The Human OCT-4 Isoforms Differ in Their Ability to Confer Self-renewal*

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OCT-4 transcription factors play an important role in maintaining the pluripotent state of embryonic stem cells and may prevent expression of genes activated during differentiation. Human OCT-4 isoform mRNAs encode proteins that have identical POU DNA binding domains and C-terminal domains but differ in their N-terminal domains. We report here the cloning and characterization of the human OCT-4B isoform. Human OCT-4B cDNA encodes a 265-amino acid protein with a predicted molecular mass of 30 kDa. Embryonic stem (ES) cell-based complementation assays using ZHBTc4 ES cells showed that unlike human OCT-4A, OCT-4B cannot sustain ES cell self-renewal. In addition, OCT-4B does not bind to a probe carrying the OCT-4 consensus binding sequence, and we demonstrate that two separate regions of its N-terminal domain are responsible for inhibiting DNA binding. We also demonstrate that OCT-4B is mainly localized to the cytoplasm. Overexpression of OCT-4B did not activate transcription from OCT-4-dependent promoters, although OCT-4A did as reported previously. Furthermore, transcriptional activation by human OCT-4A was not inhibited by co-expression of OCT-4B. Taken together, these data suggest that the DNA binding, transactivation, and abilities to confer self-renewal of the human OCT-4 isoforms differ.

The oct-4 gene, also referred to as oct-3, encodes a nuclear protein that belongs to a family of transcription factors containing the POU DNA binding domain (1–6). It is normally found in the pluripotent stem cells of pregastrulation embryos, including oocytes, early cleavage-stage embryos, and the inner cell mass of the blastocyst (1, 3, 7, 8). Its expression is downregulated during differentiation, and knock-out of oct-4 causes early lethality in mice because of the absence of an inner cell mass (9). These results suggest that OCT-4 plays a pivotal role in mammalian development (10) and in the self-renewal of embryonic stem cells (11). During human development, OCT-4 is expressed at least until the blastocyst stage where it regulates gene expression (12).

OCT-4 is a transcriptional regulator of genes involved in maintaining the undifferentiated pluripotent state and may also prevent expression of genes activated during differentiation (13). It activates transcription via octamer motifs located proximal or distal to transcriptional start sites (14). The POU domain of OCT-4 is a conserved DNA binding domain that binds as a monomer to the octamer sequence motif 5’-ATG-CAAAT-3’ (15). This cis-acting element is important in controlling the activity of many promoters and enhancers of housekeeping and cell type-specific genes (16). OCT-4-binding sites have been found in various genes, including jgf-4 (fibroblast growth factor-4), pdgfl (platelet-derived growth factor-α receptor), osteopontin, and Nanog (17–21). In addition, genes expressed in the trophectoderm but not in the embryo prior to blastocyst formation, such as IFN-γ (γ-interferon) and the α and β subunits of chorionic gonadotropin, may be targets for silencing by OCT-4 (22–24). This suggests that OCT-4 functions as a master switch during differentiation by regulating cells that have pluripotent potential or can develop such potential (25, 26).

Transcriptional regulation by OCT-4 is complex. In embryonic stem cells, the octamer sequence motif is active irrespective of its distance from the site of transcriptional initiation (2, 28). However, in differentiated cells, OCT-4 can transactivate only when the octamer motif is in a proximal position (1, 13, 29); to be active from distal sites, it requires stem cell-specific bridging factors that link it to the transcription initiation site (29). A number of factors such as Sox2, high mobility group, E7, and EIA are known to influence the ability of OCT-4 to act as activator or repressor (15, 29–32). Recently, physical association of OCT-4 with Ewing’s sarcoma protein was documented, suggesting that Ewing’s sarcoma protein may also play a role in regulating OCT-4 (35).

Although only a single form of OCT-4 mRNA has been identified in embryonic mouse tissues, two forms, i.e. OCT-4A and OCT-4B, generated by alternative splicing, were identified in the RT3-PCR products from adult human pancreatic islets (36). Compared with human OCT-4A, little is known about the properties of OCT-4B. To identify the biochemical functions of

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The abbreviations used are: RT, reverse transcription; ES, embryonic stem cells; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CTD, C-terminal domains; NTD, N-terminal domains; NLS, nuclear localization signal; GST, glutathione S-transferase.
the human OCT-4B isoform, we performed RT-PCR and sequenced OCT-4 cDNAs from human ES cells. This revealed a novel alternative spliced variant of OCT-4 mRNA in which exon 1a is replaced by exon 1b. The DNA binding and C-terminal domains of OCT-4B are identical to the corresponding domains of OCT-4A, but it lacks the sequences necessary for transactivation. Moreover, it does not bind DNA and mainly localizes to the cytoplasm. We also found that, unlike OCT-4A (35), it cannot stimulate transcription from OCT-4-dependent promoters, nor does it antagonize the induction of gene expression by OCT-4A. In addition, ectopic expression of human OCT-4B in ZHBTc4 ES cells, unlike that of human OCT-4A, was not sufficient to maintain stem cell self-renewal and permit them to display differentiated ES cell phenotypes. These data imply that the DNA binding and transactivation properties of the human OCT-4 isoforms and their abilities to confer self-renewal differ. Thus, the different polypeptides encoded by the human OCT-4 gene may have different targets as well as different roles as regulators of human ES cells.

**EXPERIMENTAL PROCEDURES**

Materials and General Methods—Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs. 

Pfu Turbo polymerase was purchased from Stratagene, and [γ-32P]ATP (3000 Ci/mmol) was obtained from PerkinElmer Life Sciences. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations, and SDS-PAGE of proteins were carried out by standard methods (37). Sub-clones generated from PCR products were sequenced by the chain termination method with double-stranded DNA templates to ensure the absence of mutations.

