transfection efficiency, using FuGENE6 transfection reagent (Roche Diagnostics, Japan), according to its instruction manual. In 48 h after transfection, surviving cells were scraped from the bottom of culture flasks using PBS buffer containing 0.02 % EDTA and harvested by centrifugation (800 rpm for 5 min) at 4 °C. The pellets were suspended in 1 mL PBS buffer and 35 µL of suspensions were removed for counting cell numbers. The remains of suspensions were again subject to centrifugation (10,000 rpm for 5 min) at 4 °C and pellets were resuspended in 200 µL buffer (0.25 M Tris-HCl pH 8.0). Cell lysis was performed by repeating incubations in liquid nitrogen for 4 min and in 37 °C water bath for 4 min by 6 cycles. For preparing clear lysates, lysis-treated samples were centrifuged at 15,000 rpm at 4 °C for 5 min. 20 µL each supernatant was removed to use for measurement of β-galactosidase activity resulting from transfection with pMH lacZ6 plasmid. After the inactivation treatment of other enzymes by heating at 65 °C for 10 min, the remains were cooled down to room temperature and immediately used for CAT assay. Using FAST CAT Assay Kit (Molecular Probes, Eugene, OR, USA), 60 µL each crude lysate was examined for CAT activity, according to the instruction manual provided by the supplier. The optical density at 420 (OD₄₂₀) and 504 nm (OD₅₀₄) were, respectively, measured for β-galactosidase and CAT activities with spectrophotometer DU640 (BECKMAN COULTER, Brea, CA, USA). Assays of β-galactosidase activities were done by standard method using ONPG as substrate. For assays of CAT activities, each reaction was evaporated, resolved in 8 µL ethyl acetate and subjected to thin layer chromatography using TLC plates (Merck, Tokyo, Japan) and chloroform/methanol mixture (in a ratio of 85:15) as solvent. After developing for approximately 30 min, signal bands corresponding to chloramphenicol and acetylated chloramphenicol were individually detected at a wavelength of 365 nm using UV transilluminator CSF-20 AF (COSMO BIO, Tokyo, Japan) to be excised from TLC plates. They were transferred into microtubes, in which chloramphenicol or acetylated chloramphenicol was recovered by extracting with

100 µL methanol for OD₅₀₄ measurement. Using measured values, each CAT activity was normalized with transfection efficiency on the basis of the following formula:

$$\text{CAT activity normalized with transfection efficiency (\%)} = \frac{\text{CAT activity measured with each sample (acetylation ratio; \%)} \times \text{Cell number of each sample}}{\beta\text{-galactosidase activity} - \frac{\text{CAT activity measured with HeLa cells (acetylation ratio; \%)} \times \text{Cell number of HeLa cells}}$$

Experimental procedure from co-transfection to calculation of CAT activity for each construct was independently conducted at least five times and each relative CAT activity against standard CAT activity was calculated as the mean value and standard error (SE). CAT activity with NUP-200 or NUP-132 was used as standard CAT activity.

Results

Expression analysis of the *HMT-1* in various human tissues

At the beginning of analysis on transcriptional regulation for the *HMT-1*, its expression profile in various tissues was briefly examined by semi-quantitative RT-PCR. As expected, the *HMT-1* transcripts were expressed in all sixteen tissues examined, indicating that human β1,4-MT is one of ubiquitous enzymes (Fig. 1a). However, as can be seen in Fig. 1a, b, the amount of the *HMT-1* fragments amplified after PCR with 30 cycles was nearly equal to or lower than that of the control housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) after PCR with 26 cycles. By PCRs with 22 or 26 cycles, no *HMT-1* fragment was amplified in all tissues (data not shown). PCRs for *HMT-1* and *G3PDH* were carried out under the same thermal and time conditions, annealing temperatures of *HMT-1*-specific forward and reverse primers (98.0 and 101 °C, respectively) were higher than those of *G3PDH*-specific forward and reverse primers (89.8 and 94.9 °C, respectively), and length of amplified fragments for *HMT-1* (161 bp) were much shorter than those for *G3PDH* (983 bp). *G3PDH* is

known to be an abundant transcript. Collectively, it could be speculated that *HMT-1* would be a medium- to low-abundant transcript in any tissue. Moreover, judging amplification efficiency from cycle number of

the PCRs, difference in abundance of the *HMT-1* mRNA among tissues examined was estimated to fall in the range by less than 16-fold (for example, compare amount of product after PCR with 34 cycles for small intestine with that after PCR with 30 cycles for testis in Fig. 1a). Notably, the *HMT-1* expression was likely to be generally upregulated in liver, kidney, spleen, pancreas, testis and ovary, possibly reflecting high demand of *N*-glycans for proteins produced in these tissues. Inversely, it seemed that its expression was downregulated in skeletal muscle, thymus, prostate and small intestine for unknown reason (Fig. 1a).

Determination of initiation site for the *HMT-1* transcription

In order to determine the transcription start site for the *HMT-1*, the primer extension assay was carried out. For this experiment, the 5'-biotinylated primer A1-12RB was designed to anneal at +95 to +118 positions downstream from initiation codon of the *HMT-1* transcript (Fig. 2a). The result shown in Fig. 2b indicated that the *HMT-1* transcription was started at the −19 position upstream from initiation codon, where five potential *cis*-acting motifs (for binding of USF, NF-1, AP-1, AP-4 and AP-2) were overlapping (see Fig. 3). The same result was also obtained from another primer extension experiment using human KB cells (data not shown). In both cases, no signal band indicating other initiation site available for the *HMT-1* transcription was detected. On the other hand, RNA protection analysis on the yeast *ALG1* gene has previously demonstrated that its transcription was started from several sites in

Fig. 1 Expression profiles of the *HMT-1* in various human tissues by semi-quantitative RT-PCR. **a** RT-PCRs were conducted using first-stranded cDNAs derived from human skeletal muscle, brain, lung, liver, kidney, heart, spleen, thymus (included in Human MTC Panel I cDNAs, Clontech) and pancreas, testis, prostate, placenta, ovary, small intestine, colon, leukocyte (included in Human MTC panel II cDNAs, Clontech)

as templates, as described in “Materials and methods”. After 30 and 34 cycles of PCRs, 10 μ L of each was analyzed by 1 % agarose gel electrophoresis. **b** Control PCRs for glycerol-3-phosphate dehydrogenase (G3PDH) with gene-specific primers were also performed under the same condition, except by 22 and 26 cycles

Fig. 2 Determination of initiation site for the *HMT-1* transcription by primer extension analysis. **a** The flanking region around initiation codon (boxed) of the *HMT-1* was illustrated. The 5'-biotinylated primer A1-12RB used in the experiment was designed to anneal at +95 to +118 position downstream from initiation codon of the *HMT-1* transcript (a horizontal arrow). **b** Product after primer extension reaction was subject to electrophoresis. Two numbers on the right side (–20 and

–15) indicate the estimated positions where products, respectively, extending from the primer to –20 and –15 positions from initiation codon will migrate. By reverse transcriptase, single-stranded cDNA fragments with length of 137 base (a horizontal arrow in **b**) were yielded. This means that the primer was extended up to –19 position upstream the start codon (a vertical arrow in **a**), as indicated by a horizontal broken arrow in **(a)**

yeast cells (Albright and Robbins, 1990). Although we have not performed similar analysis on mRNAs derived from various human tissues, these data suggest that the *HMT-1* transcription might be initiated from a single site in human cells, at least in general immortalized cells.

