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Transcriptional regulation of the *fucosyltransferase VI* gene in hepatocellular carcinoma cells

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Abstract The α1,3-fucosyltransferase VI (FUT VI) protein is a key enzyme for synthesis of sialyl Lewis X and Lewis X in epithelial cells. Despite its importance, how *FUT VI* expression is regulated has not previously been elucidated. In this work, we examined transcriptional regulation of the *FUT VI* gene in hepatocellular carcinoma HepG2 cells. 5′-Rapid amplification of cDNA ends analysis revealed transcription start sites of *FUT VI* in HepG2 cells at +65 and +278 nucleotides (nt) downstream of the position registered in the Data Base of Human Transcription Start Sites. We determined promoter regions for *FUT VI* in HepG2 cells using a luciferase reporter gene assay. The promoter activities of constructs located 5′-upstream of the transcription start site decreased when the −186 to −156 and −56 to −19 nt regions were deleted. Site-directed mutagenesis of these regions revealed that two hepatocyte nuclear factor-4α (HNF-4α) and one octamer binding transcription factor-1 (Oct-1) binding sites are essential for *FUT VI* transcription. Furthermore, transient over-expression of HNF-4α but not Oct-1 enhanced both *FUT VI* promoter activities and *FUT VI* mRNA levels in HuH-7 cells. These results suggest that two defined regions in the 5′-flanking region of the *FUT VI* transcription start site are critical for *FUT VI* transcription in HepG2 cells.

Keywords Fucosyltransferase VI · Hepatocyte nuclear factor · HepG2 cells · Transcriptional regulation

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Introduction

The glycan sialyl Lewis X (sLeX), or NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-R, and its stereoisomer sialyl Lewis A (sLeA), or NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAc-R, serve as ligands for endothelial E-selectin and are involved in the recruitment of leukocytes to sites of inflammation [1]. Cancer cells that express sialyl Lewis antigens on their cell surfaces have also been known to adhere to endothelial E-selectin and are associated with malignancy and metastasis. Two principle mechanisms for tumor-associated alteration of glycans have been proposed, incomplete synthesis and neosynthesis of glycans [2, 3].

Sialyl Lewis antigens are synthesized by α1,3/4-fucosyltransferases (FUT) activity from the precursor NeuAcα2-3Galβ1-3/4GlcNAc-R, which is synthesized via transfer of α2-3-linked sialic acid with α2,3-sialyltransferases to Galβ1-3/4GlcNAc-R. To date, six human *α1,3/4-FUT* genes, *FUT III* [4], *FUT IV* [5], *FUT V* [6], *FUT VI* [7, 8], *FUT VII* [9, 10] and *FUT IX* [11] have been identified and cloned. On the basis of acceptor specificities toward various synthetic α2-3-sialyl lacto-*N*-neotetraose analogs [12], glycoproteins and glycolipids [13], it has been shown that FUT III, IV, VI and VII can produce the sLeX antigen. Among human FUTs, FUT VI has the highest α1,3-FUT activity against glycolipids and consequently, FUT VI can synthesize the sLeX epitope faster [14]. FUT VI is believed to contribute to sLeX synthesis in the liver or hepatocellular carcinoma HepG2 cells and to be involved in cancer metastasis [15–17]. Moreover, the level of α1,3-FUT activity in plasma is elevated in various cancerous conditions [18–20]. The major α1,3-FUT activities in plasma, liver, and kidney are related to FUT VI. Additionally, a missense mutation in the *FUT VI* gene leads to a complete absence of

α1,3-FUT activity and α1-3-fucosylation of serum glycoproteins [21].

In the previous study, we used matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI–TOFMS) to demonstrate that human hepatocellular carcinoma-derived HepG2 cells constitutively secrete transferrin, α1-antitrypsin, α2-HS glycoprotein and β-glycoprotein with high levels of sLeX, and have high levels of α1,3-FUT activity, possibly attributable FUT VI, when tested against NeuAcα2-3Galβ1-4GlcNAc-R [16].

Although α1,3-FUT activity, sLeX and *FUT VI* mRNA expression have been observed to change under various pathological conditions, the detailed mechanism of constitutive expression and transcriptional regulation of *FUT VI* remain to be elucidated. In the present study, we investigated transcriptional regulation of *FUT VI* in HepG2 cells using 5′-rapid amplification of cDNA ends (5′-RACE), a dual luciferase assay for sequential deletion and site-directed mutagenesis, and transient over-expression of hepatocyte nuclear factor-4α (HNF-4α) and octamer binding transcription factor-1 (Oct-1). Our results indicate that 5′-flanking regions at positions −156 to −136 nt and −56 to −19 nt relative to the *FUT VI* gene transcription start site are critical for *FUT VI* transcription and mRNA expression in HepG2 cells.

Materials and methods

Cell lines and culture conditions

Human hepatocellular carcinoma cell lines, HepG2 and HuH-7 cells, were purchased from the Japanese Cancer Research Resources Bank/Health Science Research Resources Bank (Tokyo, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan), supplemented with 100 U/ml penicillin (Banyu Pharmaceutical Co., Tokyo, Japan), 100 μg/ml streptomycin (Meiji Seika Co., Tokyo, Japan), and 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), at 37°C in a humidified atmosphere containing 5% CO_2.

Identification of the FUT VI transcription start site using 5'-rapid amplification of cDNA ends (5′-RACE)

The 5′ end of the *FUT VI* cDNA was amplified with 5′-full RACE Core Set (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg of total RNA using the 5′-phosphorylated *FUT VI* specific primer 5′-CCGTTGCAGAACCA-3′. Template mRNA was digested with RNase H at 37°C for 30 min and the cDNA was precipitated by addition of ethanol. The single strand DNA precipitate was dissolved into the ligation buffer and incubated with T4 ligase at 16°C for 16 h. The concatemer DNA was used as a template for the first PCR amplification, using the forward primer 5′-GGTCATGCATGACTGAGTCT-3′ and the reverse primer 5′-TGTGGCTTCAATGCGATGTG-3′. PCR conditions were 94°C for 3 min, followed by 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The resulting PCR products were diluted 100-fold with sterile water, and amplified under the conditions described above, using the second forward primer 5′-CCAAATCCCTTCACCCTGTT-3′ and the reverse primer 5′-GCTTTGAGTACAAAGT GAAA-3′. PCR products were ligated into the pGL4.11 vector (Promega, Madison, WI) and sequenced using a 3730xl DNA analyzer (Applied Biosystems, Foster, CA, USA).

