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Characterization of Putative Cis-Regulatory Elements That Control the Transcriptional Activity of the Human Oct4 Promoter

Heung-Mo Yang,[1,3] Hyun-Jin Do,[1] Jong-Hyun Oh,[1] Jin-Hoi Kim,[2] Sang-Yun Choi,[3] Kwang-Yul Cha,[1] Hyung-Min Chung,[1]* and Jae-Hwan Kim[1]*

[1]Cell and Gene Therapy Research Institute, Graduate School of Life Science and Biotechnology, Pochon CHA University, 606-13, Seoul, South Korea
[2]Major of Dairy Science, Division of Applied Life Science, College of Agriculture, Gyeongsang National University, Chinju, GyeongNam, South Korea
[3]School of Life Sciences & Biotechnology, Korea University, South Korea

Abstract Octamer-binding transcription factor-4 (Oct4), a member of the POU domain transcription factors, is crucial for both early embryonic development and the maintenance of stem cell pluripotency. The human Oct4 (hOct4) 5′ upstream sequence contains four conserved regions (CR1, 2, 3, 4) that are homologous in the murine. In this study, we constructed a series of deletion mutants of the hOct4 5′ upstream region and identified cis-regulatory elements that may be important determinants for the transcriptional activity of the hOct4 promoter. Our studies showed that CR2, 3, and 4 each acted as positive cis-regulatory elements in hOct4 promoter activity. We also newly identified a putative negative cis-acting element located between CR1 and CR2. In addition, the sequence −380/−1 at CR1 that contains a GC box was sufficient to provide the minimal promoter activity. Site-directed mutagenesis and electrophoretic mobility shift assays revealed the GC box located in the −380/−1 region may play a critical role in controlling the transcriptional activity of hOct4 by the direct binding of Sp1 or Sp3 transcription factors to the GC box. An overexpression study showed that Sp1 and Sp3 positively and negatively regulate hOct4 promoter activity. Thus, the hOct4 promoter upstream region contains multiple regulatory elements, one of which, the GC box, may be an important cis-regulatory element that regulates the transcription of the hOct4 promoter by the binding of Sp family transcription factors. J. Cell. Biochem. 96: 821–830, 2005.

Key words: embryonic carcinoma cells; Oct4; promoter; Sp family transcription factor

Octamer-binding transcription factor-4 (Oct4), a member of the POU domain family of transcription factors, contains a homeodomain that binds to many generally expressed octamer sites in promoter or enhancer regions [Verrijzer and van der Vliet, 1993]. Recent studies have shown that Oct4 plays an essential role in the maintenance of stem cell potency [Niwa et al., 2000] and is gradually downregulated when stem cells undergo differentiation [Schöler et al., 1990; Yeom et al., 1996]. Consistent with Oct4 expression in the pre-implantation embryo, it is also expressed in both embryonic stem (ES) and embryonic carcinoma (EC) cells.

The cell type specific and temporal expression of Oct4 suggests that unique mechanisms regulating Oct4 gene expression may exist. These mechanisms have been studied in murine ES and EC cells [Schoorlemmer et al., 1994; Sylvester and Schöler, 1994; Minucci et al., 1996; Pesce et al., 1999; Fuhrmann et al., 2001; Gidekel and Bergman, 2002]. One of these

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*Correspondence to: Hyung-Min Chung and Jae-Hwan Kim, Cell and Gene Therapy Research Institute, Pochon CHA University College of Medicine, 606-13 Yeoksam 1 dong, Kangnam-Gu, Seoul 135-907, South Korea.
E-mail: hmchung@chacares.com and jaehwan_k@cha.ac.kr

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mechanisms involves methylation and demethylation since the reduction of Oct4 expression upon stem cell differentiation is closely associated with increased methylation and chromatin structure changes in the promoter upstream region [Ben Shushan et al., 1993]. To escape the *de novo* methylation process, the *Oct4* gene possesses specific demethylation elements, including an Oct4 proximal enhancer that is located approximately between 2 and 1.2 kb upstream from the promoter [Gidekel and Bergman, 2002]. Another molecular mechanism involves cis-acting elements within the promoter upstream region. Previous studies showed that murine Oct4 expression in EC and ES cells is controlled by the two separate enhancer elements in the 5′ upstream region [Yeom et al., 1996] and the TATA-less proximal promoter [Minucci et al., 1996]. The distal enhancer (DE) is active in undifferentiated ES cells, the inner cell mass (ICM), and primordial germ cells (PGCs), indicating that DE activity is restricted to totipotent and pluripotent cell types [Yeom et al., 1996]. The proximal enhancer (PE) is active in P19 and primitive ectoderm during embryogenesis in a cell type-specific manner and is involved in retinoic acid (RA)-mediated Oct4 down-regulation [Okazawa et al., 1991]. The Oct4 proximal promoter (PP) also includes a GC box that binds to members of the Sp family transcription factors [Pesce et al., 1999] and half-sites of the consensus hormone response element (HRE) [Sylvester and Schöler, 1994] that is regulated by members of the steroid thyroid hormone receptor family such as RA receptor and retinoid X receptor (RXR), or by orphan members of the nuclear receptor gene superfamily [Pikarsky et al., 1994; Ben Shushan et al., 1995].

Counterparts of the murine *Oct4* gene have been identified in human [Takeda et al., 1992] and bovine [van Eijk et al., 1999]. These murine Oct4 orthologs are thought to have similar expression patterns to the murine gene. However, Oct4 expression in bovine seemed to be much broader than murine Oct4 as it is found in not only pluripotent cells but also trophoblast cells of the blastocyst [van Eijk et al., 1999]. In addition, it is not clear whether the expression of the Oct4 orthologs is controlled by regulatory elements and molecular mechanisms similar to those that regulate murine Oct4. The hOct4 5′ upstream sequence has been cloned [Takeda et al., 1992] and clustered as four conserved regions (CR1∼4) that are homologous in the murine and bovine [Nordhoff et al., 2001]. However, it is not clear whether these cis-regulatory elements regulate human Oct4 transcriptional activity.