Identification of potential motifs for the *HMT-1* transcription within a 1 kb region upstream from *HMT-1* ORF

In order to identify the motifs which influence the *HMT-1* transcription, we searched for a genomic clone on

Fig. 3 Identification of potential *cis*-acting motifs within the 1 kb region upstream from the *HMT-1* ORF. Nucleotide sequence located in the 1 kb region upstream from the initiation codon of the *HMT-1* was determined from NUP-1000 subclone, as described in “Materials and methods”. Identification of potential *cis*-acting motifs was performed using TF SEARCH software and TRANSFAC database on the web, or GENETYX-MAC ver.12 software (GENETYX). Each position where a motif was located is indicated by *arrow*. One TATA box and three CAAT boxes are highlighted in *grey color*

GenBank database using nucleotide sequence of the *HMT-1* cDNA and found the 161 kb clone 165E (GenBank accession No. AC007011) derived from human chromosome 16p13.3. It contained 5' upstream region and exon 1–12 out of the total 13 *HMT-1* exons near its one end (Fig. 4a). For further analyses, an approximately 6.5 kb segment upstream from *HMT-1* ORF was first isolated by long PCR and subsequent conventional cloning. Using the obtained recombinant clone, designated ET-8, as template, a 1 kb segment upstream from *HMT-1* ORF was then amplified by PCR and subcloned into pMAMneoCAT vector (for detail, see “Materials and methods”) to generate a construct designated NUP-1000 (Fig. 4b). Sequencing of NUP-1000 revealed that it contained a large number of multiple motifs, which potentially bind specific transcription factors, as shown in Fig. 3. Although one TATA box (at –285 position) and three CAAT boxes (at –240, –323 and –356 positions) were detected within NUP-1000 sequence, they seemed to be non-functional for the *HMT-1* transcription, because they were not located within a 100 bp region upstream from transcription initiation site (at –19 position) which was determined by our primer extension analyses (Fig. 2b). Accordingly, the *HMT-1* can be considered as a TATA-less gene, and this idea agrees with the fact that many housekeeping genes are often controlled under TATA-less promoters.

Refinement of transcriptional control region of the *HMT-1*

In order to systematically detect motifs serving for controlling the *HMT-1* transcription, a series of deletants were constructed on the basis of NUP-1000. Obtained constructs, designated NUP-800, NUP-600, NUP-400 and NUP-200, include 800, 600, 400 and 200 bp regions upstream from *HMT-1* ORF, respectively (Fig. 4b). Then, CAT assays with them were conducted and subject to TLC analyses (Fig. 4c). Subsequently, each relative CAT activity used in Fig. 4d was calculated via normalization with transfection efficiency (for detail, see “Materials and methods”). HeLa cells used in our CAT assays exhibited no background CAT activity (see “Negative” in Fig. 4c), while the mock-transfectants with pMAMneoCAT vector had less than 6 % of CAT activity with NUP-200 in the absence (3.04 %, SE = 0.86) and presence (5.19 %, SE = 0.60) of the inducer dexamethasone

(Dex), due to leaky constitutive expression by RSV promoter and inducible expression by MMTV promoter, respectively (also see “pMAMneoCAT” and “pMAMneoCAT Dex” in Fig. 4c, d, respectively). In contrast to these negative controls, all NUP constructs, in which both RSV and MMTV LTR promoters were exchanged by the upstream regions of the *HMT-1* (Fig. 4b), yielded nearly equal transcriptional activity for the CAT (NUP-1000: 86.3 %, SE = 5.9; NUP-800: 79.0 %, SE = 8.6; NUP-600: 89.3 %, SE = 7.2; NUP = 400: 91.6 %, SE = 4.4; in comparison with CAT activity with NUP-200 as 100 %, also see Fig. 4c, d). In other words, stepwise deletion of a region from –1000 to –200 scarcely had significant effect on the CAT transcription activity. These observations indicate that a 200 bp region upstream from *HMT-1* ORF would be enough for sustaining the *HMT-1* transcription. Therefore, subsequent analyses were performed only in the range of this 200 bp region.

Detection of the regions that negatively or positively control the *HMT-1* transcription

As a result of scrutiny, the 200 bp region upstream from *HMT-1* ORF could be divided into eight segments, each of which might potentially bind transcription factor(s) (Fig. 5a). Thus, seven corresponding deletants (NUP-156, NUP-132, NUP-112, NUP-70, NUP-57, NUP-41 and NUP-33) were additionally constructed. As shown in Fig. 5b, CAT assays with these constructs resulted in graded reduction in CAT activity, according to shortening the length of the *HMT-1* upstream region from 200 bp, except those with NUP-132, NUP-41 and NUP-33 (NUP-156: 56.1 %, SE = 4.4; NUP-132: 146 %, SE = 2.6; NUP-112: 126 %, SE = 2.3; NUP-70: 57.0 %, SE = 9.5; NUP-57: 13.8 %, SE = 3.3; NUP-41: 37.5 %, SE = 9.1; NUP-33: 44.4 %, SE = 7.5; in comparison with CAT activity with NUP-200 as 100 %).