Cloning of sequences 5′ of the FUT VI transcription start site and construction of plasmids for luciferase assay

Regions 5′ of the *FUT VI* transcription start site, −2,067 to +1 nt (−2067_+1) and −2,067 to +213 nt (−2067_+213), were isolated using PCR from HepG2-derived genomic DNA with a forward primer for −2,067: 5′-TGAGGCT TAGA GGGACATGG-3′, and reverse primers for +1: 5′-CACTTCCTTTCCCCACCCAC-3′ and for +213: 5′-GGAGGCAGAGCTGTTGCAGA-3′. The PCR products were 5′-phosphorylated using the T4 Polynucleotide Kinase (Toyobo, Tokyo, Japan) and then ligated into pGL4.11 vector that had been digested with *EcoRV* (Toyobo) and treated with alkaline phosphatase from *E. coli* (Toyobo). The 5′ regions were then sequenced using a 3730xl DNA analyzer.

Deletion constructs used in the luciferase assay

Plasmids for pGL4/−867_+1, pGL4/−622_+1, pGL4/−297_+1, pGL4/−186_+1, pGL4/−156_+1, pGL4/−136_+1, pGL4/−106_+1 and pGL4/−56_+1 were constructed by PCR using pGL4/−2067_+1 plasmid as the PCR template, and then ligated into the pGL4.11 vector. The PCR conditions were 94°C for 2 min, followed by 30 cycles of 95°C for 0.5 min, 60°C for 5 min, and 72°C for 1 min. The specific forward primers used were 5′-TGAGGCTTAGAGGGACATGG-3′ (pGL4/−2067_+1), 5′-TGACAGAGCAAGATTCCATC-3′ (pGL4/−867_+1), 5′-GGG TTCACACCATTCTCCTG-3′ (pGL4/−622_+1), 5′-TCCCTACCTCCCAGAACCTC-3′ (pGL4/−297_+1), 5′-TGATACTTGGAGGCTGGGCT-3′ (pGL4/−186_+1), 5′-TCACC TTTGCACCCAGCTT-3′ (pGL4/−156_+1), 5′-GGA GTGAGAGAGTTTAAGGTCAT-3′ (pGL4/−136_+1), 5′-CAAAACCAGTATTCCAGATCATTT-3′ (pGL4/−106_+1) and 5′-CTCCCTGGACTTCTGCTTTG-3′ (pGL4/−56_+1). The specific reverse primer used was 5′-CACTTCCTTTCCC CACCCAC-3′.

The pGL4/−19_+1 and pGL4/−11_+1 constructs were prepared by inverse PCR and self-ligation of PCR products. The specific forward primers used were 5′-GGGT GGG GAAAGGAAGT-3′ (pGL4/−19_+1) and 5′-AAAGGAAG TATCAAGATCTGGC-3′ (pGL4/-11_+1). The specific and common reverse primer used was 5′-ATCCTCGAGGCTA GCG-3′.

Site-directed mutagenesis of FUT VI promoter regions

Site-directed mutagenesis of the 5′-flanking region of the *FUT VI* gene was carried out using the QuickChange II XL site-directed mutagenesis kit (Stratagene Co., La Jolla, CA, USA), which is based on inverse-PCR. The pGL4/−186_+1 and pGL4/−56_+1 plasmids were used as PCR templates. Primers used for site-directed mutagenesis were: 5′-GGCTGGG**G**TTTGAGCTCGGTCACCTTTGC-3′ and 5′-GCAAAGGTGACCGAG CTCAAA**C**CCCAGCC-3′ for *−186/mut1*; 5′-GGCTGGG**G**TTTGAG**G**TCGGTCACC TTTGC-3′ and 5′-GCAAAGGTGACCGA**C**CT CAAA***C***CCCAGCC-3′ for *−186/mut2*; 5′-GCCTCGAG GATCTCCCTGGACTTCTGCT**G**T**T**CACTGCCCTG-3′ and 5′-CAGGGCAGTG**A**A**C**AGCAGAAGTCCAGGGA GATCCTCGAGGC-3′ for *−56/mut1*.

PCR was performed according to the manufacturer's instructions. PCR products were digested with *DpnI* and self-ligated with DNA ligase. The recombinant plasmids were transformed into *E. Coli* XL10-Gold competent cells (Stratagene) and positive clones were confirmed by DNA sequencing using a 3730xl DNA analyzer.

Assay HNF-4α and Oct-1-responsive promoter activities

To determine the level of HNF-4α and Oct-1 responsiveness in HuH-7 cells, we used the Translucent HNF-4 reporter vector (for HNF-4α) and the Translucent Oct-1(1) reporter vector (Panomics, Fremont, CA, USA). HuH-7 cells were seeded at a density of $1–3\times10^5$ cells per well of a 48-well plate and then transfected with 2 μg of translucent HNF-4 or translucent control mutated plasmid and 0.1 μg of pRL–CMV40 as an internal control for transfection efficiency, using LipofectAmine 2000 (Invitrogen). In the pRL–CMV vector, the CMV promoter is upstream of the *Renilla* luciferase gene. After 24 h, the cells were harvested and assayed using the Dual Luciferase Assay System (Promega).

Construction of HNF-4α and Oct-1 expression plasmids pcDNA3.2/HNF-4α and pcDNA3.2/Oct-1

Human HNF-4α and Oct-1 full-length cDNAs that had been PCR-amplified from HepG2 and U937 cDNA, respectively, were cloned into the pcDNA3.2/V5 vector using pcDNA Gateway Directional TOPO expression kits (Invitrogen) and the forward and reverse primer sets 5′-CACCATGCGACTCTCCAAAACCCT-3′ and 5′-CTAGA TAACTTCCTGCTT GGT-3′ for *HNF-4α*, or 5′-CAC CATGCTGGACTGCAGTGACTA-3′ and 5′-GCCCAGCT CACTGTGCCTTG-3′ for *Oct-1*. The recombinant plasmids were transformed into *E. coli* DH5α competent cells and positive clones were confirmed using a 3730xl DNA analyzer.