To identify the *cis*-regulatory elements in the human Oct4 promoter upstream region, we constructed hOct4 5′ upstream region deletion mutants and demonstrated that each of the four conserved regions participates in controlling the expression of the h*Oct4* gene. We also newly identified a minimal promoter region and a putative negative *cis*-regulatory element between CR1 and CR2. In addition, we showed that both Sp1 and Sp3 can directly bind to the GC box in the PP, which plays critical roles in controlling the promoter activity. Transient co-transfection experiments showed that Sp1 and Sp3 positively and negatively regulate Oct4 promoter activity, respectively. Thus, the GC box and its binding proteins, Sp1 and Sp3 transcription factors may be involved in the mechanism that specifically regulates human Oct4 transcription.

MATERIALS AND METHODS

Cell Culture and Reagents

Human embryonic carcinoma (hEC; NCCIT) and human acute T cell leukemia (Jurkat) (American Type Cell Collection, Manassas, VA) cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were usually grown to confluence at 37°C in a humidified atmosphere of 5% CO_2 and subcultured every 3 days. Cells at passages 7–15 were used throughout all the experiments. The Sp1-binding oligonucleotide and the Sp1- and Sp3-specific polyclonal antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Plasmid Construction

The −2601/−1 (nucleotide positions are indicated with respect to the +1 translation start site) [Nordhoff et al., 2001] genomic fragment of the hOct4 promoter upstream region was amplified by PCR from human lymphocyte genomic DNA and cloned into the XhoI/HindIII sites of the pGL3-basic reporter plasmid (Promega, Madison, WI). Serial deletions of the Oct4

promoter upstream region (D1∼D7, D3-1∼D3-3 and D4-1∼D4-7) were generated by PCR amplification using the −2601/−1-Luc construct as a template followed by restriction enzyme digestion and ligation with the reporter plasmid digested at the same sites. The primers used in the PCRs are listed in Table I. The positive clones were selected by using the XhoI/HindIII restriction enzymes and verified by sequencing. The Sp1 and Sp3 expression vectors were gifts from Dr. Horowitz (North Carolina State University, NC [Udvadia et al., 1993, 1995]). The Sp1 and Sp3 coding sequences were inserted into pcDNA3.1+ (Invitrogen, La Jolla, CA) and the Flag epitope tag sequence was inserted into the N termini of the Sp1 and Sp3 open reading frames to produce Flag-tagged constructs. Site-directed mutagenesis was used to disrupt transcription factor-binding elements (GC boxes) in the PP region. Mutant promoter constructs were generated using −380-Luc (D5) as a template. Mutagenesis was carried out by using the "QuickChange" method (Stratagene, La Jolla, CA). The primers used to generate mutations in GC box-1 and -2 sequences (mutant bases are shown in lower case) also introduced an EcoRI or PstI restriction site (underlined) that could be used to select the positive clones. These primers are as follows: 5′-TCCACCC-ATCCAtGaattcGGGCCAGAGGTC-3′ (mutant GC-1) and 5′-CCCTCATTTCACtgcagtCCCG-GCTTGGGG-3′ (mutant GC-2). The mutated nucleotide sequences of all mutant constructs were confirmed by DNA sequencing.

Transient Transfection and Reporter Assays

Transient transfection was performed in 3×10^5 NCCIT cells in 60-mm cell culture dishes by using the ExGen500 in vitro Transfection Reagent (Fermentas, Hanover, MD) according to the manufacturer's protocols. 3.4 μg plasmid DNA in total was used per dish. To measure luciferase activity, the plates were rinsed with phosphate-buffered saline and the cells were harvested 48 h after transfection. The cells were lysed with passive lysis buffer (Promega) and the lysates were collected by centrifugation at 4°C for 15 min at 12,000*g*. One hundred microliters of supernatant was then used for reporter assays employing the Bright-Glo Luciferase Assay System (Promega) and a Genios luminometer (TECAN, Salzburg, Austria). The luciferase activity from the reporter plasmid was normalized relative to the β-galactocidase activity of the co-transfected internal control plasmid, pcDNA3.1/Hygro/LacZ (Invitrogen).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of NCCIT and Jurkat cells were isolated by the method of Andrew and Faller [1991]. Binding reactions were performed for 20 min on ice with 8 μg of total nuclear protein in 15 μl of 10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 10% glycerol, 1 μg of poly(dI-dC), 1 mM dithiothreitol, 1 nM phenylmethylsulfonyl fluoride, and 20,000 cpm of ^{32}P-labeled oligonucleotide. The DNA–protein complexes were separated from the free probe