When NUP-156 was used instead of NUP-200 (namely, the segment I from –200 to –157 was deleted), CAT activity decreased by 44 %, suggesting the segment I might play a role in enhancement of transcription (Fig. 5b). Conversely, CAT activity increased by 46 % when NUP-132 was used instead of NUP-200 (Fig. 5b). This observation strongly suggested that the segment II from –156 to –133 should possess powerful repressive activity in transcription. CAT activity with NUP-112 slightly

Fig. 4 CAT assays for the 1 kb region upstream from the *HMT-1* ORF. **a** Schematic representation of human genomic clone 165E7 (GenBank accession No. AC007011) derived from chromosome 16p13.3. It contains 5'-upstream region and exons 1–12 out of 13 *HMT-1* exons near one end. As illustrated, long PCR with A1-G1F and A1-13R primers produced 6.5 kb fragment from HeLa genomic DNA, which was subsequently cloned into pUC118 vector as ET-8 construct (as described in “Materials and methods”). **b** For the first round of CAT assays, the 1 kb region upstream from the *HMT-1* ORF was re-amplified from ET-8 by standard primers using indicated primers, subcloned into pMAMneoCAT vector as NUP-1000 construct. Then, using NUP-1000, truncated subclones NUP-800, -600, -400 and -200 were constructed by the same procedure as preparation for NUP-1000, described in “Materials and methods”. **c** After preparing lysates from transfectants with NUP construct series and performing CAT assays, thin layer chromatography (TLC) of CAT reactions was carried out for approximately 30 min, using

ethyl acetate as solvent. The negative control on TLC analysis (“Negative” in figure) was prepared by CAT reaction with lysate from HeLa cells, while positive indicator for acetyl-chloramphenicol (“Standard” in figure) was provided from the kit. **d** Results of CAT activities obtained after normalization are shown. As the *CAT* gene on pMAMneoCAT vector exhibits leaky expression due to distal RSV promoter and inducible expression due to proximal MMTV LTR promoter with dexamethasone (Dex), background levels of CAT activities with mock transfectants in the absence and presence of Dex have been also individually validated in parallel, as shown in (c) and (d). **d** Relative CAT activity with each construct was calculated against that with NUP-200 as 100 %. Each CAT activity was measured, normalized according to a formula shown in “Materials and methods” and averaged in at least five independent experiments. Each bar in the graph indicates standard error (SE)

decreased in comparison to that with NUP-132 (20 %), indicating weak enhancing effect of the segment III from –132 to –113. In contrast, CAT activity with NUP-70 greatly reduced to approximately same level of that with NUP-156, i.e. approximately 60 % of that with NUP-200 (Fig. 5b). These observations implied that the segment III plus IV (a region ranging from –132 to –71) might have enhancer effect more than the segment I, and that total enhancing effect by the segment III plus IV might

bring into balance with silencing effect by the segment II.

Assays with NUP-57 and NUP-41 yielded 13.8 and 37.5 % of CAT activity with NUP-200, respectively (Fig. 5b). These results suggested that a region consisting of the segment V from –70 to –58 also might have an enhancer motif, whereas that the segment VI from –57 to –42 would have silencer effect on transcription. In respect of enhancer in the segment V and silencer in the segment VI, the

Fig. 5 CAT assays for the 200 bp region upstream from the *HMT-1* ORF. **a** Redrawing of potentially *cis*-acting elements localized within the 200 bp region upstream from the *HMT-1* ORF. Based on these positions, the region is divided into eight segments, as indicated by vertical broken lines. Seven constructs where a segment was deleted one by one from upstream side were additionally prepared for the second round of CAT assays (NUP-156, -132, -112, -70, -57, 41 and -33). **b** Results of the second round of CAT assays with these constructs are shown. Relative CAT activity with each construct was calculated against that with NUP-200 as 100 %. Each CAT activity was measured, normalized according to a formula shown in “Materials and methods” and averaged in at least five independent experiments. Each bar in the graph indicates standard error (SE)

strongest candidates are an Sp1 and AP-4 motifs, respectively (Fig. 5a), although it has not been so far verified whether they indeed function.

Deletion of the segment VII (from -41 to -34) from NUP-41 did not result in significant change of CAT activity (NUP-41: 37.5 %, SE = 9.1; NUP-33: 44.4 %, SE = 7.5; also see Fig. 5b), demonstrating that this segment, just including an Ets-1 binding motif, has no effect on the *HMT-1* transcription. In addition, it is noteworthy that NUP-33 still maintained more than 40 % of transcriptional activity with NUP-200 (Fig. 5b). This fact means that 33 bp region upstream from *HMT-1* ORF (i.e. the segment VIII which contains an USF/NF-1/AP-1/AP-4/AP-2 multiple overlapping motif) would be enough for minimum level of the *HMT-1* expression, and therefore support basal level of the *HMT-1* transcription.

Characterization of two overlapping motifs around -132 position from *HMT-1* ORF

For more detailed investigation, we focused on a region around -132 position, because two functionally reciprocal segments are flanking there, one of which (the segment II) negatively regulates the *HMT-1* transcription, whereas the other (the segment III) positively regulates (Fig. 5b). Interestingly, both are potentially bound with two kinds of transcription factors, one of which is commonly AP-2 (Fig. 5a). As seen in Figs. 3 and 6a, there are an NF-1/AP2 overlapping motif at -148 to -134 position in the segment II, and an NF-kB/AP-2 overlapping motif at -130 to -116 position in the segment III. In addition, there exists an AP-2 motif at -135 to -129 position in the boundary between the segment II and III, partially overlapping with the NF-kB/AP-2 overlapping motif (Fig. 6a). In order to confirm which transcription factor preferentially *trans*-acts these motifs, site-directed mutagenesis was performed against the two overlapping motifs.

As shown in Fig. 6a, two variants of NUP-156, NUP-156/*mutN* and NUP-156/*mutA*, were constructed. In NUP-156/*mutN*, two guanosine residues 5'-¹⁴⁷GG⁻¹⁴⁶-3', which are essential for NF-1 binding, were disrupted without effect on AP-2 binding. On the other hand, NUP-156/*mutA* had disruption of 5'-¹³⁸GG⁻¹³⁷-3', which interferes with binding of both. In CAT assays with these disruptants (Fig. 6b), the control CAT activity with NUP-156 was 35.6 %, SE = 2.0 of that with NUP-132 (100 %), which well accorded with a result shown in Fig. 5b (38.4 %, calculated from NUP-156: 56.1 %, SE = 4.4 versus NUP-132: 146 %, SE = 2.6). CAT activity obtained with NUP-156/*mutN* (67.2 %, SE = 5.1) was elevated by twofold, compared to that with the original NUP-156 (35.6 %, SE = 2.0), while CAT activity with NUP-156/*mutA* (91.6 %, SE = 4.4) was elevated by threefold, nearly equal to that with NUP-132 (Fig. 6b). These observations support the possibility that both NF-1 and AP-2 should substantially function as repressors of the *HMT-1* transcription. In addition, we inferred that the AP-2 motif at -135 to -129 boundary position has little effect on transcriptional repression, from results of CAT activities with NUP-156/*mutA* (91.6 %, SE = 4.4) and NUP-132 (Fig. 6a, b).