FUT VI promoter assay

For the *FUT VI* promoter assay, the relevant plasmids were transiently transfected into HepG2 and HuH-7 cells using LipofectAmine 2000. Cells were seeded at a density of approximately $1–3\times10^5$ cells per well of a 48-well plate, and then transfected with 2 μg of pGL4 constructs and 0.1 μg of pRL–CMV. After 24 h, the cells were harvested and lysed. Activity of the firefly and *Renilla* luciferases was determined using the Dual Luciferase Assay System.

For the *FUT VI* promoter assay in the presence of HNF-4α and Oct-1, the following plasmids were used: pGL4/−186_+1(three HNF-4α and one Oct-1 binding sites), pGL4/−156_+1 (two and one), and pGL4/−56_+1 (one and one) and pGL4/−19_+1 (none and none). The plasmids were transiently transfected into HuH-7 cells, which have low HNF-4α and Oct-1 promoter activities, using Lipofect Amine 2000. For each assay, 2 μg of a pGL4 construct, 0.1 μg of pRL–CMV, and 0.05 μg of either pcDNA3.2/HNF-4α or pcDNA3.2/Oct-1 were transfected into pre-seeded HuH-7 cells in 48-well dish. After 24 h, the cells were harvested and lysed, and the firefly and *Renilla* luciferase activities were determined as described above.

Real-time PCR determination of FUT VI mRNA levels

Total RNA was extracted from HepG2 and HuH-7 cells that had been transiently transfected with pcDNA3.2/HNF-4α using TRIzol regent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA (20 μl) was synthesized from 5 μg of total RNA using ReverTra Ace reverse transcriptase (Toyobo) and an oligo $(dT)_{20}$ primer, followed by DNase I treatment, according to the manufacturer's instructions.

The cDNA (5 μl) was then used for quantitative real-time PCR using SYBR-Green PCR Master Mix (Toyobo) and the ABI Prism 7000 Detection system (Applied Biosystems) in a 96-well plate according to the manufacturer's instructions. PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 63°C for 60 s, and 72°C for 60 s. The amount of *FUT VI* transcript was determined for each sample and normalized to *GAPDH* levels. The specific forward and reverse oligonucleotide

primers used were 5′-CAGCCTCAAGAT CATCAGCA-3′ and 5′-ACAGTCTTCTGGGTGGCAGT-3′ for *GAPDH* (GenBank accession no. *M33197*), and 5′-CAAAGCCA CATCGCATTGAA-3′ and 5′-ATCCCCGTTGCAGA ACCA-3′ for *FUT VI* (NM000150). The oligonucleotide primer sequences were designed using Primer Express software version 2.0.0.

Reverse transcription–PCR for COUP-TF I, COUP-TF II and HNF-4α

Total RNA was extracted from HuH-7 and HepG2 cells using TRIzol reagent. Next, cDNA (0.5 μl) was amplified in a PC-812 thermal cycler (Astec Co., Fukuoka, Japan) using KOD Dash (Toyobo) with specific forward and reverse primers according to the manufacturer's instructions. The PCR conditions for amplification of chicken ovalbumin upstream promoter transcription factor (*COUP-TF*) *I*, *COUP-TF II* and *HNF-4α* were as follows: 95°C for 2 min, followed by 30 cycles of 98°C for 20 s and 60°C for 5 s. For GAPDH, 20 cycles of 72°C for 30 s and 72°C for 5 min were performed. Specific primers for *GAPDH*, *HNF-4α*, *COUP-TF I* and *COUP-TF II* were as follows: 5′-CCTGGCCAAGGTCATCCATG-3′ and 5′-GGAAGGCC ATGCCAGTGAGC-3′ for *GAPDH* (GenBank accession no. ***M33197***); 5′-GGAGATGACTTGAGGCCTTACT-3′ and 5′-GGGGAATCGTTTCCAAGGCCTC-3′ for *HNF-4α* (***NM_178849***); 5′-GCCTGTGCCATTTCTGATTT-3′ and 5′-CTCGCCAGCTGCTAACTACC-3′ for *COUP-TF I* (***NM_005654***); and 5′-TCTGATGTAGCCCATGTGGA-3′ and 5′-GCAAGTTGTTCTGACCGACA-3′ for *COUP-TF II* (***NM_021005***).

Primer sequences were designed using Primer Express software version 2.0.0.

Results

Transcription start sites for the *FUT VI* gene

In previous reports [16], we demonstrated that hepatoma-derived HepG2 cells have robust and constitutive α1,3-FUT activity; that remarkably high levels of sLeX are detectable on the cell surface; and that a high proportion of glycoproteins secreted by these cells have sLeX on highly branched complex types of *N*-glycans. To better understand the nature of high and constitutive expression of *FUT VI* in HepG2 cells, we determined the transcription start sites of *FUT VI* using 5′-RACE. The results revealed the presence of two transcripts, 650 and 450 bp (Fig. 1a). Based on sequence analysis, these gene products are transcribed from position +65 and +278 nt, respectively, relative to the position registered in the Data Base of Human Transcription Start Sites (DBTSS). To confirm the transcription start site at +65 nt from the position registered in DBTSS, we carried out 5′-RACE analysis using different 5′-RACE primers, of almost the same position or upstream of its position. These results indicated that transcription start site at around +65 nt from the position registered in DBTSS (Data not shown). In this paper, we set the transcription start site at the +65 nt relative to the position in DBTSS as the revised transcription start site and refer to this position henceforth as position +1 nt (Fig. 1b).