TABLE I. Primer Sequences Used for PCR Cloning

Designation	Sequence
−2601	5′-CGCCTCGAGAGGATGGCAAGCTGAGAAACAC-3′
−1*	5′-TATAAGCTTGGGGAAGGAAGGCGCCCCAAG-3′
−2329 (D1)	5′-TCCCTCGAGCTTGTAGACCTTCCGGCAGAC-3′
−1844 (D2)	5′-CTGCTCGAGTGAGCCTCAGGATACTCAGGC-3′
−1588 (D3)	5′-GGACTCGAGGAGGCTCAGTCTTTGAGGGGA-3′
−1019 (D4)	5′-CGGCTCGAGGGAGTCTGATTCTGGAAGACG-3′
−380 (D5)	5′-GGCCTCGAGAGGATTGCTTTGGCCCAGTAG-3′
−130 (D6)	5′-TTACTCGAGCCTCTCCTCCACCCATCCAGG-3′
Δ130 (D7)*	5′-TATAAGCTTGATGGGTGGAGGAGAGGGAGG-3′
−1423 (D3-1)	5′-AATCTCGAGGGTTAGAGCTGCCCCCTCTGG-3′
−1291 (D3-2)	5′-TGACTCGAGTTTTCCCCACCCAGGGCCTAG-3′
−1111 (D3-3)	5′-ATTCTCGAGGGTTGGGAAACTGAGGCCCAG-3′
−946 (D4-1)	5′-GCACTCGAGGCAGTCTACTCTTGAAGATGG-3′
−871 (D4-2)	5′-ATTCTCGAGCTGGATTTGGCCAGTATCGGG-3′
−782 (D4-3)	5′-ATTCTCGAGGGCTGTTGATGCATTGAGGGA-3′
−700 (D4-4)	5′-ATTCTCGAGCACAGTGCCAGAGGTCTGTGG-3′
−634 (D4-5)	5′-ATTCTCGAGGAGTGATTCCAGACAGCTGGG-3′
−548 (D4-6)	5′-CACCTCGAGCGCCTTTAATCATGACACTGG-3′
−484 (D4-7)	5′-ACACTCGAGAGCCTGGGTAACATAGCAAGG-3′

The asterisks indicate reverse primers for PCR cloning and underlines are restriction site (XhoI and HindIII) introduced at the 5′ ends of primer for subcloning.

on a native 5% polyacrylamide gel. The gels were vacuum-dried and visualized by autoradiography. The oligonucleotide sequences used as EMSA probes are as follows: 5′-CATCCA-GGGGGCGGGGCCAG-3′ (GC-1), 5′-CATCCAt-GaattcGGGCCAG-3′ (mutant GC-1). The mutated sequences are shown in lower case.

Statistical Analysis

The data for promoter analysis were analyzed by ANOVA using General Linear Models procedures of the Statistical Analysis System (SAS Institute, Gary, NC). Means were compared by Duncan's multiple range procedure for multiple comparisons. In all experiments, $P < 0.05$ was considered statistically significant.

RESULTS

Transcriptional Activity of the Deletion Mutants of the hOct4 Promoter Upstream Region

It has been recently reported in human, bovine, and murine genomes that Oct4 5′ upstream regions contain four conserved regions denoted as CR1-4 [Nordhoff et al., 2001]. The human 5′ upstream region is depicted schematically in Figure 1A. To identify the cis-acting elements that regulate the transcription of *Oct4* gene, a series of deleted fragments of the 5′ upstream region from the translation start were cloned into the promoter-less luciferase reporter vector pGL3-basic. The lengths of the deletion constructs from the 5′ and 3′ ends of the Oct4 promoter were based on the CR1-4 containing putative enhancer sequences (Fig. 1A). Each deletion construct was transiently transfected into NCCIT cells along with the pcDNA3.1/Hygro/LacZ plasmid to normalize transfection efficiency for a reporter assay (Fig. 1B). The entire −2601 fragment showed about 350-fold higher promoter activity than the empty vector. Deletion of the CR4 that contains a putative DE (the D1 mutant) reduced the promoter activity by half. The additional deletions of CR3 and CR2, which each contains a putative PE (the D2, D3, and D4 mutants),

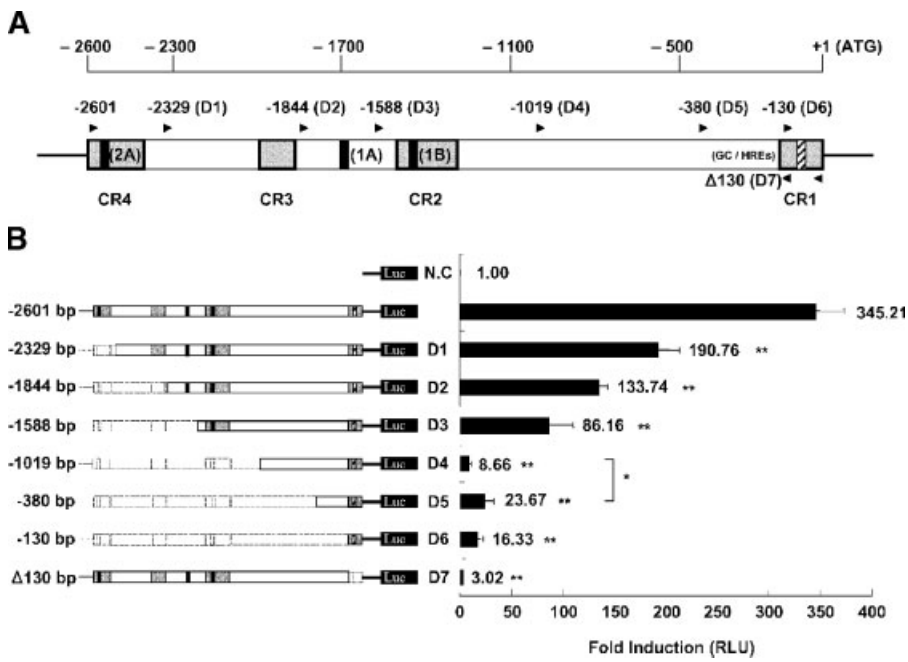

Fig. 1. Effect of deleting parts of the hOct4 promoter on its transcriptional activity. **A**: Schematic depiction of the hOct4 5′ upstream region. The nucleotide numbers represent the distances from the translation start site (+1). Each conserved region (CR1-4) is bolded and boxed. The dark and hatched boxes indicate putative enhancer regions and the GC box/HREs, respectively. The locations of the primers used to construct the deletion mutants are depicted by arrowheads. **B**: The plasmids containing the luciferase gene flanked with various lengths of the hOct4 5′ upstream region (3 μg) were transfected into the human EC cell line NCCIT grown on 60-mm dishes, as described in the ''Materials and Methods.'' The transfection efficiency was normalized relative to the β-galactosidase activity obtained by co-transfecting pcDNA3.1/hyg/LacZ (0.4 μg). The luciferase activity of the 5′ upstream regions is shown as fold induction relative to that of the empty pGL3-basic plasmid. The data shown are the mean and SEM from three independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity of the deletion constructs to that of −2601/−1-Luc construct. $^{**}P < 0.01$, $^{*}P < 0.05$. The dot-lines indicate deleted promoter regions.