Plasmid Construction—To construct the human OCT-4 isoforms, total RNA was prepared from human ES cells, and cDNA was synthesized using the Superscript™ first-strand synthesis system (Invitrogen), as described previously (35). The construction of pcDNA3-hOCT-4A has been described previously (35). To construct pcDNA3-OCT-4B, the human ES cell-derived cDNAs were amplified with primer 5'-hoCT4B-1 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4B-265 (5'-GATCGAATTCTCAGTTTGAATGCGATATGG-3', an AgeI site is underlined) and cloned into the same sites of pEGFP N1 to generate pEGFP-hOCT-4A. This in turn was digested with SalI and NotI and cloned into the Xhol and NotI sites of vector pcDNA3-IP.

For pCAG-IP/mOCT-4-EGFP, human OCT-4B was amplified from pcDNA3-mOCT-4B by PCR using primers 5'-hoCT4B-1 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4B-351 (5'-GATCGGATCCATGCACTTCTACAGACTATGGG-3', an AgeI site is underlined), digested with BamHI and AgeI, and cloned into the same sites of pEGFP N1 to generate pEGFP-mOCT-4. This was then digested with Xhol and NotI and cloned into the same sites of vector pCAG-IP. (c) For pCAG-IP/hOCT-4A-EGFP, human OCT-4A was amplified from pcDNA3-hOCT-4A by PCR using primers 5'-hoCT4A-1 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4A-351, digested with BamHI and AgeI, and cloned into the same sites of pEGFP N1 to generate pEGFP-hOCT-4A.

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GST fusion OCT-4A and GST fusion OCT-4B, and their derivative plasmids GST-OCT-4A(POU), GST-OCT-4B(POU), GST-OCT-4B(NPB), GST-OCT-4B(PBC), GST-OCT-4A(NPB), GST-OCT-4B(NPB), GST-OCT-4A(NPB), GST-OCT-4B(NPB)Δ(21–40), and GST-OCT-4B(NPB)Δ(1–20), were generated by the following steps. (a) For GST-OCT-4A, plasmid pcDNA3/OCT-4A was digested with BamHI and EcoRI and cloned into the same sites of pGEX (4T-1) vector (Amersham Biosciences). (b) For GST-OCT-4B, plasmid pcDNA3/OCT-4B was digested with BamHI and EcoRI and cloned into the same sites of pGEX (4T-1). (c) For GST-OCT-4A (POU), the POU domain of OCT-4A was amplified from pcDNA3/OCT-4A by PCR using primers 5'-hoCT4A-134 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4A-289 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', an EcoRI site is underlined), digested with BamHI and EcoRI, and cloned into the same sites of pGEX (4T-1). (d) For GST-OCT-4B (POU), the POU domain of OCT-4B was amplified from pcDNA3/OCT-4B by PCR using primers 5'-hoCT4B-41 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4B-351, digested with BamHI and EcoRI, and cloned into the same sites of pGEX (4T-1). (e) For GST-OCT-4B (POU), the N-terminal and POU domain of OCT-4B was amplified from pcDNA3/OCT-4B by PCR using primers 5'-hoCT4B-1 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4B-360 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', an EcoRI site is underlined), digested with BamHI and EcoRI, and cloned into the same sites of pGEX (4T-1). (f) For GST-OCT-4B (PBC), the N-terminal and POU domain of OCT-4B was amplified from pcDNA3/OCT-4B by PCR using primers 5'-hoCT4B-1 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', a BamHI site is underlined) and 3'-hoCT4B-360 (5'-GATCGGATCCATGCACTTCTACAGACTATTCCTTGGGGCC-3', an EcoRI site is underlined), digested with BamHI and EcoRI, and cloned into the same sites of pGEX (4T-1). (g) For GST-OCT-4A(NPB), the N-terminal domain of OCT-4B or the POU and C-terminal domains of OCT-4A were amplified from pcDNA3/OCT-4B or pcDNA3/OCT-4A.
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by PCR using primers 5'-hOCT4B-1 and 3'-hOCT4B-40 (5'-CTCCTCTGGATTTTAAAGGCAGAAGA-3') or primers 5'-hOCT4A-134B (5'-ATCCAGGGAGGTCCCGAGCATCAAAG-3') and 3'-hOCT4A-360, respectively. The secondary PCR was performed using gel-purified primary PCR products as templates and primers 5'-hOCT4B-1 and 3'-hOCT4A-360. The secondary PCR products were digested with BamHI and EcoRI and cloned into the same sites of pGEX (4T-1). (e) For GST-OCT-4B(NP9B), the N-terminal domain of OCT-4A or the POU and C-terminal domains of OCT-4B were amplified from pcDNA3/OCT-4A or pcDNA3/OCT-4B using primers 5'-hOCT4A-1 and 3'-hOCT4A-137 (5'-CTGGGACGGGTGTGCTCCAGCTTCC-3') or primers 5'-hOCT4B-41B (5'-AAACCCGTCCAGGACATCAAAGCTCTGC-3') and 3'-hOCT4A-360, respectively. The secondary PCR was performed using gel-purified primary PCR products as templates and primers 5'-hOCT4A-1 and 3'-hOCT4A-360. The secondary PCR products were digested with BamHI and EcoRI and cloned into the same sites of pGEX (4T-1). (f) For GST-OCT-4B(NP9B)Δ(21–40), a fragment of the human OCT-4B sequence extending from the N-terminal domain through the POU DNA binding domain was cloned into pKSI1 vector (Stratagene). A deletion of amino acids 21–40 was introduced by site-directed mutagenesis using a mutagenic primer set (5'-CTTGATATGCGAGGTGGGATGGGAATCCCGAATGTTGAGCTCCAGGAGATCAAACGGTGCTC-3') and 5'-GAGCTTTTGGATGTCCTGGAGTTTTCACAGGATTCCAG-3') and the QuikChange kitTM (Stratagene). Then the OCT-4B (NP9B)Δ(21–40) fragment of the pKSI1-OCT-4B (NP9B)Δ(21–40) construct was digested with BamHI and EcoRI and cloned into the same sites of pGEX (4T-1). (g) For GST-OCT-4B (NP9B)Δ(1–20), the (NP9B)Δ(1–20) fragment of OCT-4B was amplified from pcDNA3/OCT-4B by PCR using primers 5'-hOCT4B-21 (5'-GATCGAGTCCGAGGAGATTGATACGG-3', a BamHI site is underlined) and 3'-hOCT4B-289, digested with BamHI and EcoRI, and cloned into the same sites of pGEX (4T-1).