Effects of deletion of specific *FUT VI* 5′-flanking regions on transcriptional activity

We PCR-amplified two 5′-flanking regions of the *FUT VI* gene, −2,067 to +1 nt (−2067_+1) and −2,067 to +213 nt (−2067_+213), from genomic DNA and cloned the fragments into the luciferase expression vector pGL4.11, resulting in pGL4/−2067_+1 and pGL4/−2067_+213 (Fig. 2a). The vectors were subsequently transfected into HepG2 cells and luciferase activity was determined (Fig. 2b). The activity of pGL4/−2067_+213 was almost at the same as that observed for the control plasmid, pGL4/empty. However, the activity of pGL4/−2067_+1 was approximately three times higher than the control. Three constructs in which portions of the region had been deleted (pGL4/−867_+213, pGL4/−297_+213 and pGL4/−19_+213) also had no observable promoter activity. These results strongly suggest that transcription of *FUT VI* is primarily regulated in the region −2,067 to +1.

We next tested if the deletions affect transcriptional activity by introducing plasmids carrying the deletions in to HepG2 cells (see "Materials and methods"). After transfection of the deletion constructs into HepG2 cells, luciferase activities were determined. As shown in Fig. 2b, luciferase activity was significantly lower when the −186 to −156 or −56 to −19 nt regions had been deleted, suggesting that transcription of *FUT VI* is regulated by factors that bind in these regions. By contrast, deletion of the −2,067 to −662 nt region enhanced luciferase activity, suggesting that the region contains binding site(s) for one or more negative regulatory factor or decrease in transfection efficiency by insert length of DNA in pGL4.11 vector. We next asked if there are conserved domains or motifs in the region −286 to +1 nt of 5′ of the *FUT VI* transcription start site. We performed a similarity search using Match program (http://www.gene-regulation.com/index.html) and the results revealed the presence of consensus transcription factor binding sites. Specifically, HNF-4α consensus sites are detectable in the −186 to −156 region and HNF-4α and/or Oct-1 consensus sites are detectable in the −56 to −19 region (Fig. 3).

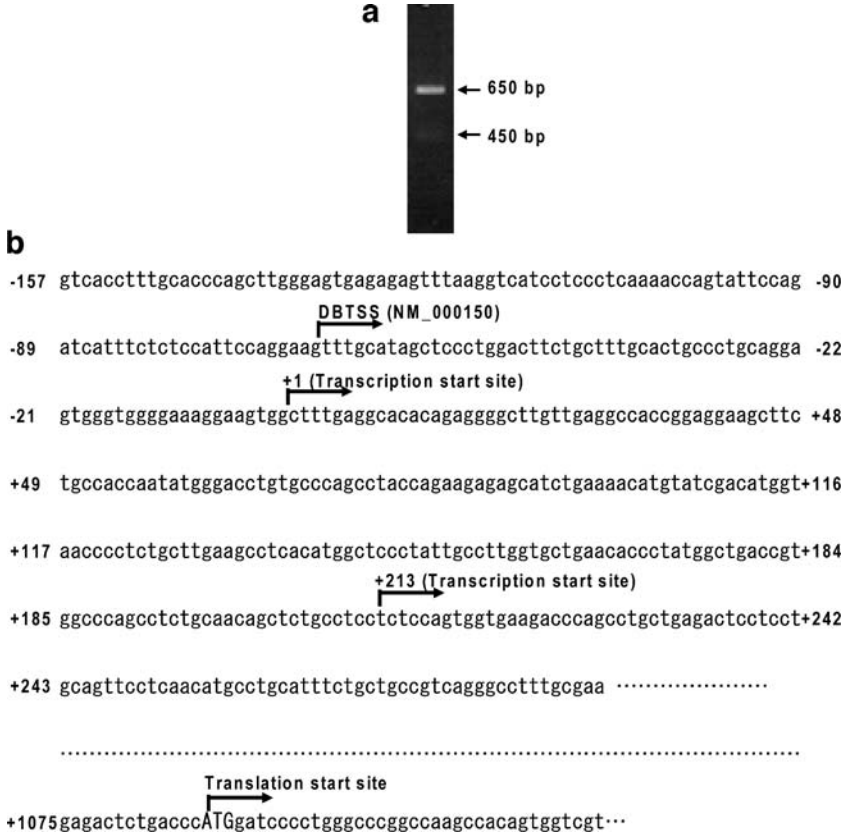

Fig. 1 The transcription start site and 5′-untranscribed region of *FUT VI* genomic DNA. (**a**) The results of 5′-RACE of *FUT VI* mRNA. mRNA from HepG2 cells was subjected to 5′-RACE. PCR products were separated by 2% agarose gel electrophoresis and DNA was detected with ethidium bromide. (**b**) Nucleotides are numbered relative to the transcription start site obtained from 5′-RACE analysis (*i.e.* the observed transcription start site was set to +1). The position of the *FUT VI* transcription start site registered in the Data Base of Human Transcription Start Sites (DBTSS) is at −65 relative to our experimentally defined start site (+1). The putative translation start site for *FUT VI* is at +1,088

Site-directed mutagenesis of *FUT VI*

To determine if HNF-4α or Oct-1 can up-regulate *FUT VI* gene transcription, we prepared constructs of in which the consensus sequence for HNF-4α (CTTTG) [22] in the −186 to −156 and −56 to −19 nt regions and for Oct-1 (TTTGC) [23] in the −56 to −19 nt region had been mutated. The mutant constructs were then transfected into HepG2 cells and we determined luciferase activities of each (Fig. 4).

In terms of the −186 to −156 nt region (Fig. 4a), promoter activities of constructs carrying a one-base substitution (pGL4/−186_+1/mut1) or a two-base substitution (pGL4/−186_+1/mut2) were significantly lower than what is observed for the unmodified reporter construct (pGL4/−186_+1/wild) and similar to that observed for pGL4/−156_+1. In terms of the −56 to −19 nt region (Fig. 4b), promoter activity of pGL4/−56_+1/mut1 was significantly lower than that observed for the unmodified construct (pGL4/−56_+1/wild); notably, however, the HNF-4αconsensus sequence of (CTTTG) in this region overlaps with the Oct-1 consensus sequence (TTTGC). These results are consistent with the idea that that HNF-4α alone or both HNF-4α and Oct-1are important for *FUT VI* transcription in two promoter regions, −186 to −156 (HNF-4α) and −56 to −19 nt (HNF-4α and Oct-1).