significantly reduced the Oct4 promoter activity further, indicating that the CR2, 3 and 4 sequences bearing putative PEs and a DE act as positive regulators. However, further deletion of the $-1019 \sim -380$ internal region between CR2 and CR1 (the D5 mutant) caused the promoter activity to recover by up to threefold, which suggests that this region may contain a negative cis-acting element(s) that regulates transcription activity. Continued 5′ end deletion showed that the $-380/-1$ region that includes CR1 possesses the minimal promoter activity. However, deletion of the -130 bp from the 3′ end of the Oct4 promoter that contains the GC box and the HREs (the D7 mutant) almost abolished this promoter activity, which suggests that the 130 bp upstream from the translation start site may be important for the transcription of the *hOct4* gene.

To clarify the existence of the closely located positive and negative cis-regulatory elements between -1588 and -380 bp of the Oct4 promoter region, a series of ten additional deletion constructs between the D3 and the D5 mutants were generated (Fig. 2a), and transient transfection and luciferase assay were carried out in NCCIT cells (Fig. 2b). The deletion of the putative PE 1B sequence (the D3-1 mutant) significantly decreased the promoter activity compared to the -1588 bp region of the D3 mutant and further deletions had no effect on the decreased hOct4 promoter activity. However, the decreased promoter activity was restored when the region between -548 and -380 bp was deleted (the D4–6 and D5 mutants), suggesting that this region may play a role in the negative regulation of *Oct4* gene transcription.

One of the GC Boxes Is Critical for the Transcriptional Activity of the Oct4 Minimal Promoter

To identify critical *cis*-acting elements in the PP region that shows minimal promoter activity, we aligned the sequences of the human and murine Oct4 promoters that contain the PP region and found that two GC boxes ($-119\sim -110$ bp; GC-1, $-33\sim -25$ bp; GC-2 in hOct4 CR1) and HREs, which overlap with chicken ovalbumin upstream promoter-transcription factor-binding sites are completely conserved

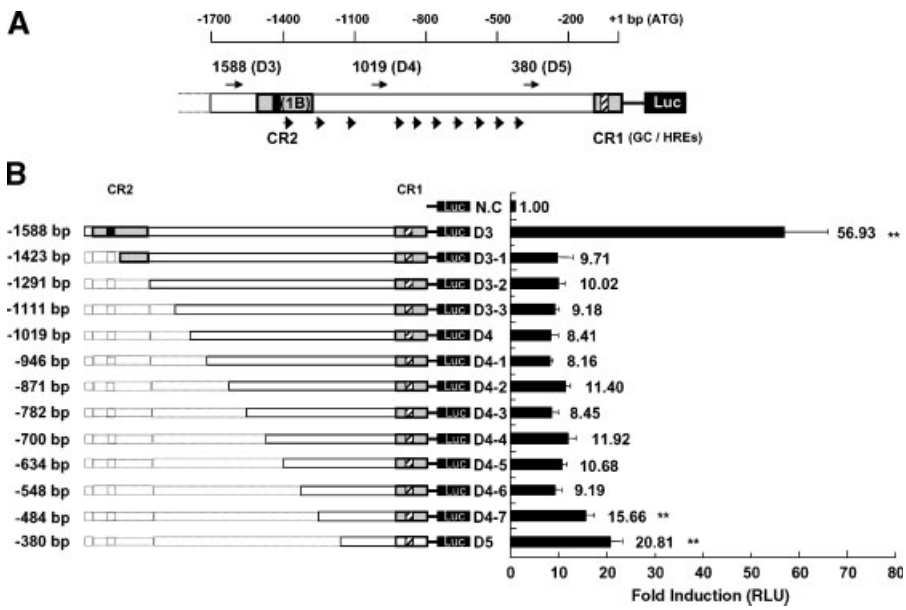

Fig. 2. Mutational analysis of the hOct4 5′ upstream region between D3 and D5. **A**: The schematic structure of the D3 deletion mutant construct. **B**: The luciferase activity of the mutant constructs examining the region between D3 and D5. The primers used to construct the previous deletion mutants (the D3, D4, and D5 mutants) are indicated by arrows while the oligonucleotide primer sets used to construct the additional deletion mutants are indicated by arrowheads. The dark and hatched boxes indicate putative enhancer regions and the GC box/HREs, respectively and the dot-lines indicate deleted promoter regions. The plasmids containing the luciferase gene flanked by each mutant construct (3 μg) were co-transfected into NCCIT cells grown on 60-mm dishes along with pcDNA3.1/hyg/LacZ (0.4 μg). The luciferase activity of the 5′ upstream regions is shown as fold induction relative to that of the empty pGL3-basic plasmid. The data shown are the mean and SEM from three independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity of the deletion constructs to that of D3-1 construct. $^{**}P < 0.01$.

in both the human and murine Oct4 proximal promoter regions (Fig. 3A). The two GC boxes within CR1 were disrupted by site-directed mutagenesis to test the role the GC boxes play in sustaining the minimal promoter activity. The minimal promoter activities of the wild type and GC box mutants are shown in Figure 3B. The mutants of GC-1 or both GC-1 and GC-2 showed significant loss of promoter activity, while mutation of GC-2 only slightly reduced the promoter activity. Thus, the loss of promoter activity by the disruption of the GC-1 within Oct4 proximal promoter implies GC-1 may play a critical role in Oct4 expression.