Fig. 6 CAT assays for mutagenized NF-1/AP-2 and NF-kB/AP-2 overlapping motifs. **a** Detailed information of nucleotide sequences for NF-1/AP-2 and NF-kB/AP-2 overlapping motifs. In upper figure, putative binding sites of NF-1, AP-2 and NF-kB are indicated by horizontal arrows and precise positions of nucleotide residues matching with their consensus sequences (boxed) are represented by vertical lines. In lower figure, positions of substitutional mutations introduced into NUP-156 and NUP-132 constructs are shown in small letters. **b** Results of CAT assays with four mutants, as well as those with NUP-156 and NUP-132 as controls. Relative CAT activity with each construct was calculated against that with NUP-132 as 100 %. Each CAT activity was measured, normalized according to a formula shown in “Materials and methods” and averaged in at least five independent experiments. Each bar in the graph indicates standard error (SE)

For analysis on an NF-kB/AP-2 overlapping motif, two variants of NUP-132, NUP-132/mutK and NUP-132/mutA, were prepared. NUP-132/mutK was mutated in which only an NF-kB binding site is not available, whereas NUP-132/mutA was mutated so that only an AP-2 binding site is invalid (Fig. 6a). In this CAT assay, NUP-132/mutK exhibited 152 %, SE = 1.1 of CAT activity with the control NUP-132 (Fig. 6b). This result means that disruption of the NF-kB motif caused upregulation, which seemed to be in contradiction with data obtained from the first round of CAT assays for NUP-132 and NUP-112 (Fig. 5b)

indicating that the segment III had enhancer activity (regarding reasons for this discrepancy, see “Discussion”). On the other hand, NUP-132/mutA yielded 106 %, SE = 2.0 activity nearly equal to NUP-132 (Fig. 6c), suggesting that AP-2 binding to this motif would originally have little inhibitory effect on the CAT transcription, or that NF-kB binding would have little enhancing effect.

Discussion

For proper accomplishment of protein N-glycosylation, early assembly of DLO on cytoplasmic side of the rER membrane must progress at a rate greater than subsequent flipping of DLO into luminal side. To assure this, all activities of five glycosyltransferases involved in early DLO assembly are required in addition to their substrates. If any of them is diminished, eukaryotic cell would not be able to survive. In fact, an antibiotic tunicamycin prevents cell division and consequently kills yeast and mammalian cultured cells by blocking activity of GlcNAc-1-P transferase, which is involved in the first step of DLO assembly (Elbein 1984). Therefore, transcription of genes encoding these enzymes might be rather fundamental event in eukaryotes, particularly multicellular organisms including mammals. Nevertheless, any potential motif that exists in upstream regions of their genes and might control their transcription positively or negatively has not been almost investigated or characterized except that of the *DPAGT* gene encoding GlcNAc-1-P transferase. As for the *DPAGT1*, it has been demonstrated that transcription factors STAT5a (Zhang et al. 2003) and TCF (Sengupta et al. 2010) indeed bind to an upstream promoter region of the *DPAGT1*, and that its transcriptional regulation should be tightly associated with Wnt signaling (Varelas et al. 2014). In this study, we investigated on a region which would participate in transcriptional regulation of the *HMT-1* gene encoding human β 1,4-MT involved in the third step of DLO assembly. With respect to direct analysis of *cis*-elements functioning in transcriptional regulation of an enzyme acting on early DLO assembly, this is the first report as far as we know.

Our results of semi-quantitative RT-PCRs suggest that the *HMT-1* could be regarded as one of house-keeping genes which are expressed at medium or low level (Fig. 1a, b). Western blot analyses by Rind et al.

(2010) revealed that protein expression level of α 1,2-MT encoded by the *hALG11* was lower than β -actin, an abundant protein ubiquitously expressed, and that protein expression level of β 1,4-MT encoded by the *HMT-1* was further lower than α 1,2-MT. Therefore, it is reasonable that expression of the *HMT-1* transcripts might be downregulated and restricted to relatively lower level. Data in Fig. 1a also revealed the difference in *HMT-1* requirement among human tissues, possibly due to necessity for protein *N*-glycosylation. The expression profile of the *HMT-1* among various tissues resembles that of the *hALG2* (our unpublished data).

Analysis of nucleotide sequence of a 1 kb region upstream from *HMT-1* ORF (a clone NUP-1000) uncovered existence of various motifs which potentially regulate the *HMT-1* transcription positively or negatively, by binding of transcription factors (Fig. 3). As described in “Results”, a TATA box and CAAT boxes found in nucleotide sequence of NUP-1000 were not located within each region suitable for promoting *HMT-1* transcription. Of course, our determination of initiation site for the *HMT-1* transcription has not excluded the possibility that other sites might be available for starting transcription. However, at least in HeLa cells, the *HMT-1* transcription should be regulated by TATA-less promoter to initiate RNA synthesis from a single site. This idea prompted us to explore other *cis*-acting elements for the *HMT-1* transcription.

Our data obtained from CAT assays using a series of deletants (Figs. 4d, 5b) demonstrated that a 200 bp region upstream from *HMT-1* ORF was likely to be necessary and sufficient for the *HMT-1* expression and that seven of eight segments within the 200 bp region (Fig. 5a) contribute to controlling the *HMT-1* transcription, positively or negatively; five segments I, III, IV, V and VIII contain enhancer motifs, whereas two segments II and VI have silencer motifs. Taken together, via the 200 bp region, the *HMT-1* gene transcription might be controlled at mainly four levels. Its basal level would be maintained by the segment VIII (−33 to −1), which contains an USF/NF-1/AP-1/AP-4/AP-2 multiple overlapping motif (Fig. 5a, b). It is likely that the segment V (−70 to −58), which contains an Sp1 motif, might reinforce the transcription by 30 % (Fig. 5B). Moreover, the recruitment of the segment IV (from −112 to −71) containing a GATA-1 motif yields nearly 300 % of the basal level

transcription. However, this enhancement is fully suppressed by existence of the segment II (−156 to −133), which possesses an NF-1/AP-2 overlapping motif. Finally, the segment I containing a GATA-1 motif up-regulates transcriptional activity to the level about twofold higher than that accomplished by the segment VIII only. From our results of semi-quantitative RT-PCRs, it was estimated that the differences in amount of the *HMT-1* transcripts among all tissues examined are ranged within about tenfold (Fig. 1a). This variation could be explained in part by the regulation of transcriptional activity via the 200 bp region.