The reporter construct pOCT-4(10x)TATAluc has been described (35). The FOR promoter (–508 to +1) was cloned by PCR amplification from pGL3-FOR (3.5 kb).4 The primers used for this purpose were 5'-FOR(–508) (5'-GATCGGTACCCCATGCCCCCTT-3', a KpnI site is underlined) and 3'-FOR(+1) (5'-GATCTTGGATAAACCCTATTTG-3', a HindIII site is underlined). The PCR product was digested with KpnI and HindIII and cloned into the same sites of the promoterless pGL3 basic vector (Promega).

Cell Culture—Human ES cells (Miz-hES1, SNU-hESC3, and Cha-hESC3) were grown as described previously (38, 39). Briefly, they were cultured in Dulbecco’s modified Eagle’s medium/F12 medium with 20% knock-out serum replacement, 1 mm glycerol, 1% nonessential amino acids, 0.1 mm B-mercaptoethanol, and 4 ng/ml basic fibroblast growth factor (Invitrogen) on mitomycin C-treated mouse embryonic fibroblast feeders at 37 °C and 5% CO₂. After 5 days of culture, colonies were detached mechanically from the feeder cells with a micropipette, and individual colonies were mechanically divided into four or five pieces. These ES cell clumps were then separately plated on fresh feeder cell layers. HEK293T or NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum (Invitrogen), penicillin, and streptomycin.

RT-PCR and Alkaline Phosphatase Staining—Total RNA was prepared from human ES cells using an RNasey mini kit (Qiagen) with on-column DNase treatment, and messenger RNA purified with an Oligodex-dT mRNA mini kit (Qiagen) followed by cDNA synthesis using a Superscript first-strand synthesis system for RT-PCR (Invitrogen), as instructed by the manufacturers. To detect human OCT-4 isoforms in human ES cells, human OCT-4A and OCT-4B cDNAs were amplified with human OCT-4A primers [5'-e1a (5'-GATCGGTACCTGATTGGGACGGGACACCTGGCT-3') and 3'-e1 (5'-CCTTCCCCAAATAAGAACCC-3')], and human OCT-4B primers [5'-e1b (5'-GATCGTGATCATGCGATTCCTTCAGACTATTCTCTTGAGGCC-3') and 3'-e1], respectively.

RT-PCRs for OCT-4 downstream target genes and differentiation marker genes were performed with gene-specific primer sets as described previously (9, 11, 30, 41). Alkaline phosphatase was stained with an AP staining kit (Sigma).

Quantitative Real Time PCR—Quantitative real time PCR was performed with an Applied Biosystems 7500 Fast real time PCR system (Applied Biosystems) and SYBR Green Master Mix (Applied Biosystems), as described previously (43). As a control, the level of GAPDH mRNA was determined in the real time PCR assay of each RNA sample and was used to correct for experimental variation. The following primer sequences were used: hOCT-4A forward primer was 5'-CTCCTGGAGGGCCAGGATC-3', and hOCT-4A reverse primer was 5'-CCAATCGGCGCTGTATAT-3'. The hOCT-4B forward primer was 5'-ATGCAGTTGCTGAAACAG-3', and the hOCT-4B reverse primer was 5'-CCACATCGGCCCTGTATAT-3'. The GAPDH forward primer was 5'-GAAGGTGAAGGTTGAGTTC-3', and the GAPDH reverse primer was 5'-GAAGGATGGTGATTGCAATTCG-3'. Quantitation of the relative expression levels of the human OCT-4 isoforms was achieved by normalizing for the endogenous GAPDH using the ΔCₚ method of quantitation.

ES Cell-based Complementation Assay—ES cell-based complementation assays were performed with ZHBTc4 ES cells as described previously (44). Fluorescence was detected with a fluorescence microscope (Olympus, 1X51) equipped with a CoolSNAP digital camera (Olympus).

Expression and Purification of GST Fusion Proteins—GST-OCT-4 proteins were generated in Escherichia coli as described previously (45). After binding to glutathione-Sepharose, the proteins were washed and eluted with reduced glutathione (Sigma). Protein concentrations were determined by the method of Bradford (Bio-Rad). The purity and size of the eluted proteins were evaluated by Coomassie staining of SDS-polyacrylamide gels.

Western Blot Analysis—Western blot analysis was performed using an anti-OCT-4 antibody (C-20; Santa Cruz Biotechnology), and reactive bands were detected by chemiluminescence using Western Lightening (PerkinElmer Life Sciences).

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Electrophoretic Mobility Shift Assays (EMSAs)—Probes for EMSAs were prepared from synthetic oligonucleotides whose sequences have been described previously (46). The probes were prepared by end labeling annealed complementary oligonucleotides with [γ-32P]ATP using T4 polynucleotide kinase. EMSAs were performed with recombinant GST-OCT-4 proteins (OCT-4A, OCT-4B, and their derivatives) for 30 min at 4 °C in binding buffer containing 20 mM HEPES (pH 7.4), 40 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 0.1% Nonidet P-40, and 0.2 μg of poly(dI-dC)-(dI-dC). Following binding, the reaction mixtures were run on 4% polyacrylamide gels (acrylamide/bisacrylamide, 1 mM EDTA) buffer at 150 V for 2–3 h at 4 °C. The gels were dried and exposed to Kodak X-Omat film at −70 °C.