Promoter activities of HNF-4α and Oct-1 in HuH-7 and HepG2 cells

To further investigate the idea that HNF-4α and Oct-1 regulate *FUT VI* gene transcription, we determined the responsiveness of human hepatocyte HuH-7 and HepG2 cells to HNF-4α and Oct-1 using HNF-4 and Oct-1 binding site reporter plasmids in each of the two cell lines, which have low (HuH-7) or high (HepG2) endogenous levels of expression of *FUT VI* mRNA. The relative activity of an Oct-1 reporter in HuH-7 and HepG2 cells were 1.6±0.7 and 1.3±0.1 (Oct-1 reporter vector/control vector, n=3), respectively. The relative activity of the HNF-4α reporter in HuH-7 and HepG2 cells were 1.2±0.2 and 5.0±0.7 (HNF-4

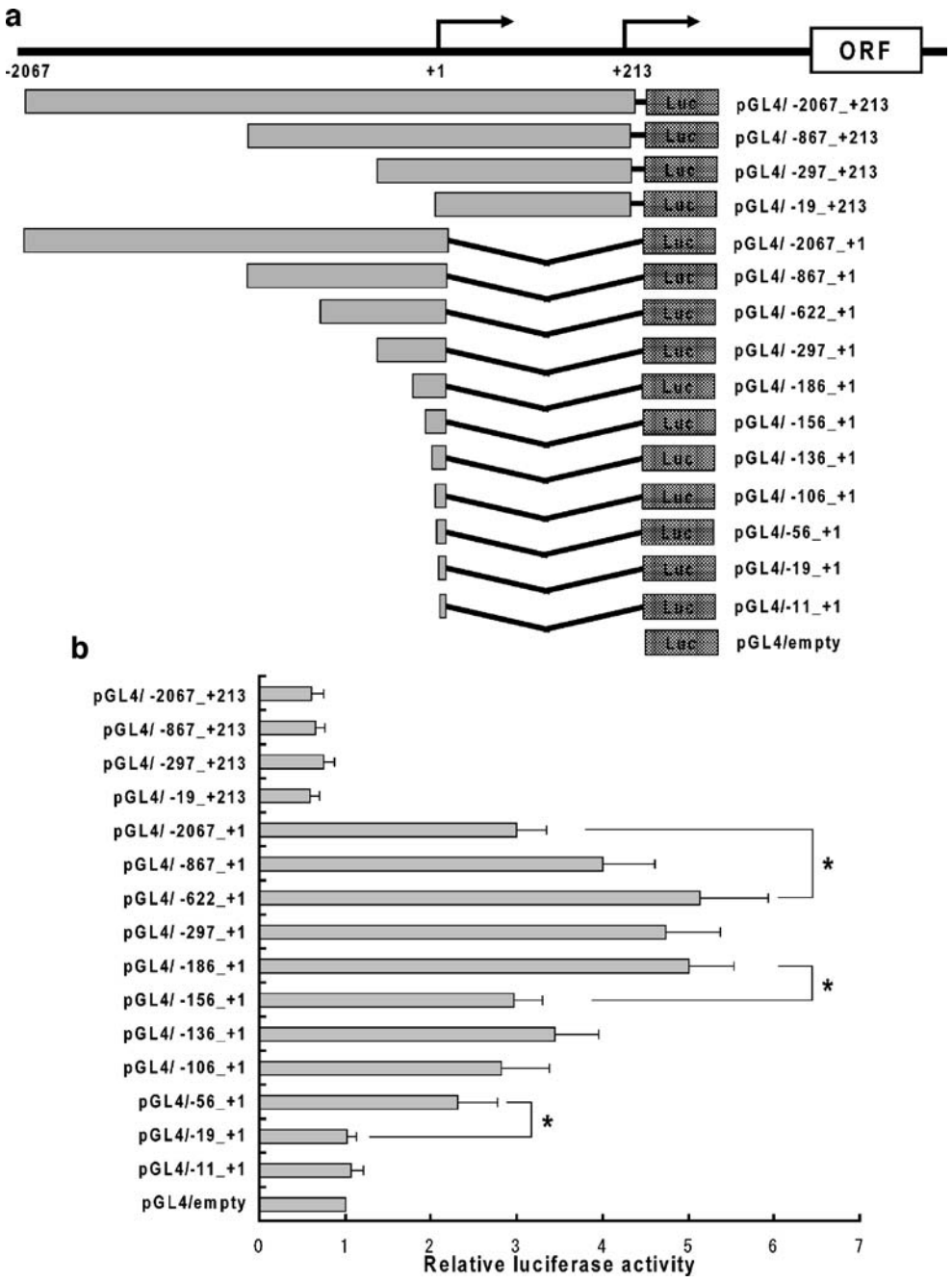

Fig. 2 Plasmids used to assay the responsiveness of *FUT VI* promoter regions and the effect of deletion of conserved motifs on the response. Luciferase reported plasmids containing the specific promoter regions indicated were constructed from HepG2 genomic DNA by PCR and ligation (**a**). In each case, 2 μg of each plasmid construct and 0.1 μg of pRL–CMV were co-transfected into HepG2 cells and luciferase activity was determined in a dual-luciferase assay 24 h post-transfection (**b**). In each case, firefly luciferase activities were normalized to *Renilla* luciferase activity to correct for differences in transfection efficiency. The results obtained in three independent experiments are expressed as mean ± SD. Significant differences ($p<0.05$) are indicated by *asterisks*

reporter vector/control vector, $n=3$), respectively. The results suggest that Oct-1 is not active in either cell line and that HNF-4 is active in HepG2 but not Huh-7 cells.

Expression of *FUT VI* in HuH-7 cells that over-express HNF-4α or Oct-1

As shown above, human hepatoma cell line HuH-7 cells have relatively low HNF-4α promoter activity and low *FUT VI* mRNA expression (Fig. 5b). We next over-expressed HNF-4α and Oct-1 in HuH-7 cells and examined asked if HNF-4α and/or Oct-1 can regulate the *FUT VI*. As shown in Fig. 5a, promoter activity for the constructs pGL4/−186_+1, pGL4/−156_+1, and pGL4/−56_+1 (see Figs. 2 and 3), which have three HNF-4α, two HNF-4α, and one HNF-4α and/or one Oct-1 binding site(s), respectively, increased significantly when HuH-7 cells were also transfected with pcDNA3.2/HNF-4α. On the other hand, the activity of pGL4/−19_+1, which lacks HNF-4α binding sites, was not affected by exogenous expression of HNF-4α.