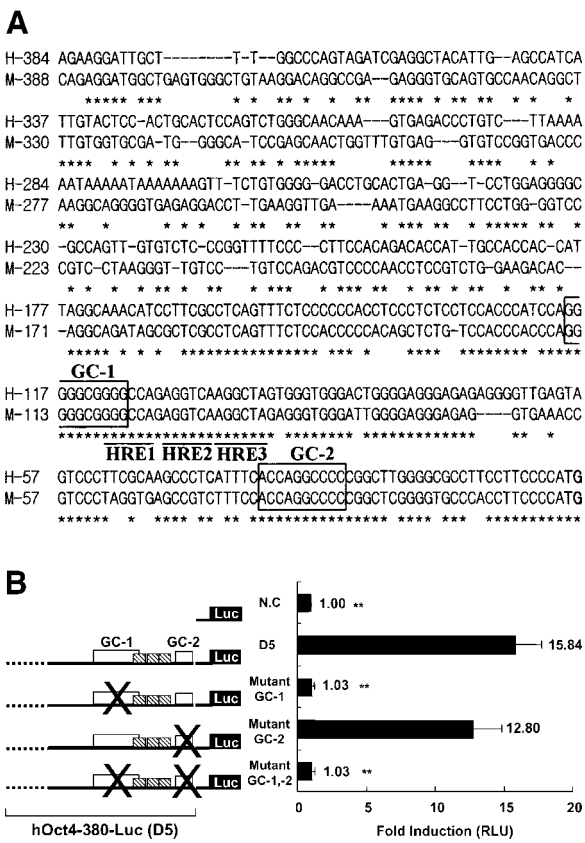

Fig. 3. The GC box is critical for the transcriptional activity of the Oct4 minimal promoter. **A**: Sequence alignment of the human and murine Oct4 promoter CR1 regions. Putative GC boxes are indicated by white boxes and three HREs are underlined. The bold letters indicate the translation start site. **B**: The diagram depicts the location of the mentioned major cis-regulatory elements in the hOct4 proximal promoter. The GC boxes are indicated by white boxes while the HREs are indicated by hatched boxes. X indicates the disruption of the transcription factor-binding site by site-directed mutagenesis. The plasmids containing the luciferase gene flanked by each construct (3 μg) were co-transfected into NCCIT cells grown on 60-mm dishes along with pcDNA3.1/hyg/LacZ (0.4 μg) and luciferase assays were performed. The luciferase activity of the 5′ upstream regions is shown as fold induction relative to that of the empty pGL3-basic plasmid. The data shown are the mean and SEM from three independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity of the deletion constructs to that of D5. $^{**}P < 0.01$.

Protein-Binding Activity in the GC-1 of hOct4 Promoter

To determine whether the nuclear proteins can bind to the GC-1, an oligonucleotide designed to span the GC-1 sequence was synthesized and EMSA was carried out. When the nuclear extract from NCCIT cells was added to the ^{32}P-labeled GC-1 probe, five protein–DNA complexes denoted C1∼C5 were observed (Fig. 4A). To confirm the sequence specificity of this protein–DNA complex formation, a labeled mutated GC-1 oligonucleotide was used as a probe (lane 3) or competition experiments using a 100-fold molar excess of unlabeled GC-1 probe were performed (lane 4). Whereas no complexes were observed when the labeled mutant GC-1 probe or the excess unlabeled GC-1 probe were used, the use of a 100-fold molar excess of unlabeled mutant GC-1 probe did not alter the binding activity of the nuclear proteins (lane 5). Thus, multiple protein–DNA complexes were formed in a GC-1 sequence-specific manner. Notably, a similar pattern of protein–DNA complexes was also formed when the commercial Sp1-binding oligonucleotide (Santa cruz) was used as a probe (Fig. 4A, lane 6), or when a Jurkat cell nuclear extract, which contains the Sp family transcription factors [Halim et al., 2001], was used as a protein pool (Fig. 4A, lane 7). This suggests that Sp family transcription factors may be present in the DNA–protein complexes formed between the GC-1 oligonucleotide and nuclear extracts prepared from NCCIT cells.

To further investigate the identities of the proteins that bind to the Oct4 GC-1, we performed supershift experiment with different Sp protein-specific antibodies (Fig. 4B). The Sp1-specific antibody supershifted the C1 complex (lane 4) while the Sp3-specific antibody supershifted C2 complex (lane 5). Thus, the Sp1 and Sp3 transcription factors are present in the NCCIT nuclear extract and can directly interact with the hOct4 promoter element, GC-1.

Effect of Sp1 and Sp3 on hOct4 Promoter Activity

To determine the effect of the Sp1 and Sp3 transcription factors on Oct4 promoter activity,