Transfection and Reporter Assays—Cells were transiently transfected by electroporation with the Gene Pulser II RF module system (Bio-Rad), as instructed by the manufacturer. Luciferase assays were performed with the dual-luciferase assay system (Bio-Rad), as instructed by the manufacturer. Luciferase activity was used to normalize transfection efficiencies.

Subcellular Localization Experiments—Full-length human OCT-4A or OCT-4B cDNAs were subcloned into the BamHI/EcoRI sites of pBabePuro. NIH3T3 cells were plated on glass coverslips and infected with high titer retroviral stocks produced by transient transfection of Phoenix cells (47). Immunocytochemical analyses were performed as described previously (45). Briefly, the infected cells were washed in phosphate-buffered saline (PBS) and fixed for 10 min at −20 °C with acetone/methanol (1:1, v/v). To detect human OCT-4A or OCT-4B, we used anti-OCT-4 antibody (C-20; Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and fluorescence was detected with a confocal laser scanning microscope (LSM5 Pascal; Carl Zeiss Co., Ltd.). To localize the human OCT-4 isoforms in ES cells, pCAG-IP/EGFP, pCAG-IP/mOCT-4-EGFP, pCAG-IP/hOCT-4A-EGFP, or pCAG-IP/hOCT-4B-EGFP-transfected ZHBTc4 ES cells were washed in PBS and fixed for 10 min at −20 °C in a mixture of acetone and methanol (1:1, v/v). The coverslips were mounted with 50% glycerol/PBS, and green fluorescence was detected with a fluorescence microscope (IX51; Olympus, Tokyo, Japan) equipped with a CoolSNAP digital camera (Olympus).

RESULTS

Identification of OCT-4 Isoforms in Human Embryonic Stem Cells—Transcripts of the human OCT-4 isoforms (OCT-4A and OCT-4B) were first observed as RT-PCR products during a search for transcription factors containing POU-domains in adult human pancreatic islets (36). We examined OCT-4 isoforms in human embryonic stem cells by RT-PCR using total human ES cell (Miz-hES1) RNA with specific oligonucleotide primers flanking the coding region corresponding to the N-terminal domain of OCT-4 proteins. As shown in Fig. 1A, OCT-4A mRNA was generated from exon 1a (labeled E1a), the 3′-half of exon 1b (E1b), and exons 2–4 (E2, E3, and E4) using an internal splicing acceptor site in exon 1b. OCT-4B mRNA contains exons 1b to 4 (Fig. 1A). PCR products of the expected size of 532 and 247 bp derived from human OCT-4A (Fig. 1B, lane 1) and OCT-4B (lane 2) mRNA, respectively, were observed using human ES cell RNA. These RT-PCR products were sequenced and confirmed to represent nucleotides 1–532 and 1–247 of the reported human OCT-4A or OCT-4B cDNA sequences, respectively (data not shown). These results point to the existence of two isoforms of human OCT-4 differing in...
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Differential abilities of the human OCT-4 isoforms to rescue self-renewal of embryonic stem cells. A, Western blot analysis of OCT-4 expression in ZHBTc4 ES cells harboring the mouse Oct-4 gene under the control of the inducible tetracycline promoter. Total cell extracts were prepared from ZHBTc4 ES cells 0, 12, 24, 36, and 48 h following doxycycline treatment. The extracts were resolved by 15% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and immunoblotted with anti-OCT-4 antibody (C-10; Santa Cruz Biotechnology). B, schematic representation of the OCT-4 isoform EGFP fusions used in this study. Expression vectors pCAG-IP/mOCT-4-EGFP, pCAG-IP/hOCT-4A-EGFP, and pCAG-IP/hOCT-4B-EGFP correspond to mouse OCT-4, human OCT-4-A, and human OCT-4-B fused to EGFP, respectively. The pCAG-IP/EGFP expression vector was used as a control. CAG expression units (CAGs) (44) are indicated by shaded boxes, and mouse OCT-4, human OCT-4-A, and human OCT-4-B are represented by white boxes, and EGFP is indicated by solid boxes. C, human OCT-4-B is defective in ability to confer self-renewal. ZHBTc4 ES cells were transfected with pCAG-IP/EGFP (vector), pCAG-IP/mOCT-4-EGFP (mOCT-4), pCAG-IP/hOCT-4A-EGFP (hOCT-4-A), or pCAG-IP/hOCT-4B-EGFP (hOCT-4-B), and cultured in the absence (+Dox, left two panels) or in the presence (+Dox, right two panels) of doxycycline. Phase-contrast (panels a–d and i–l) and fluorescence views (panels e–h and m–p) are shown.

FIGURE 2. Differential abilities of the human OCT-4 isoforms to rescue self-renewal of embryonic stem cells. A, Western blot analysis of OCT-4 expression in ZHBTc4 ES cells harboring the mouse Oct-4 gene under the control of the inducible tetracycline promoter. Total cell extracts were prepared from ZHBTc4 ES cells 0, 12, 24, 36, and 48 h following doxycycline treatment. The extracts were resolved by 15% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and immunoblotted with anti-OCT-4 antibody (C-10; Santa Cruz Biotechnology). B, schematic representation of the OCT-4 isoform EGFP fusions used in this study. Expression vectors pCAG-IP/mOCT-4-EGFP, pCAG-IP/hOCT-4A-EGFP, and pCAG-IP/hOCT-4B-EGFP correspond to mouse OCT-4, human OCT-4-A, and human OCT-4-B fused to EGFP, respectively. The pCAG-IP/EGFP expression vector was used as a control. CAG expression units (CAGs) (44) are indicated by shaded boxes, and mouse OCT-4, human OCT-4-A, and human OCT-4-B are represented by white boxes, and EGFP is indicated by solid boxes. C, human OCT-4-B is defective in ability to confer self-renewal. ZHBTc4 ES cells were transfected with pCAG-IP/EGFP (vector), pCAG-IP/mOCT-4-EGFP (mOCT-4), pCAG-IP/hOCT-4A-EGFP (hOCT-4-A), or pCAG-IP/hOCT-4B-EGFP (hOCT-4-B), and cultured in the absence (+Dox, left two panels) or in the presence (+Dox, right two panels) of doxycycline. Phase-contrast (panels a–d and i–l) and fluorescence views (panels e–h and m–p) are shown.