Furthermore, the *FUT VI* promoter regions carrying deletions of conserved binding motifs were unaffected by overexpression of Oct-1 via transfection with pcDNA3.2/

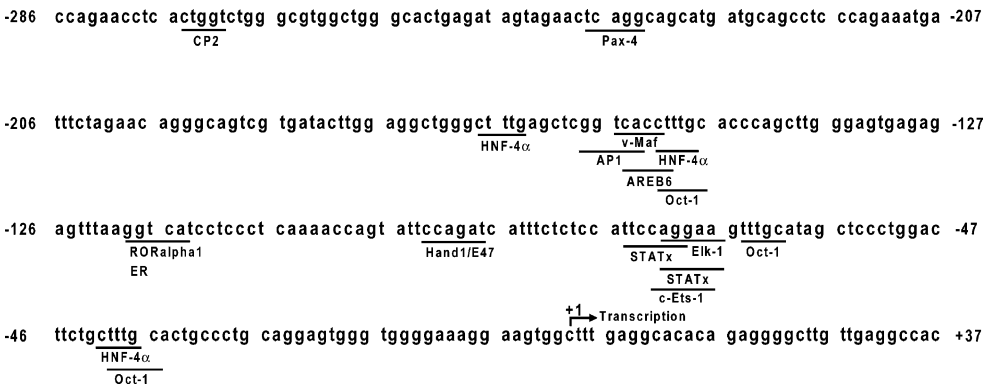

Fig. 3 Consensus transcription factor binding sites at −186 to +1 of the *FUT VI* promoter region. Consensus transcription factor binding motifs are detectable in the 5′-flanking region of *FUT VI* (*underlined*). The sequences are numbered relative to the transcription start site (*i.e.* start site=+1)

Oct-1. We next determined *FUT VI* mRNA expression levels in HepG2, HuH-7, and HuH-7 cells transfected with the pcDNA3.2/HNF-4α expression vector by real-time PCR. As shown in Fig. 5b, *FUT VI* mRNA levels increased in HuH-7 cells transfected with pcDNA3.2/HNF-4α as compared with non-transfected HuH-7 cells. Together with previous results, the data show that *FUT VI* transcription is up-regulated in HuH-7 cells by HNF-4α . Moreover, *FUT VI* mRNA levels were higher in HepG2 cells than in HNF-4α-transfected HuH-7 cells (Fig. 5b). By contrast, responsiveness of the HNF-4α reporter construct in HepG2 cells (5.0±2.7) was lower than that observed in HuH-7 cells exogenously expressing HNF-4 (15.3±2.7).

We next examined the levels of mRNA for *HNF-4α* and *COUP-TF I* and *II* in HuH-7 and HepG2 cells. *COUP-TF I* and *II* act as regulatory factors to mediate HNF-4α-induced promoter activity (Fig. 5c). Although the activity of HNF-4α in HepG2 cells is higher than that in HuH-7 cells, RT-PCR revealed similar *HNF-4α* mRNA expression levels in both HuH-7 and HepG2 cells. As shown in Fig. 5c, *COUP-TF I* mRNA was not detected in either HuH-7 or HepG2 cells, but *COUP-TF II* mRNA levels in HuH-7 cells was

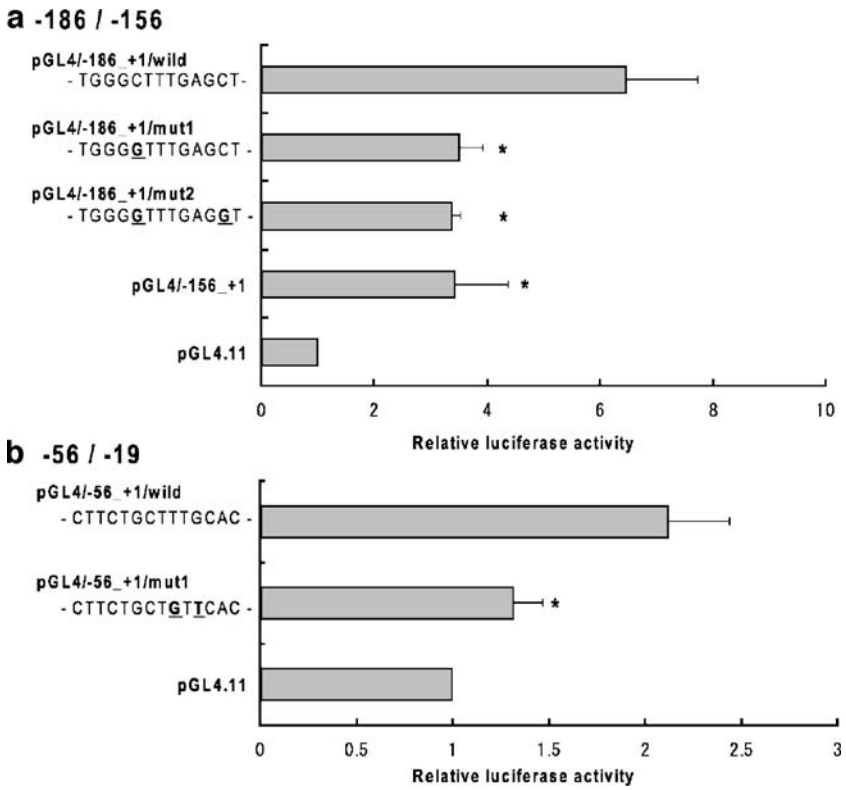

Fig. 4 Effect of site-directed mutagenesis on responsiveness of conserved regions of the *FUT VI* promoter. **a** Mutagenesis in the region −186 to −156. **b** Mutagenesis in the region −56 to −19 of the *FUT VI* promoter. In each case, 2 μg of the mutant construct and 0.1 μg of pRL–CMV were co-transfected into HepG2 cells and luciferase activity was determined 24 h post-transfection. In each case, firefly luciferase activity was normalized to *Renilla* luciferase activity to correct for differences in transfection efficiency. The results obtained in three independent experiments are expressed as mean ± SD. Significant differences ($p<0.05$) as compared with the equivalent wild-type reporter construct