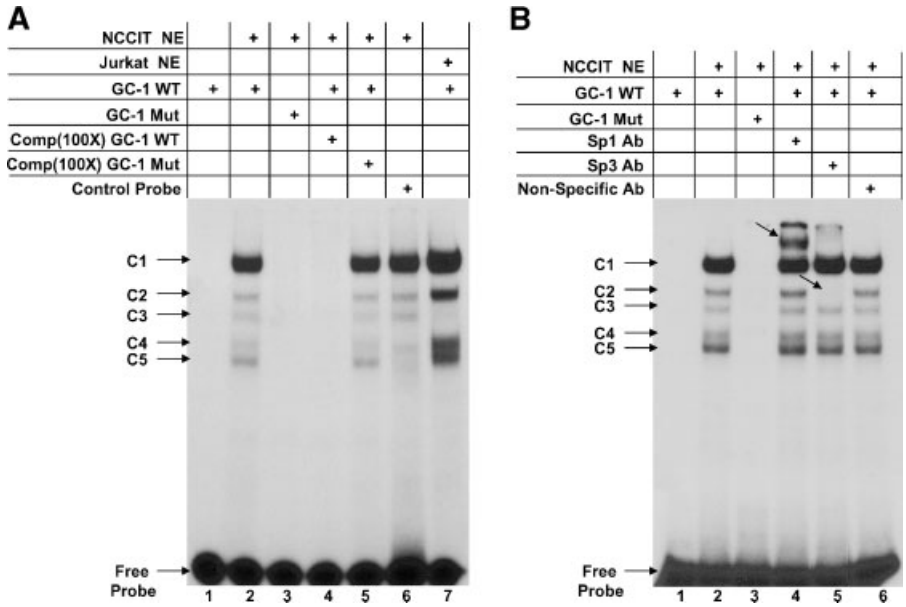

Fig. 4. Binding of nuclear proteins from NCCIT cells to the GC-1 in hOct4 proximal promoter. **A**: The ^{32}P-radiolabeled 20 bp oligonucleotides GC-1 WT (**lane 2**), GC-1 mutant (**lane 3**), or Control probe (Santa Cruz Sp1 oligo) (**lane 6**) were incubated with a nuclear extract of NCCIT cells (lanes 2–6) or Jurkat cells (**lane 7**) with or without a 100-fold molar excess of the unlabeled GC-1WT (**lane 4**) or GC-1 mutant (**lane 5**) probe as a competitor. Protein–DNA complexes (C1–C5) were indicated by the arrows. **B**: An EMSA supershift experiment was performed using 1 μg polyclonal rabbit antibodies specific for Sp1 (**lane 4**) or Sp3 (**lane 5**) or nonspecific polyclonal rabbit antibody (**lane 6**). The supershifted bands are indicated by diagonal arrows (lanes 4 and 5).

transient transfection and reporter assays were performed using the −2601-luc and −380-luc constructs. As shown in Figure 5, while Sp3 was slightly repressive, Sp1 enhanced the activities of the hOct4 −2601-luc and −380-luc reporters. Although the trans-activational activity of Sp1 or Sp3 was slightly less with regard to the −380-luc construct compared to the −2601-luc construct, the effect (patterns) of trans-activational activities of Sp1 and Sp3 on the −2601-luc and −380-luc constructs did not differ. Taken together, the binding of Sp1 and Sp3 to Oct4

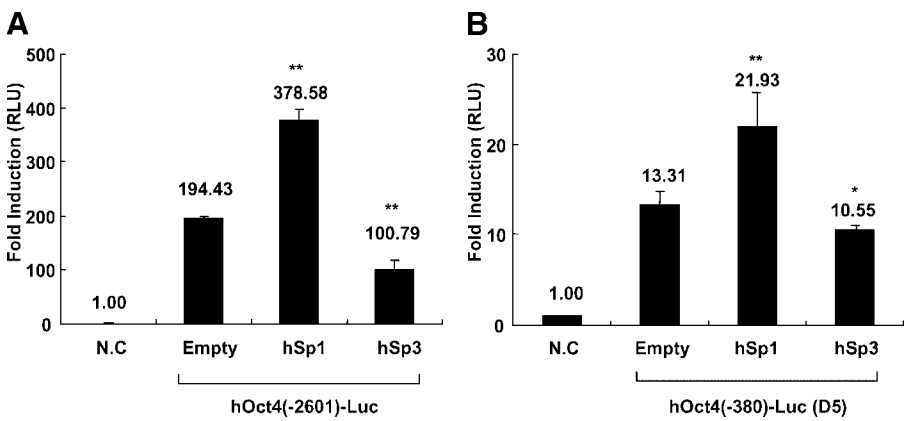

Fig. 5. Effect of Sp1 and Sp3 on hOct4 transcription activity in NCCIT cells. The hOct4 promoter constructs −2601-luc (**A**) and D5 (**B**) (1.5 μg) were co-transfected into NCCIT cells along with 1.5 μg of Sp1 and Sp3 expression vectors and luciferase activity was measured. Transfection efficiency was normalized according to the β-galactosidase activity obtained by co-transfecting pcDNA3.1/hyg/LacZ (0.4 μg). The fold induction relative to the luciferase activity of empty pGL3-basic plasmid is shown. The data shown are the mean and SEM from five independent experiments with duplicates for each experiment. Statistical analysis was performed comparing the activity obtained from co-transfection with Sp family expression vector to that of empty vector. $^{**}P < 0.01$, $^{*}P < 0.05$.

GC-1 and the ability of transiently overexpressed Sp1 and Sp3 to trans-activate hOct4 promoter constructs suggest that these transcription factors may play key roles as major transcriptional regulators of hOct4 gene expression.

DISCUSSION

The pluripotent and fetal cell type-specific expression pattern of Oct4 may be regulated by specific mechanisms. The presence of conserved elements in the Oct4 promoter of different species further suggests that this mechanism may involve similar regulatory elements and factors. Several studies have characterized the cis-regulatory elements in the murine Oct4 upstream region. These elements include the TATA-less minimal promoter containing the GC box [Okazawa et al., 1991] and two enhancer elements [Yeom et al., 1996]. In this study, we identified the regions involved in regulating human Oct4 transcription and demonstrated that multiple cis-acting elements, including putative enhancer regions, act positively in Oct4 promoter activity. In addition, we revealed for the first time that the region between −548 and −380 bp may act as a negative regulatory element for Oct4 transcription and that the promoter region between nucleotides −380 and −1 containing GC boxes and HREs is sufficient to provide minimal promoter activity in NCCIT cells. Moreover, site-directed mutagenesis and EMSA suggested that GC-1 plays a critical role in Oct4 transcription, and overexpression studies demonstrated that Sp1 and Sp3 can regulate Oct4 promoter activity positively and negatively, respectively, by binding to GC-1.