To test whether the stem cell phenotype can be rescued by transfections of human OCT-4 isoforms, OCT-4-A or OCT-4-B cDNAs under the control of the constitutive CAG expression unit were transfected into ZHBTc4 ES cells, respectively. To identify the transfected ES cells, we constructed plasmids expressing green fluorescent protein fusions of the human OCT-4 isoforms. The structures of expression vectors used are shown schematically in Fig. 2B. Consistent with the previous reports (11, 44), the mouse OCT-4-transfected ZHBTc4 ES cells were able to differentiate because of superthreshold production of OCT-4 (Fig. 2C, panels b and f). In addition, growth of the transfecants in the presence of doxycycline rescued their self-renewal ability and stem cell phenotype (Fig. 2C, panels i and n). As a control, ZHBTc4 ES cells transfected with EGFP and cultured in the presence of doxycycline underwent differentiation (Fig. 2C, panels i and m).

To investigate the behavior of the OCT-4 gene products of different species, we introduced an expression construct for human OCT-4-A into the ZHBTc4 ES cells and performed the
ES cell-based complementation assay. The ZHBTc4 ES cells cultured in the absence of doxycycline again differentiated because of superthreshold production of OCT-4 (Fig. 2C, panels c and g), and their self-renewal ability was rescued in the presence of doxycycline, indicating that human OCT-4A protein is active in mouse ZHBTc4 ES cells (Fig. 2C, panels k and o). However, contrary to expectation, when human OCT-4B was introduced into ZHBTc4 ES cells, it failed to rescue stem cell renewal in the presence of doxycycline (Fig. 2C, panels l and p). These data indicate that the abilities of human OCT-4A and OCT-4B isoforms to confer self-renewal on ES cells differ.

Different Abilities of Human OCT-4 Isoforms to Maintain the Undifferentiated State—The flat morphology of doxycycline-treated ZHBTc4 ES cells expressing human OCT-4B (Fig. 2C) suggested failure to maintain the undifferentiated state of the ES cells. To verify this hypothesis, we examined known molecular markers of undifferentiated ES cells. To stain for alkaline phosphatase activity (a marker of pluripotent cells of embryonic origin) (48), the pCAG-IP/EGFP, pCAG-IP/mOCT-4A-EGFP, and pCAG-IP/EGFP constructs (Fig. 2B) were linearized with PvuI, and 50 μg of each linearized plasmid DNA was transfected into ZHBTc4 ES cells expressing vector, mouse OCT-4, or human OCT-4 isoforms. Hprt was used as a control to quantify the RT-PCR results. Following amplification, an aliquot of each product was analyzed by staining the gel with ethidium bromide. The ES cell lines from which the input RNAs used in the RTs are derived are shown above the panel.

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We also evaluated the expression of markers of differentiated cells. As shown in Fig. 3B, expression of Cdx-2 mRNA, which is implicated in trophoblast differentiation (52), was detected in ZHBTc4 ES cells expressing human OCT-4B or vector but was not present in ZHBTc4 ES cells expressing mouse OCT-4 or human OCT-4A. Fgf-5 mRNA, a marker of primitive endoderm (53), also appeared in ZHBTc4 ES cells expressing human OCT-4B, indicating formation of the endoderm lineage. These properties all suggest that human OCT-4A-expressing ES cells expanded normally and remained in an undifferentiated status, whereas human OCT-4B-expressing ES cells did not.

DNA Binding Properties of Human OCT-4 Isoforms—Because the results presented above indicate that human OCT-4B is not able to induce stem cell self-renewal, we examined its biochemical characteristics. In order see whether it can bind to an authentic OCT-4 DNA recognition sequence, we studied the DNA binding properties of the human OCT-4 isoforms using 4B-EGFP-transfected ZHBTc4 cells, 4 × 10^3 transfectants were seeded onto 35-mm dishes containing mitomycin C-treated mouse embryonic fibroblast feeder layers. After 2 days of culture, the cells were transferred to medium containing 1 μg/ml doxycycline to repress the tetracycline-repressible mouse Oct-4 transgene. After another 4 days, colonies resistant to puromycin were tested for OCT-4-EGFP protein expression by examining green fluorescence and staining the cells for alkaline phosphatase. ZHBTc4 ES cells expressing human OCT-4A contained alkaline phosphatase activity (Fig. 3A, panels a and b) comparable with that in ZHBTc4 ES cells expressing mouse OCT-4 (Fig. 3A, panels c and d). However, the clones expressing human OCT-4B (Fig. 3A, panels g and h) or EGFP alone (Fig. 3A, panels a and b) had lost this characteristic, pointing to failure to maintain the undifferentiated state.