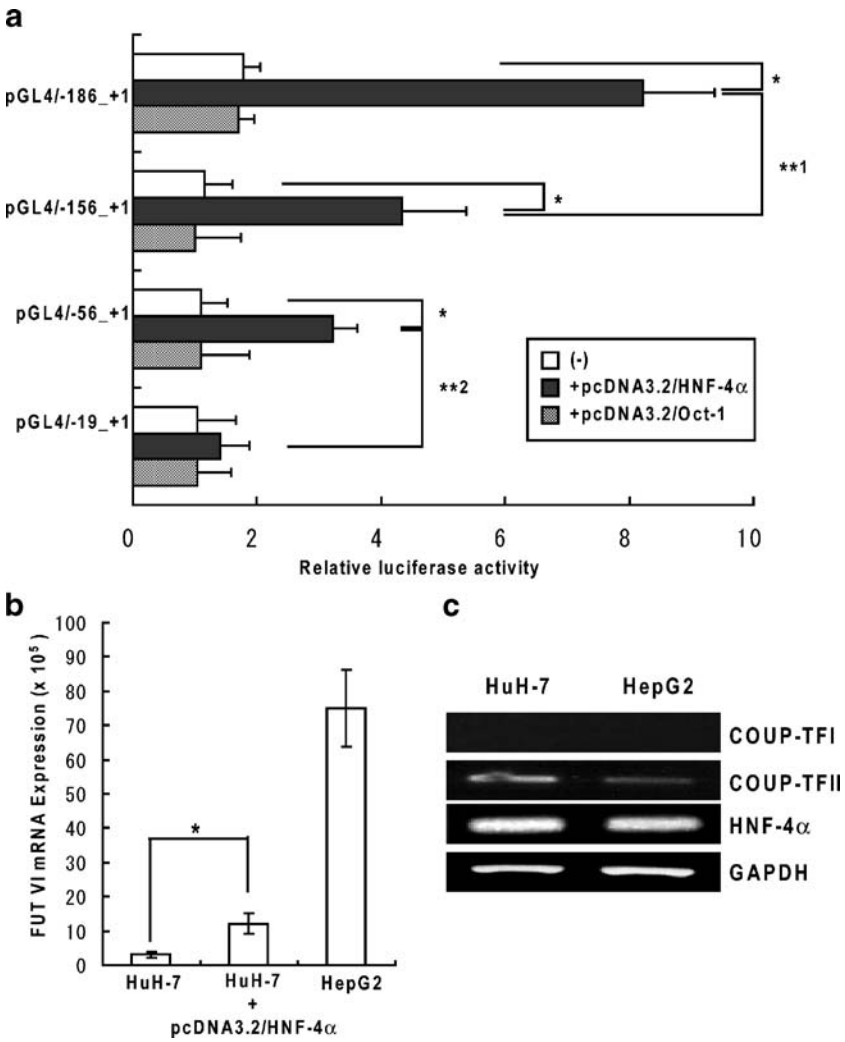

Fig. 5 Effect of HNF-4α and Oct-1 overexpression on expression of *FUT VI* and detection of putative transcriptional mediators expressed in HuH-7 and HepG2 cells. **a** Luciferase activities of deletion constructs of *FUT VI* 5′-flanking region in HuH-7 and HuH-7 cells transfected with HNF-4α or Oct-1. In each case, 2 μg of the plasmid construct (pGL4/−186_+1, pGL4/−156_+1, pGL4/−56_+1 or pGL4/−19_+1; see Fig. 2) and 0.1 μg of pRL–SV40 were co-transfected into HuH-7 cells, with or without 50 ng of pcDNA3.2/HNF-4α or pcDNA3.2/Oct-1. Luciferase activities were determined by a dual-luciferase assay 24 h post-transfection. Firefly luciferase activity was normalized to *Renilla* luciferase activity to correct for differences in transfection efficiency. The results obtained in three independent experiments are expressed as mean ± SD. Significant differences ($p<0.05$) relative to HuH-7 cells lacking the overexpression constructs are indicated by *asterisks* against. *Double asterisks* indicate significant differences ($p<0.05$) as compared with HuH-7 cells co-transfected with pGL4/−186_+1 and pcDNA3.2/HNF-4α (*1*) or pGL4/-56_+1 and pcDNA3.2/HNF-4α ($p<0.05$) are indicated by asterisks. **b** FUT VI mRNA expression levels in HuH-7 cells, HuH-7 cell transfected with pcDNA3.2/HNF-4α and HepG2 cells. FUT VI and GAPDH mRNA expression levels were determined by real-time PCR using specific primer sets described in the text. The results obtained in three independent experiments are expressed as mean ± SD. Significant differences ($p<0.05$) are indicated by asterisks. **c** *HNF-4α*, *COUP-TF I*, *II* and *GAPDH* mRNA levels in HuH-7 cells and HepG2 cells. RT-PCR was carried out for HuH-7 and HepG2 cDNAs using specific oligonucleotide primer sets (see "Materials and methods")

higher than that in HepG2 cells, suggesting that COUP-TF II negatively regulates HNF-4α activity in HuH-7 cells.

Discussion

In mammalian cells, the FUT VI enzyme plays an important role in sLeX biosynthesis, particularly in epithelial cells. Moreover, high levels of expression of FUT VI are essential for synthesis of sLeX in human colon carcinoma KM12 cells [24]. The results of immuno-histochemical and RNA blot analyses have shown that sLeX in primary liver cancer cells is primarily synthesized by FUT VI and is involved in cancer metastasis [17]. However, a detailed mechanism that explains constitutive expression and transcriptional regulation of the *FUT VI* gene has not been revealed.