Next, we focused on the segments II and III, which include silencer and enhancer motifs respectively. CAT activity with NUP-156/*mutN* (67.2 %, SE = 5.1) or NUP156/*mutA* (91.6 %, SE = 4.4) was drastically increased as compared to that with NUP-156 (35.6 %, SE = 2.0, Fig. 6b), suggesting the importance of an NF-1/AP-2 overlapping motif in the segment II. Moreover, by comparing three CAT activities with NUP-156, NUP-156/*mutN* and NUP-156/*mutA* mutually, it was estimated that NF-1 and AP-2 have similar repressive effect. Perhaps both are competitive for binding to this overlapping motif. If so, results of our CAT assays with two mutants suggested that NF-1 rather than AP-2 could preferentially bind to the motif, and that repressive effect by NF-1 might be greater than that by AP-2, because CAT activity with NUP-156/*mutN*, where binding of NF-1 is exclusively abolished and consequently AP-2 is capable of binding noncompetitively, was not maintained on the same level of CAT activity with NUP-156 (Fig. 6b). In other words, if AP2 preferentially bound to the motif and completely compensated for repression by NF-1, CAT assay with NUP-156/*mutN* would yield the same activity with NUP-156. This observation suggests that NF1 is one of the most effective repressors for the *HMT-1* expression. Of course, AP-2 also apparently serves as a negative regulator, considering that CAT activity with NUP-156/*mutN* disrupting only a NF-1 binding site was still lower than that with NUP-132 (Fig. 6b).

Contrary to our expectation for an NF-kB/AP-2 overlapping motif in the segment III as an enhancer motif, CAT activity with NUP-132/*mutK* (152 %, SE = 1.1) was greatly increased against that with NUP-132 (Fig. 6b). As described in “Results”, NUP-132/*mutK* was designed for preventing only NF-kB

from binding to the motif. However, from later validation, it was revealed that the substitutional mutation in NUP-132/*mutK* for this purpose unexpectedly resulted in bearing a novel TCF-1 binding site (5′-⁻¹²⁸aaaAG⁻¹³²-3′) in antisense chain of this motif (Fig. 6a). In HeLa cells, TCF-1 might be activated much greater than NF-κB, and its binding to the mutated motif does not compete with AP-2 binding. Therefore, it is possible that the great increase in CAT activity with NUP-132/*mutK* could be principally due to noncompetitive binding of TCF-1 instead of NF-κB competing with AP-2, and that enhancing effect by TCF-1 would greatly overcome repressive effect by AP-2. Accordingly, in the CAT assay with this mutant, we were not successful in precisely assessing silencer activity of an AP-2 binding site in the overlapping motif. We, however, concluded that it has little repressive effect, because noncompetitive binding of AP-2 could not suppress action of transactivator such as TCF-1, and exclusive removal of the motif from NUP-132 (i.e. NUP-112) ultimately resulted in reduction of CAT activity (Fig. 5b).

On the other hand, CAT activity with NUP-132/*mutA*, in which only an AP-2 binding site was abolished, was slightly upregulated against that with NUP-132 (106 %, SE = 2.0, Fig. 6b), suggesting that an NF-κB binding site would not be so effective for enhancing in HeLa cells. This result, however, may not reflect true enhancing activity of this site, because in NUP-132, NF-κB and AP-2 are thought to be competitive for binding to the motif, whereas not in NUP-132/*mutA*. Rather, the difference in CAT activities between NUP-132/*mutA* (106 %, SE = 2.0) and NUP-112 (126 %, SE = 2.3) might more strictly reflect net enhancer activity of the NF-κB binding site itself.

Taking into consideration, it is likely that function of the NF-κB/AP-2 overlapping motif in the segment III as enhancer would be far less than that of the segment IV (Fig. 5b). However, it would be very interesting to regard the motif as a modulator, which is able to associate with both negative (AP-2) and positive (NF-κB) transcription factors for tuning up the transcription level.

In this study, we identified several *cis*-acting motifs critical for the *HMT-1* transcription within the 200 bp region upstream from *HMR-1* ORF. One of them is the NF-1/AP-2 overlapping motif, located at −148 to

−134 position in the segment II, which strongly represses the transcription possibly by binding both NF-1 and AP-2. In human, NF-1A, NF-1B, NF-1C and NF-1X have been characterized as members of NF-1 family (Gronostajski 2000). According to their mRNA expression profiles represented by BioGPS (URL: <http://biogps.org/>; Wu et al. 2009) using Human U133A/GNF1H Gene Atlas (Su et al. 2004) as a dataset, abundance in *NF-1A* and *NF-1X* transcripts is not so varied in many human tissues, whereas *NF-1B* and *NF-1C* transcripts are expressed in a tissue-specific manner. Interestingly, the *NF-1B* transcription is downregulated in liver, kidney, pancreas, testis and ovary, and upregulated in prostate and small intestine. This expression pattern of *NF-1B* seems to be inversely proportional to that of *HMT-1* (Fig. 1a). Furthermore, *NF-1C* transcription is upregulated in skeletal muscle, where that of *HMT-1* is downregulated. For AP-2, there are five paralogous genes in human genome (Eckert et al. 2005). In their expression profiles by BioGPS, the *AP-2A* transcription is specifically activated in prostate, in which *HMT-1* transcription is downregulated. These observations raise the possibility that both NF-1B and/or AP-2α proteins should substantially downregulate the *HMT-1* expression in certain tissues such as prostate. In fact, both have been already reported to serve as repressors in several cases (Liu et al. 1997; Mitchell and DiMario 2010; Finlay-Schultz et al. 2011). Another silencer localized in the 200 bp region is AP-4 binding motif in the segment VI, as demonstrated by difference in CAT activities with NUP-57 and NUP-41 (Fig. 5b). As well as NF-1 and AP-2, AP4 functions as both trans-activator and repressor according to cellular genes (Cui et al. 1998; Kim et al. 2006; Jung et al. 2008; Ku et al. 2009; Ma et al. 2012). Judging from its expression profile by BioGPS, AP-4 is ubiquitously expressed at a similar level in most tissues. Hence, it does not seem to be associated with variation of transcriptional level of the *HMT-1* observed in our RT-PCR analyses (Fig. 1a).

As substantial enhancers for the *HMT-1* transcription, we detected three candidates, two GATA-1 motifs and one Sp1 motif, in our experiments. The first GATA-1 motif located at −192 to −182 position in the segment I might upregulate *HMT-1* transcription up to 30 % degree, whereas the second one located at −96 to −82 position in the segment IV up to more than 200 %. The Sp1 motif located at −69 to −58 position

in the segment V might also potentially elevate transcription twice. Interestingly, in the case of the *HMT-1* transcription, it is possible that the region ranging from −96 to −58, which includes the second GATA-1 motif and an Sp1 motif, mainly functions as promoter, instead of TATA box or CAAT box. This idea agrees well with several demonstrations that the GATA-1 and Sp1 have been reported to cooperatively activate transcription (Fischer et al. 1993; Merika and Orkin 1995; Furusawa et al. 2003). In fact, deletion of this region caused drastic reduction in transcription activity (compare NUP-112 with NUP-57 in Fig. 5b). Hence, precise function of these motifs remained to be further investigated in more detail.