To investigate further the features of the ZHBTc4 ES cells transfected with the human OCT-4 isoforms, we investigated levels of expression of known OCT-4 downstream target genes such as Fgf-4 (20), Rex-1 (50), Sox-2 (51), and Nanog (17, 18) by RT-PCR. As shown in Fig. 3B, expression of all four OCT-4 downstream target genes was detected in the tetracycline-treated ZHBTc4 ES cells transfected with mouse OCT-4 (2nd lane) or human OCT-4A (3rd lane) cDNAs. However, these genes were down-regulated in tetracycline-treated ZHBTc4 ES cells expressing human OCT-4B (Fig. 3B, 4th lane). Tetracycline-treated ZHBTc4 ES cells expressing EGFP served as a negative control (Fig. 3B, 1st lane).
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EMSAs. An oligonucleotide containing the consensus OCT-4 DNA-binding sequence was synthesized and used as target in the binding reactions. The structures of the OCT-4 isoform GST fusions used in this study are shown schematically in Fig. 4A. They were expressed in E. coli, purified, and coupled to glutathione-Sepharose beads. The affinity-purified OCT-4 isoforms were fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunoblotted with an anti-OCT-4 antibody (C-20; Santa Cruz Biotechnology) to quantify the amount of GST fusion proteins. The GST fusion OCT-4 isoforms used in this study. Constructs GST-OCT-4A and GST-OCT-4B represent the GST domain fused to the OCT-4A and OCT-4B, respectively. The functional domains of the OCT-4 isoforms are also shown.

Inhibiting OCT-4B DNA Binding

EMSAs were performed with the concentration of the OCT-4 probe kept constant, and the amount of input protein varied. GST-OCT-4A bound to the DNA (Fig. 4C, lanes 5–7), and binding was specific as it was displaced by an excess of cold oligonucleotide containing the OCT-4-binding site but not by an excess of cold mutant oligonucleotide containing a mutant OCT-4-binding sequence not recognized by OCT-4 (data not shown). Therefore, the OCT-4B isoform hardly bound at all (Fig. 4C, lanes 8–10). These results indicate either that the N-terminal domain of OCT-4A is required for efficient DNA binding or that the N-terminal sequence of the OCT-4A POU domain (total 154 amino acids) lacks two amino acids of the N-terminal sequence of the OCT-4B POU domain (total 156 amino acids) (Fig. 1D) (36). EMSAs with the POU domains of OCT-4A and OCT-4B on their own revealed that both bound the probe (Fig. 5B, lanes 5–7 and 8–10). Moreover, the addition of the C-terminal domain of OCT-4B to the POU DNA binding domain of OCT-4B (OCT-4B (P^6)) produced a recombinant protein still capable of forming protein-DNA complex (Fig. 5C, lanes 8–10), whereas the addition of the N-terminal domain of OCT-4B (OCT-4B (N^P^6)) essentially abolished DNA binding (Fig. 5C, lanes 5–7). These results show that the N-terminal domain of OCT-4B inhibits POU DNA binding.

To further test the contribution of the N-terminal domain of OCT-4B to DNA binding, we generated a series of chimeric proteins in which the order of the domains remained constant, i.e. NTD-POU-CTD, but domains were swapped between OCT-4A and OCT-4B. As shown in Fig. 5D, OCT-4A (N^P^6) was unable to bind the OCT-4 probe, whereas OCT-4B (N^P^6) was able to bind it. This confirms that the N-terminal domain of OCT-4B contains some sequence or sequences that inhibit DNA binding and that this is active on POU^A as well as POU^B.

Two Separate Regions in OCT-4B NTD Are Responsible for Inhibiting OCT-4B DNA Binding—Fusion of the N-terminal domain of OCT-4B to the POU and C-terminal domains of OCT-4A revealed inhibition of DNA binding by the 40-amino acid sequence of OCT-4B (Fig. 5D). To define the minimum inhibitory domain, we tested partial deletions of the N terminus in which either amino acids 21–40 or 1–20 were deleted. The structure of the OCT-4B deletion mutants used is shown in Fig. 6A. These truncation mutants were expressed as GST fusion proteins in E. coli and purified to near homogeneity in roughly equal yield (Fig. 6B). As shown in Fig. 6C, neither partial deletion mutant was able to bind DNA. These results demonstrate that the N-terminal domain of OCT-4B possesses at least two independently acting sequences that inhibit POU DNA binding.

OCT-4A Is Nuclear whereas OCT-4B Is Cytoplasmic—We also determined the intracellular locations of OCT-4A and OCT-4B. For this purpose, we infected NIH3T3 cells with a retroviral expression construct, pBabePuro, containing the retroviral expression construct, pBabePuro, containing the