It has been recently reported that Oct4 5′ upstream regions contain four conserved regions (CR1-4) on the basis of homologous sequences between human, bovine and murine *Oct4* genes [Nordhoff et al., 2001]. Since the Oct4 5′ upstream enhancer regions have been shown to be important for murine and bovine Oct4 transcription [Yeom et al., 1996; van Eijk et al., 1999], we investigated the function of the hOct4 5′ upstream enhancer region by introducing a series of deletion mutants into NCCIT cells (Figs. 1 and 2). The decrease of hOct4 promoter activity caused by the loss of each conserved region suggested that each conserved region, which harbor putative enhancer elements (the DE 2A in CR4 and the PEs 1A, 1B in CR2), may be important in Oct4 transcription activity. Furthermore, we identified a putative negative regulatory element within the 5′ upstream region between nucleotides −548/−380 (Fig. 2). Although the precise molecular mechanism behind this sequence-specific repression is still unknown, the fact that this region shows less sequence conservation than the other conserved regions [Nordhoff et al., 2001] suggests the possibility of human-specific mechanisms that repress hOct4 activity; these mechanisms may operate along with other known Oct4 transcription-repressive mechanisms in the murine, such as those involving germ cell nuclear factor or RA-mediated mechanisms [Schoorlemmer et al., 1994; Minucci et al., 1996].

The human Oct4 proximal promoter has typical TATA-less and GC-rich sequences whose G + C contents are approximately 68% [Nordhoff et al., 2001]. These sequences consist of GC-1 (5′-GGGGCGGGG-3′), GC-2 (5′-CCAGGCCC C-3′), and repeated half-HREs (5′-AGGTCA-3′) (Fig. 3A). Generally, GC-rich promoters are known to interact with zinc finger transcription factors; one of these zinc finger transcription factors, Sp1, is closely related to TATA-less promoter activity [Emami et al., 1998]. Site-directed mutagenesis experiments indicate that GC-1 plays an essential role in Oct4 promoter activity, whereas GC-2 has only minor effects (Fig. 3B). EMSA experiments showed that the GC boxes, especially GC-1, bind to various nuclear proteins in NCCIT cells (Fig. 4A). It has been shown that in mouse ES cells, the transcription factors Sp1 and Sp3 can bind to GC-1 in the Oct4 proximal promoter [Pesce et al., 1999]. Since the human and mouse GC-1 sequences share 100% identity, supershift experiments using Sp1/Sp3-specific antibodies were performed to identify the GC-1-bound nuclear proteins. This revealed that Sp1 and Sp3 can bind to human Oct4 GC-1 (Fig. 4B) and suggested the trans-activational effects of Sp1 and Sp3 in hOct4 transcription (Fig. 5). However, sequence alignment analysis (Fig. 3A) revealed that apart from the −130/−1 sequence that contains the completely conserved GC box, hOct4 minimal promoter sequence possessed different putative transcription factor-binding sites compared to the equivalent murine sequence. This also suggests that some of the mechanisms regulating hOct4 expression differ from those controlling murine Oct4 expression.

The transcription factors, Sp1 and Sp3 belong to the Sp family of transcription factors, which are characterized by three conserved zinc fingers that form a DNA-binding domain and recognize GC boxes [Philipsen and Suske, 1999] show the most homology to each other with, regard to their structure and DNA-binding affinity. They also have been shown to have additive [Ihn and Trojanowska, 1997], synergistic [Bigger et al., 1997] and reciprocally repressive [Majello et al., 1997; Yu et al., 1999] effects on the activity of specific promoters. Whereas the studies on GC box in Oct4 proximal promoter have been reported in murine ES and EC cells, the transcription factors interacting with the GC box are little known. It has been only demonstrated that in $Sp1^{-/-}$ mouse ES cells, Sp1 acts as a mild activator of Oct4 promoter and suggested the functional loss of Sp1 may be compensated by Sp3 [Pesce et al., 1999]. To examine the effect of Sp1 and Sp3 on hOct4 promoter transcription activity, co-transfection and reporter assays were performed. They revealed that Sp1 and Sp3 regulate hOct4 transcription in opposite ways , and human EC, NCCIT cells maybe provide the circumstance for the opposite function of Sp1 and Sp3 (Fig. 5).

Multiple regulatory elements existing in the Oct4 promoter upstream region [Nordhoff et al., 2001] suggest that a multi-channel regulatory mechanism may participate in *Oct4* gene regulation. Thus, not only the promoter itself but also the cellular context may regulate the expression of Oct4. Our results strongly support the notion that the Oct4 GC box and the Sp family transcription factors play an important role in hOct4 transcription. In addition, these results suggest that the mechanism regulating hOct4 expression is probably a very complicated event cascade that involves, both cis-regulatory elements and trans-activators.

In conclusion, we examined for the first time the roles the conserved regions within the 5′ upstream sequence of hOct4 play in regulating transcription. We identified a putative negative cis-regulatory element in the 5′ upstream sequence and discovered the central role of GC-1 box in the minimal promoter activity of hOct4. Moreover, the GC box, a cis-regulatory element, can be bound by Sp1 and Sp3, which act as positive and negative regulators, respectively. Further studies analyzing the molecular mechanism involving the putative positive and negative cis-elements in the hOct4 5′ upstream sequence are currently in progress. In addition, the remaining unknown transcription factors that bind to the GC box and the importance of the HREs in hOct4 promoter activity remain to be determined. Our observations will provide the basis for understanding the global regulation mechanism of hOct4 expression in not only hEC but also hES cells.