In this work, we used 5′RACE to identify two transcription start sites of the *FUT VI* gene, which we number +1 and +213, that are different from what is reported in DBTSS (Fig. 1b). Regions 5′ of the transcription start sites we identified do not contain canonical TATA-, CAAT-, or GC-boxes. Moreover, neither TATA- nor

CCAAT-boxes could be identified in the promoter regions of other human *FUT* genes, *FUT III* [25], *IV* [26] and *VII* [27].

We next determined what regions are essential for constitutive expression of *FUT VI* in HepG2 using a luciferase reporter gene assay (Fig. 2b) and site-directed mutagenesis (Fig. 4). The results indicate that transcription of *FUT VI* is regulated by HNF-4α and Oct-1, as binding sites for one or more these factors are detected in the regions −186 to −156 and −56 to −19 nt. In an attempt to detect direct binding of HNF-4α to the *FUT VI* promoter, we performed an electorophoretic mobility shift assay using double-stranded DNA probes corresponding to the putative HNF-4α binding regions as a probe incubated with nuclear extract from HepG2 cells. We were able to confirm the presence of specific shifted bands; however, a super-shift assay with an anti-HNF-4α monoclonal antibody did not result in a super-shifted band, such that we could not confirm a direct binding interaction. HNF-4α is a member of the so-called orphan nuclear receptor superfamily and contains a putative zinc-finger. Additionally, HNF-4α participates in regulation of many genes involved in diverse metabolic pathways, including glucose, cholesterol and fatty acid metabolism pathways; in the synthesis of blood coagulation factors; and in the development of the hepatic phenotypes. Oct-1 is a ubiquitous transcription factor expressed in all eukaryotic cells that can participate in positive or negative regulation of target gene transcription [28–30].

The response to Oct-1 was low for both HuH-7 and HepG2 cells. However, response to HNF-4α differed between the two cell types. Specifically, HepG2 cells were approximately five times more responsive to HNF-4α than HuH-7 cells. Over-expression of HNF-4α led to up-regulation of *FUT VI* promoter response and increased levels of *FUT VI* mRNA levels (Fig. 5b) as compared with mock-transfected cells. Interestingly, although HNF-4α-responsiveness of HepG2 cells was less robust than that of HuH-7 cells that exogenously express HNF-4α as measured by the luciferase reporter assay, *FUT VI* mRNA levels were higher in HepG2 cells than in HuH-7 cells that exogenously express HNF-4α. It is known that many modulator, co-activator and repressor molecules modify activation by HNF-4α. It follows, then, that the presence or absence of co-factors for HNF-4α may influence HNF-4α promoter activity and thus, affect *FUT VI* mRNA expression in HepG2 and HNF-4α−transfected HuH-7 cells. This could explain our finding that although HNF-4α mRNA is detected in HepG2 cells, HNF-4α activity was not in this cell line. Indeed, the results suggest that repressors of HNF-4α promoter activity are expressed in HuH-7 cells.

The DNA-binding activity of HNF-4α is modulated by homo-dimerization, post-translational phosphorylation, CBP-mediated acetylation, and binding of agonistic and antagonistic ligands [31]. Furthermore, HNF-4α promoter activity is affected by certain activator and/or repressor molecules, including the short heterodimer partner (SHP) protein, COUP-TF I, COUP-TF II and HNF-1α [31]. COUP-TFs are strong suppressors of HNF-4α−mediated promoter activity and/or enhance HNF-4α-dependent HNF-1α promoter activity as compared with the effects of HNF-4α alone [32]. Although expression the *COUP-TF I* gene is not detected in HuH-7 or HepG2 cells, *COUP-TF-II* mRNA is detected. Moreover, *COUP-TF-II* mRNA levels are higher in HuH-7 cells than in HepG2 cells (Fig. 5c). We have also demonstrated that the responsiveness of the *FUT VI* promoter region in HepG2 cells to HNF-4α decreases after transfection with a COUP-TF II expression plasmid (unpublished data). It is reasonable that COUP-TF II may also affect the promoter activity of HNF-4α in HuH-7 cells. COUP-TFs have been reported to be expressed in some tumor cell lines [33], lung carcinoma [34, 35], endometrial cancer cells [36], and adrenal tumors [37], but not in terminally differentiated epithelial cells. Furthermore, COUP-TFs affect the expression of carcinogenesis-related molecules, *e.g.*, by altering human telomerase reverse transcriptase (*hTERT*) promoter activity [38, 39]. However, ligands of COUP-TFs have been not identified and its transcriptional regulation remains unknown.

In previous studies, we stimulated human hepatocellular carcinoma-derived HuH-7 cells with proinflammatory cytokines and investigated sLeX expression on α1-acid glycoprotein (AGP) secreted into a medium or on the cell surface. Our results indicated that interleukin-1β (IL-1β) enhances expression of sLeX on AGP [40] and the cell surface and additionally, that α2,3-sialyltransferase IV and FUT VI were involved in the synthesis of sLeX in HuH-7 cells [41]. Nevertheless, consensus sequences for IL-1β-inducible transcriptional elements, nuclear factor kappa-B (NF-κβ), activator protein-1 (AP-1) or nuclear factor IL-6 (NF IL-6) binding are not detected in the promoter regions of the *FUT VI* and *HNF-4α* genes. Match analysis (http://www.gene-regulation.com/) using >75% match reveals that *COUP-TF II* promoter regions contain one NF-kB, four AP-1 and no NF-IL-6 binding sites in the region 1,000 nt 5′ of the transcription start site registered in DBTSS. Elevation of *FUT VI* mRNA levels in HuH-7 cells in response toIL-1β-stimulation may be a result of increases in HNF-4α-responsive promoter activity via binding of NF-kB or AP-1 to *COUP-TF* promoter regions or binding of unidentified ligands to COUP-TF II, resulting in a subsequent decrease in COUP-TF II expression.

In conclusion, we assayed transcriptional regulation of the *FUT VI* gene and demonstrated that HNF-4α, and/or Oct-1, binding sites are present in two regions, −186 to −156 and −56 to −19 nt 5′ of the *FUT VI* transcription start site. Moreover, the regions are important for *FUT VI*

gene transcription, and we found that HNF-4α plays a critical role in regulation of *FUT VI* transcription in HepG2 cells.

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