FIGURE 4. Binding of human OCT-4 isoforms to oligonucleotides. A, a schematic representation of the GST fusion OCT-4 isoforms used in this study. Constructs GST-OCT-4A and GST-OCT-4B represent the GST domain fused to the OCT-4A and OCT-4B, respectively. The functional domains of the OCT-4 isoforms are also shown. B, immunoblot analysis of OCT-4 isoforms to quantify the amounts of GST fusion proteins. The GST fusion OCT-4 isoforms used in this study. Constructs GST-OCT-4A and GST-OCT-4B represent the GST domain fused to the OCT-4A and OCT-4B, respectively. The functional domains of the OCT-4 isoforms are also shown. C, DNA binding by the OCT-4 isoforms. Following preparation of radiolabeled probes, EMSAs were performed with either no recombinant protein (lane 1), recombinant GST (lane 2; 50 ng; lane 3, 100 ng; lane 4; 150 ng), GST-OCT-4A [lane 5; 50 ng; lane 6, 100 ng; lane 7; 150 ng], or GST-OCT-4B (lane 8, 50 ng; lane 9, 100 ng; lane 10, 150 ng) as described under “Experimental Procedures.” The recombinant proteins used in each EMSA is indicated above the gel. Protein-DNA complexes were resolved on nondenaturing 4% polyacrylamide (acrylamide:bisacrylamide ratio, 37:1) gels run at 4 °C in 0.5 × TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA). The positions of the free probe and protein-DNA complexes are indicated.
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FIGURE 5. Deletion of the N-terminal domain permits OCT-4B to bind DNA. A, a schematic representation of the GST fusion OCT-4A, GST fusion OCT-4B, and derivatives in which domains were deleted or swapped and their ability to bind to DNA. The functional domains located within the OCT-4 isoforms are indicated. (NTD indicates the N-terminal domain of OCT-4A, etc). The + or − symbols to the right refer to the binding ability of the GST fusions to DNA (+, binding; −, no binding). B, DNA binding activity of OCT-4A (POUA) and OCT-4B (POUB). EMSAs were performed with either no recombinant protein (lane 1), recombinant GST (lane 2, 50 ng; lane 3, 100 ng; lane 4, 150 ng), GST-OCT-4A (POUA) (lane 5, 50 ng; lane 6, 100 ng; lane 7, 150 ng), or GST-OCT-4B (POUB) (lane 8, 50 ng; lane 9, 100 ng; lane 10, 150 ng) as described under “Experimental Procedures.” The recombinant proteins used in the EMSAs are indicated above the gel. Protein-DNA complexes were resolved as in Fig. 4. C, DNA binding activity of OCT-4B deletion mutants. EMSAs were performed with either no recombinant protein (lane 1), recombinant GST (lane 2, 50 ng; lane 3, 100 ng; lane 4, 150 ng), GST-OCT-4B (NPPB) (lane 5, 50 ng; lane 6, 100 ng; lane 7, 150 ng), or GST-OCT-4B (POUB) (lane 8, 50 ng; lane 9, 100 ng; lane 10, 150 ng) as described under “Experimental Procedures.” D, DNA binding of OCT-4A (NPPC) and OCT-4B (NPPPC) chimeric molecules. EMSAs were performed with either no recombinant protein (lane 1), recombinant GST (lane 2, 50 ng; lane 3, 100 ng; lane 4, 150 ng), GST-OCT-4A (NPPC) (lane 5, 50 ng; lane 6, 100 ng; lane 7, 150 ng), or GST-OCT-4B (NPPPC) (lane 8, 50 ng; lane 9, 100 ng; lane 10, 150 ng) as described under “Experimental Procedures.”

entire OCT-4 coding region. A pBabePuro retroviral vector lacking OCT-4 cDNA sequences was used as negative control. The cells were infected with either empty pBabePuro expression vector (data not shown), pBabePuro-OCT-4A (Fig. 7A, panels a and b), or pBabePuro-OCT-4B (Fig. 7A, panels c and d) and processed for immunofluorescence. We previously showed that mouse OCT-4 protein is localized to the nucleus (35). In accordance with this previous result, human OCT-4A was clearly localized to the nucleus (Fig. 7A, panel d). However, contrary to our expectation, we found that most of the human OCT-4B protein was cytoplasmic (Fig. 7A, panel c). We also transfected COS-7 cells with pcDNA3-OCT-4B or pEGFP-OCT-4B with similar results (data not shown).

To confirm these results in cells possessing stem cell properties, the subcellular distribution of the human OCT-4 isoforms was also determined in ZHBTc4 ES cells (Fig. 7B). pCAG-IP/EFGP, pCAG-IP/hOCT-4A-EGFP, and pCAG-IP/hOCT-4B-EGFP constructs that express EFGP, OCT-4A-EGFP (GFP fusion human OCT-4A), and OCT-4B-EGFP (GFP fusion human OCT-4B), respectively (Fig. 2B), were transfected into ZHBTc4 ES cells, and the subcellular locations of these proteins were detected by fluorescence microscopy. In the ZHBTc4 ES cells, EFGP-tagged OCT-4A was clearly localized to the nucleus (Fig. 7B, panels c and d), whereas most of the EFGP-tagged OCT-4B was cytoplasmic (Fig. 7B, panels e and f). EFGP alone was found in both the nucleus and cytoplasm of ES cells (Fig. 7B, panels a and b). Taken together, these data suggest that OCT-4A is a nuclear protein whereas OCT-4B is a cytoplasmic protein.

OCT-4B Is Unable to Activate Transcription of OCT-4-responsive Genes—OCT-4B lacks the N-terminal sequence that mediates the transcriptional activity of OCT-4 (13). In addition, its N-terminal domain inhibits DNA binding (Figs. 5 and 6), and it is cytoplasmic in location (Fig. 7). All these properties suggest that it may not be a transcriptional activator. To address this question, we performed transient transfection assays. 293T cells were transfected with a luciferase reporter plasmid...
OCT-4B and OCT-4A with an OCT-4-dependent reporter gene. In the absence of OCT-4B, OCT-4A increased reporter gene activity severalfold, as expected (Fig. 8C, bar 2), and increasing input levels of OCT-4B had no inhibitory effect (Fig. 8C, bars 3 and 4). Hence, we may conclude that overexpression of OCT-4B does not interfere with transactivation by OCT-4A.

**DISCUSSION**

Numbers of stem cells, and their decisions to differentiate, must be tightly controlled during embryonic development and in the adult animal to avoid premature senescence or tumor formation. Embryonic and adult stem cells share the properties of self-renewal and multiple developmental potential, suggesting the presence of common cellular machinery. Thus, greater understanding of the molecular determinants responsible for these properties is desirable. Accordingly, there is growing interest in the functional characterization of OCT-4 in embryonic stem cells.

The results reported here begin to characterize the human OCT-4 isoforms. Two isoforms of human OCT-4 are generated by alternative splicing (Fig. 1A). Oct-4 encodes a POU transcription factor that is expressed by all pluripotent cells during embryogenesis and is also abundantly expressed by ES, embryonic germ, and embryonic carcinoma cell lines (2, 3, 7, 57, 58). Differentiation of pluripotent cells to somatic lineages occurs at the blastocyst stage and during gastrulation, coincident with down-regulation of OCT-4. The unique oct-4 expression pattern in the mouse embryo also leads to the hypothesis on the pluripotent cycle (27). Consistent with this speculation, oct-4-null embryos die at implantation because of a failure to form the inner cell mass (9). These results demonstrate that oct-4 is required to prevent somatic differentiation of the inner cell mass and is important for maintaining the undifferentiated state during embryonic development.