In addition to our analysis of the upstream region of the *HMT-1*, that of downstream region from its initiation codon should be required, because most housekeeping genes which have no TATA box possess upstream promoter sequences such as initiator and GC box, or downstream promoter sequences (Cui et al. 1998). As pointed out above, GATA-1 and Sp1 motifs at −96 to −58 possibly serve as alternative upstream promoter, while unidentified promoter sequence might exist in the downstream region. In this regard, we have already cloned the 1 kb downstream region from start codon of the *HMT-1*, and analyses with CAT assay are now in progress.

Finally, we refer to the possibility that certain mutations occurring in regulatory region for the *HMT-1* transcription might cause congenital disorder of glycosylation type Ik (CDG-Ik, also termed as CDG-ALG1). At least seven cases of CDG-Ik have been so far reported, and they have been all demonstrated to be caused by one of several missense mutations resulting in substitution of amino acid such as S258L (Schwarz et al. 2004; Kranz et al. 2004; Grubenmann et al. 2004), E342P (Kranz et al. 2004), S150R (Grubenmann et al. 2004), M377V (Dupré et al. 2010), G145D (Dupré et al. 2010) and R276W (Dupré et al. 2010), or a nonsense mutation resulting in truncated termination such as C396X (Dupré et al. 2010), in the coding region of the *HMT-1*. No mutation within intronic or 5'- and 3'-untranslated regions has been identified as being responsible for CDG-Ik. If the null mutation occurs in *cis*-acting motifs critical for maintaining *HMT-1* transcription, several tissues which need production of large amount of *N*-glycans will be primarily affected, which perhaps include liver kidney, spleen, pancreas, testis and ovary, as observed in

our RT-PCR (Fig. 1a). In other words, even if *HMT-1* ORF is not mutated at all, repression of the *HMT-1* transcription itself would cause significant reductions in β -1,4-MT activity and subsequently biosynthesis of DLO, leading to shortage of *N*-glycans on glycoproteins. This might further facilitate dysfunction of basic cellular activity such as intracellular signaling or mitosis. So far, no CDG-Ik patient with such type of mutation has been reported, but it remains possible for the mutation to be characterized in a case with relatively mild symptoms of the disease.

In conclusion, our analyses demonstrate that the 200 bp region upstream from *HMT-1* ORF is associated with regulation for *HMT-1* transcription, although the possibility that there still exist other cryptic motifs which influence the transcription in a region from −1000 to −200 is not excluded. From our results, it is speculated that NF-1 and AP-2 play a crucial role in the negative regulation, whereas GATA-1 and Sp1 might contribute to the positive regulation, although the possibility that unknown motifs located in other regions and related transcription factors might affect the *HMT-1* transcription has not been excluded. Notably, we also have detected the physical interactions among three mannosyltransferases involved in early DLO assembly, including yeast and human β 1,4-MT (Gao et al. 2004; Takahashi and Gao 2012). It is possible that these interactions among enzymes modulate the productivity of DLO at post-translational level. In fact, a physical interaction between Alg7p and Alg14p, which participate in early assembly of DLO as GlcNAc-1-P transferase and GlcNAc transferase subunit, respectively, has been recently observed in yeast (Noffz et al. 2009; Lu et al. 2012) and human (Takahashi and Gao 2012), and their activities have been previously demonstrated to be controlled by feedback regulation using chicken microsomes (Kean et al. 1999). If such post-translational control exists in parallel with the transcriptional control, for expression of β 1,4-MT activity, the question which control more critically contributes to the regulation of β 1,4-MT function would consequently raise. Alternatively, these two controls might be properly used for different purposes or situations, such as constitutive maintenance of the β 1,4-MT or acute responses to intracellular signaling. Therefore, further investigations are required for both transcriptional control of the *HMT-1* and physical interactions of human β 1,4-MT, in order to consider which regulation has more profound effect on performance of the human β 1,4-MT.

Moreover, as described above, regulation of the *DPAGT1* transcription has been clearly demonstrated to be closely related to Wnt signaling. Interestingly, Wnt3a signaling protein induced increased level of the *DPAGT1* transcripts, but not of the *HMT-1* (Sengupta et al. 2010). Additionally, there is no motif potential for binding transactivator related to Wnt signaling, such as TCF, in the 1 kb region upstream from the *HMT-1* (Sengupta et al. 2010; also see Fig. 3). These observations apparently indicate that the *HMT-1* is not direct target in Wnt signaling pathway. Nevertheless, it has been reported that the *HMT-1* expression is upregulated when the *DPAGT1* transcription is enhanced (Sengupta et al. 2013). This mechanism for transcriptional co-activation of the *HMT-1* coordinated with the *DPAGT1* has not been so far elucidated. Thus, further exploration for transcriptional control of the *HMT-1* and other genes participating in DLO biosynthetic pathway will also provide insight into comprehensive understanding of regulatory mechanism at transcriptional level for the pathway.