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REFERENCES

Andrew NC, Faller DV. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 20:5519–5525.

Ben Shushan E, Pikarsky F, Klar A, Bergman Y. 1993. Extinction of Oct-3/4 gene expression in embryonal carcinoma X fibroblast somatic cell hybrids is accompanied by changes in the methylation status, chromatin structure, and transcriptional activity of the Oct-3/4 upstream region. Mol Cell Biol 13:891–901.

Ben Shushan E, Sharir H, Pikarsky F, Bergman Y. 1995. A dynamic balance between ARP-1/COUP-TFII, EAR-3/Coup-TFI, and retinoic acid receptor:retinoid X receptor heterodimers regulates Oct3/4 expression in embryonal carcinoma cells. Mol Cell Biol 15:1034–1048.

Bigger CB, Melnikova IN, Gardner PD. 1997. Sp1 and Sp3 regulate expression of the neuronal nicotinic acetylcholine receptor beta4 subunit gene. J Biol Chem 272: 25976–25982.

Emami KH, Burke TW, Smale ST. 1998. Sp1 activation of a TATA-less promoter requires a species-specific interaction involving transcription factor IID. Nucleic Acids Res 26:839–846.

Fuhrmann G, Chung AC, Jackson KJ, Hummelke G, Baniahmad A, Sutter J, Sylvester I, Schöler HR, Cooney AJ. 2001. Mouse germline restriction of *Oct4* expression by germ cell nuclear factor. Dev Cell 1:377–387.

Gidekel S, Bergman Y. 2002. A unique developmental pattern of *Oct*-3/4 DNA methylation is controlled by a *cis*-demodification element. J Biol Chem 277:34521–34530.

Halim A, LeGros HL, Chamberlin ME, Geller A, Kotb M. 2001. Distint patterns of protein binding to the MAT2A promoter in normal and leukemia T cells. Biochim Biophys Acta 1540:32–42.

Ihn H, Trojanowska M. 1997. Sp3 is a transcriptional activator of the human alpha2(I) collagen gene. Nucleic Acids Res 25:3712–3717.

Majello B, De Luca P, Lania L. 1997. Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains. J Biol Chem 272: 4021–4026.

Minucci S, Botquin V, Yeom YI, Dey A, Sylvester I, Zand DJ, Ohbo K, Ozato K, Schöler HR. 1996. Retinoic acid-mediated down-regulation of Oct3/4 coincides with the

loss of promoter occupancy in vivo. EMBO J 15:888–899.

Niwa H, Miyazaki J-I, Smith AG. 2000. Quantitative expression of oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24:372–376.

Nordhoff V, Hübner K, Bauer A, Orlova I, Malapetsa A, Schöler HR. 2001. Comparative analysis of human, bovine and murine Oct-4 upstream promoter sequences. Mammal Genome 12:309–317.

Okazawa H, Okamoto K, Ishino F, Ishino-Kaneko T, Takeda S, Toyoda Y, Muramatsu M, Hamada H. 1991. The *oct3* gene, a gene for an embryonic transcription factor, is controlled by a retinoic acid repressible enhancer. EMBO J 10:2997–3005.

Pesce M, Marin Gomez M, Philipsen S, Schöler HR. 1999. Binding of Sp1 and Sp3 transcription factors to the Oct-4 gene promoter. Cell Mol Biol 45:709–716.

Philipsen S, Suske G. 1999. A tale of three fingers: The family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res 27:2991–3000.

Pikarsky E, Sharir H, Ben-Shushan E, Bergman Y. 1994. Retinoic acid represses Oct-3/4 gene expression through several retinoic acid-responsive elements located in the promoter-enhancer region. Mol Cell Biol 14:1026–1038.

Schoorlemmer J, van Puijenbroek A, van Den Eijnden M, Jonk L, Pals C, Kruijer W. 1994. Characterization of a negative retinoic acid response element in the murine *Oct4* promoter. Mol Cell Biol 14:1122–1136.

Schöler HR, Dressler GR, Balling R, Rohdewohld H, Gruss P. 1990. Oct-4: A germline-specific transcription factor mapping to the mouse t-complex. EMBO J 9:2185–2195.

Sylvester I, Schöler HR. 1994. Regulation of the *Oct-4* gene by nuclear receptors. Nucleic Acids Res 22:901–911.

Takeda J, Seino S, Bell GI. 1992. Human Oct3 gene family: cDNA sequences, alternative splicing, gene organization, chromosomal location, and expression at low levels in adult tissue. Nucleic Acids Res 20:4613–4620.

Udvadia AJ, Rogers KT, Higgins PDR, Murata Y, Martin KH, Humphrey PA, Horowitz JM. 1993. Sp-1 binds promoter elements regulated by the RB protein and Sp-1 mediated transcription is stimulated by RB coexpression. Proc Natl Acad Sci 90:3265–3269.

Udvadia AJ, templeton DJ, Horowitz JM. 1995. Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: Superactivation by Rb requires amino acids necessary for growth suppression. Proc Natl Acad Sci 92:3953–3957.

van Eijk MJ, van Rooijen MA, Modina S, Scesi L, Folkers G. 1999. Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. Biol Reprod 60: 1093–1103.

Verrijzer CP, van der Vliet PC. 1993. POU domain transcription factors. Biochim Biophys Acta 1173:1–21.

Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hübner K, Schöler HR. 1996. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122:881–894.

Yu JH, Schwartzbauer G, Kazlman A, Menon RK. 1999. Role of the Sp family of transcription factors in the ontogeny of growth hormone receptor gene expression. J Biol Chem 274:34327–34336.