Despite the fact that OCT-4A function is critical for maintaining the pluripotency of embryonic stem cells and promoting tumorigenesis in human tissues, little is known of the biological properties of the OCT-4B isoform. First, we investigated the existence of the two OCT-4 isoforms in human ES cells by RT-PCR, because although two human OCT-4 isoforms have been reported to be expressed in adult human pancreatic islets (36), it was not known whether they were also expressed in human ES cells. Two OCT-4 isoforms were indeed detected in the human ES cells. In addition, human OCT-4A was more abundant in human ES cells than OCT-4B (Fig. 1).

OCT-4 is structurally and functionally divided into three domains (see Fig. 1D). The N-terminal 133 amino acid residues of OCT-4A encompass a transcriptional activation region that is active in various cultured cell types. Amino acid residues 134–289 form the central POU domain of the protein that binds to DNA in a sequence-specific fashion. The third domain between amino acid residues 290 and 360 also controls the transactivation function of OCT-4, but its activity is cell type-specific (13). As shown in Fig. 1D, human OCT-4A and OCT-4B mRNA encode proteins that share POU DNA binding and C-terminal domains but differ in sequence at their N termini. Because the N-terminal domain of OCT-4 functions as a transactivation domain, we measured the transactivation potential of OCT-4B. OCT-4A functioned as a transcriptional activator as reported previously (33), whereas OCT-4B did not appear to do so (Fig. 8).

We also demonstrated that the N-terminal domain of OCT-4B inhibits the sequence-specific DNA binding of OCT-4 protein, via the central POU domain (Figs. 4 and 5). Moreover, regions containing amino acids 1–20 and 21–40 both inhibited POU sequence-specific DNA binding, indicating that both regions independently maintain OCT-4B in the latent state with little or no affinity for its target sequences (Fig. 6).
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FIGURE 7. Subcellular localization of OCT-4 isoforms in cells. A, subcellular distribution of human OCT-4 isoforms in NIH3T3 cells. NIH3T3 cells grown on coverslips were infected with a retroviral expression construct, pBabePuro, containing the OCT-4A (panels a and b) or OCT-4B (panels c and d) coding regions. The subcellular distributions of OCT-4A (panel a) or OCT-4B (panel c) in infected NIH3T3 cells were analyzed with anti-OCT-4 antibody (C-20; Santa Cruz Biotechnology). B, subcellular distribution of human OCT-4 isoforms in ES cells. ZHBTc4 ES cell lines expressing EGFP (panels a and b), human OCT-4A-EGFP (panels c and d), or human OCT-4B-EGFP (panels e and f) were cultured on coverslips under low density conditions. The cells were fixed with an acetone/methanol mixture and analyzed for EGFP by fluorescence microscopy.

FIGURE 8. Transactivation properties of the OCT-4 isoforms. A, transcriptional activation by the OCT-4 isoforms using a pOCT-4(10x)TATAluc reporter vector. 293T cells were co-transfected with expression vectors encoding the OCT-4A (black bars) or OCT-4B (white bars) isoforms, the pOCT-4(10x)TATA-luc reporter vector, and the Renilla luciferase normalizing vector. Firefly luciferase activity was normalized with Renilla luciferase activity to correct for transfection efficiencies. Each transfection was performed at least three times independently, and the mean value is plotted with the standard deviation (vertical bars). Fold induction is relative to the empty expression vector. B, transcriptional activation by the human OCT-4 isoforms using pFORluc reporter vector. 293T cells were co-transfected with expression vectors encoding the OCT-4A (black bars) or OCT-4B (white bars) isoforms, the pFORluc reporter vector, and the Renilla luciferase normalizing vector. Firefly luciferase activity was normalized with Renilla luciferase activity to correct for transfection efficiencies. Each transfection was performed at least three times independently, and the mean value is plotted with the standard deviation (vertical bars). Fold induction is relative to the empty expression vector. C, human OCT-4B isoform does not interfere with transcriptional activation by OCT-4A. The pOCT-4(10x)TATAluc reporter was co-transfected into 293T cells with 0.25 μg of human OCT-4A and the indicated amounts of OCT-4B isoform. The average fold induction of transcription (normalized firefly luciferase activity) and standard error of three independent experiments are presented.
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Classical NLS sequences contain regions rich in basic amino acids and generally conform to one of three motifs (34). The first type of NLS consists of a continuous stretch of four basic amino acids (lysine or arginine) or three basic amino acids together with a histidine or proline. The second type of NLS starts with a proline and is followed within three residues by an amino acid sequence containing three out of four basic residues. The third type of NLS, known as a bipartite motif, consists of two basic amino acids, a 10 amino acid spacer and a 5 amino acid sequence containing at least three basic residues. It has been reported recently that OCT-4A harbors a conserved acid sequence containing at least three basic residues. It has been reported that OCT-4 exists as a phosphoprotein in embryonic carcinoma cells (13). Thus, it will be important to assess whether human cytoplasmic OCT-4B is involved in signal transduction.

In a broader context, our findings suggest that the OCT-4B isoform may play a different role than the unique function of the OCT-4A protein in self-renewal. Consistent with this speculation, expression of human OCT-4B alone is not sufficient to maintain stem cell self-renewal (Fig. 2C) and undifferentiated state (Fig. 3). These failures can be explained by the loss of transactivation ability (Fig. 8), which can in turn be accounted for by its localization in the cytoplasm (Fig. 7) as well as its inability to bind to the OCT-4 consensus motif (Fig. 4). Because OCT-4 is expressed in human testicular germ cell tumors and its expression transforms and endows tumorigenicity in nude mice (42, 56), it would also be interesting to determine whether OCT-4B can collaborate with OCT-4A in transforming nontumorigenic cells.

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