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References

- Adamowicz M, Chmielinska E, Kaluzny L, Bittner G, Sarnowska-Wroczynska I, Timal S, Morava E, Lehle L, Wevers RA, Lefeber DJ, Sykut-Cegielska J (2011) Clinical and biochemical characterization of the second CDGII (*DPAGT1*-CDG) patient. *J Inherit Metab Dis* 34:S181
- Aebi M (2013) *N*-linked protein glycosylation in the ER. *Biochim Biophys Acta* 1833:2430–2437
- Albright CF, Robbins PW (1990) The sequence and transcript heterogeneity of the yeast gene *ALG1*, an essential mannosyltransferase involved in *N*-glycosylation. *J Biol Chem* 265:7042–7049
- Basiri K, Belaya K, Liu WW, Maxwell S, Sedghi M, Beeson D (2013) Clinical features in a large Iranian family with a limb-girdle congenital myasthenic syndrome due to a mutation in *DPAGT1*. *Neuromuscul Disord* 23:469–472
- Buczowska A, Swiezewska A, Lefeber DJ (2015) Genetic defect in dolichol metabolism. *J Inherit Metab Dis* 38:157–169
- Cantagrel V, Lefeber DJ (2011) From glycosylation disorders to dolichol biosynthesis defects: a new class of metabolic diseases. *J Inherit Metab Dis* 34:859–867
- Carrera IA, Matthijs G, Perez B, Cerdá CP (2012) *DPAGT1*-CDG: report of a patient with fetal hypokinesia phenotype. *Am J Med Genet A* 158A:2027–2030
- Couto JR, Huffaker TC, Robbins PW (1984) Cloning and expression in *Escherichia coli* of a yeast mannosyltransferase from the asparagine-linked glycosylation pathway. *J Biol Chem* 259:378–382
- Cui Y, Narayanan CS, Zhou J, Kumar A (1998) Exon-I is involved in positive as well as negative regulation of human angiotensinogen gene expression. *Gene* 224:97–107
- de Koning TJ, Toet M, Dorland L, de Vries LS, van den Berg IET, Duran M, Poll-The BT (1998) Recurrent nonimmune hydrops fetal is associated with carbohydrate-deficient glycoprotein syndrome. *J Inherit Metab Dis* 21:681–682
- Dupré T, Vuillaumier-Barrot S, Chantret I, Yayé HS, Le Bizec C, Afenjar A, Altuzarra C, Barnérias C, Burglen L, de Lonlay P, Feillet F, Napuri S, Seta N, Moore SEH (2010) Guanosine diphosphate-mannose:GlcNAc₂-PP-dolichol mannosyltransferase deficiency (congenital disorders of glycosylation type Ik): five new patients and seven novel mutations. *J Med Genet* 47:729–735
- Eckert D, Buhl S, Weber S, Jäger R, Schorle H (2005) The AP-2 family of transcription factors. *Genome Biol* 6:246.1–246.8
- Elbein AD (1984) Inhibitors of the biosynthesis and processing of *N*-linked oligosaccharides. *CRC Crit Rev Biochem* 16:21–49
- Finlay-Schultz J, Canastar A, Short M, El Gazzar M, Coughlan C, Leonard S (2011) Transcriptional repression of the $\alpha 7$ nicotinic acetylcholine receptor subunit gene (*CHRNA7*) by activating protein-2 α (AP-2 α). *J Biol Chem* 286:42123–42132
- Fischer KD, Haese A, Nowock J (1993) Cooperation of GATA-1 and Sp1 can result in synergistic transcriptional activation or interference. *J Biol Chem* 268:23915–23923
- Furusawa M, Taira T, Iguchi-Arigo SMM, Ariga H (2003) Molecular cloning of the mouse *AMY-1* gene and identification of the synergistic activation of the *AMY-1* promoter by GATA-1 and Sp1. *Genomics* 81:221–233
- Gao X-D, Nishikawa A, Dean N (2004) Physical interactions between the Alg1, Alg2, and Alg11 mannosyltransferases of the endoplasmic reticulum. *Glycobiology* 14:559–570
- Gronostajski RM (2000) Roles of the NFI/CTF gene family in transcription and development. *Gene* 249:31–45
- Grubenmann CE, Frank CG, Hülsmeier AJ, Schollen E, Matthijs G, Mayatepek E, Berger EG, Aebi M, Hennet T (2004) Deficiency of the first mannosylation step in the *N*-glycosylation pathway causes congenital disorder of glycosylation type Ik. *Hum Mol Genet* 13:535–542
- Haeuptle MA, Hennet T (2009) Congenital disorders of glycosylation: an update on defects affecting the biosynthesis of dolichol-linked oligosaccharides. *Hum Mutat* 30:1628–1641
- Helander A, Stöberg T, Jaeken J, Matthijs G, Eriksson M, Eggertsen G (2013) Dolichol kinase deficiency (DOLK-CDG) with a purely neurological presentation caused by a novel mutation. *Mol Genet Metab* 110:342–344
- Helenius A, Aebi M (2001) Intracellular functions of *N*-linked glycans. *Science* 291:2364–2369
- Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* 96:23–28
- Jaeken J (2010) Congenital disorders of glycosylation. *Ann NY Acad Sci* 1214:190–198

- Jung P, Menssen A, Mayr D, Hermeking H (2008) AP4 encodes a c-MYC-inducible repressor of p21. *Proc Natl Acad Sci USA* 105:15046–15051
- Kean EL, Wei Z, Anderson VE, Zhang N, Sayre LM (1999) Regulation of the biosynthesis of *N*-acetylglucosaminylpyrophosphoryldolichol, feedback and product inhibition. *J Biol Chem* 274:34072–34082
- Kim MY, Jeong BC, Lee JH, Kee HJ, Kook H, Kim NS, Kim YH, Kim JK, Ahn KY, Kim KK (2006) A repressor complex, AP4 transcription factor and geminin, negatively regulates expression of target genes in nonneuronal cells. *Proc Natl Acad Sci USA* 103:13074–13079
- Kranz C, Denecke J, Lehle L, Sohlbach K, Jeske S, Meinhardt F, Rossi R, Gudowius S, Marquardt T (2004) Congenital disorder of glycosylation type Ik (CDG-Ik): a defect of mannosyltransferase I. *Am Hum Genet* 74:545–551
- Kranz C, Jungeblut C, Denecke J, Erlekotte A, Sohlbach C, Debus V, Kehl HG, Harms E, Reith A, Reichel S, Grobe H, Hammersen G, Schwarzer U, Marquardt T (2007) A defect in dolichol phosphate biosynthesis causes a new inherited disorder with death in early infancy. *Am J Hum Genet* 80:433–440
- Ku WC, Chiu SK, Chen YJ, Huang HH, Wu WG, Chen YJ (2009) Complementary quantitative proteomics reveals that transcription factor AP-4 mediates E-box-dependent complex formation for transcriptional repression of HDM2. *Mol Cell Proteomics* 8:2034–2050
- Kukuruzinska MA, Bergh MLE, Jackson BJ (1987) Protein glycosylation in yeast. *Ann Rev Biochem* 56:915–944
- Lennon K, Pretel R, Kesselheim R, te Heesen S, Kukuruzinska MA (1995) Proliferation-dependent differential regulation of dolichol pathway genes in *Saccharomyces cerevisiae*. *Glycobiology* 5:633–642
- Lieu MT, Ng BG, Rush JS, Wood T, Basehore MJ, Hegde M, Chang RC, Abdenur JE, Freeze HH, Wang RY (2013) Severe, fatal multisystem manifestations in a patient with dolichol kinase-congenital disorder of glycosylation. *Mol Genet Metab* 110:484–489
- Liu Y, Bernard HU, Apt D (1997) NFI-B3, a novel transcriptional repressor of the nuclear factor I family, is generated by alternative RNA processing. *J Biol Chem* 272:10739–10745
- Lu J, Takahashi T, Ohoka A, Nakajima K, Hashimoto R, Miura N, Tachikawa H, Gao X-D (2012) Alg14 organizes the formation of a multi-glycosyltransferase complex involved in initiation of lipid-linked oligosaccharide biosynthesis. *Glycobiology* 22:504–516
- Ma SL, Tang NL, Tam CW, Lui VW, Lam LC, Chiu HF, Driver JA, Pastorino L, Lu KP (2012) A PIN1 polymorphism that prevents its suppression by AP4 associates with delayed onset of Alzheimer's disease. *Neurobiol Aging* 33:804–813
- Marek KW, Vijay IK, Marth JD (1999) A recessive deletion in the GlcNAc-1-phosphotransferase gene results in peri-implantation embryonic lethality. *Glycobiology* 9:1263–1271
- Merika M, Orkin SH (1995) Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Kruppel family proteins Sp1 and EKLF. *Mol Cell Biol* 15:2437–2447
- Mitchell DL, DiMario JX (2010) AP-2 α suppresses skeletal myoblast proliferation and represses fibroblast growth factor receptor 1 promoter activity. *Exp Cell Res* 316:194–202
- Morava E, Vodopiutz J, Lefeber DJ, Janecke AR, Schmidt WM, Lechner S, Item CB, Sykut-Cegielska J, Adamowicz M, Wierzbicka J, Zhang ZH, Mihalek I, Stockler S, Bodamer OA, Lehle L, Wevers RA (2012) Defining the phenotype in congenital disorder of glycosylation due to *ALG1* mutations. *Pediatrics* 130:e1034–e1039
- Noffz C, Keppler-Ross S, Dean N (2009) Hetero-oligomeric interactions between early glycosyltransferases of the dolichol cycle. *Glycobiology* 19:472–478
- Regal L, van Hasselt PM, Foulquier F, Cuppen I, Prinsen HCMT, Jansen K, Keldermans L, De Meirleir L, Matthijs G, Jaeken J (2015) ALG11-CDG: three novel mutations and further characterization of the phenotype. *Mol Genet Metab Rep* 2:16–19
- Rind N, Schmeiser V, Thiel C, Absmanner B, Lübbehusen J, Hocks J, Apeshiotis N, Wilichowski E, Lehle L, Körner C (2010) A severe human metabolic disease caused by deficiency of the endoplasmic mannosyltransferase hALG11 leads to congenital disorder of glycosylation-Ip. *Hum Mol Genet* 19:1413–1424
- Rohlfing AK, Rust S, Reunert J, Tirre M, Du Chesne I, Wemhoff S, Meinhardt F, Hartmann H, Das AM, Marquardt T (2014) ALG1-CDG: a new case with early fatal outcome. *Gene* 534:345–351
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, NY
- Schwarz F, Aepli M (2011) Mechanisms and principles of *N*-linked protein glycosylation. *Curr Opin Struct Biol* 21:576–582
- Schwarz M, Thiel C, Lübbehusen J, Dorland B, de Koning T, von Figura K, Lehle L, Körner C (2004) Deficiency of GDP-Man:GlcNAc2-PP-dolichol mannosyltransferase causes congenital disorder of glycosylation type Ik. *Am J Hum Genet* 74:472–481
- Sengupta PK, Bouchie MP, Kukuruzinska MA (2010) *N*-Glycosylation gene *DPAGT1* is a target of the Wnt/ β -catenin signaling pathway. *J Biol Chem* 285:31164–31173
- Sengupta PK, Bouchie MP, Nita-Lazar M, Yang HY, Kukuruzinska MA (2013) Coordinate regulation of *N*-glycosylation gene *DPAGT1*, canonical Wnt signaling and E-cadherin adhesion. *J Cell Sci* 126:484–496
- Snow TM, Woods CW, Woods AG (2012) Congenital disorder of glycosylation: a case presentation. *Adv Neonatal Care* 12:96–100
- Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB (2004) A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci USA* 101:6062–6067
- Takahashi T, Gao X-D (2012) Physical interactions among human glycosyltransferases involved in dolichol-linked oligosaccharide biosynthesis. *Trends Glycosci Glycotechnol* 24:65–77
- Takahashi T, Honda R, Nishikawa Y (2000) Cloning of the human cDNA which can complement the defect of the yeast mannosyltransferase I-deficient mutant *alg1*. *Glycobiology* 10:321–327
- Thiel C, Schwarz M, Peng J, Grzmil M, Hasilik M, Braulke T, Kohlschütter A, von Figura K, Lehle L, Körner C (2003) A new type of congenital disorders of glycosylation (CDG-II)

- provides new insights into the early steps of dolichol-linked oligosaccharide biosynthesis. *J Biol Chem* 278:22498–22505
- Thiel C, Rind N, Popovici D, Hoffmann GF, Hanson K, Conway RL, Adamski CR, Butler E, Scanion R, Lambert M, Apeshiotis N, Thiel C, Matthijs G, Körner C (2012) Improved diagnostics lead to identification of three new patients with congenital disorder of glycosylation-I_p. *Hum Mutat* 33:485–487
- Timal S, Hoischen A, Lehle L, Adamowicz M, Huijben K, Sykut-Cegielska J, Paprocka J, Jamroz E, van Spronsen FJ, Körner C, Gilissen C, Rodenburg RJ, Eidhof I, Van den Heuvel L, Thiel C, Wevers RA, Morava E, Veltman J, Lefeber DJ (2012) Gene identification in the congenital disorders of glycosylation type I by whole-exome sequencing. *Hum Mol Genet* 21:4151–4161
- Varelas X, Bouchie MP, Kukuruzinska MA (2014) Protein *N*-glycosylation in oral cancer: dysregulated cellular networks among *DPAGT1*, E-cadherin adhesion and canonical Wnt signaling. *Glycobiology* 24:579–591
- Varki A (1993) Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* 3:97–130
- Vieira J, Messing J (1987) Production of single-stranded plasmid DNA. *Methods Enzymol* 153:3–11
- Wu X, Rush JS, Karaoglu D, Krasnewich D, Lubinsky MS, Waechter CJ, Gilmore R, Freeze HH (2003) Deficiency of UDP-GlcNAc:dolichol phosphate *N*-acetylglucosamine-1 phosphate transferase (*DPAGT1*) causes a novel congenital disorder of glycosylation type I_j. *Hum Mutat* 22:144–150
- Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, Hodge CL, Haase J, Janes J, Huss JW, Su AI (2009) BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 10:R130.1–R130.8
- Würde AE, Reunert J, Rust S, Hertzberg C, Haverkämper S, Nürnberg G, Nürnberg P, Lehle L, Rossi R, Marquardt T (2012) Congenital disorder of glycosylation type I_j (CDG-I_j, *DPAGT1*-CDG): extending the clinical and molecular spectrum of a rare disease. *Mol Genet Metab* 105:634–641
- Zhang XL, Qu XJ, Vijay IK (2003) *STAT5a* regulates the GlcNAc-1-phosphate transferase gene transcription and expression. *Cell Physiol Biochem* 13:85–92