Octamer and Sox Elements Are Required for Transcriptional *cis* Regulation of *Nanog* Gene Expression

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The pluripotential cell-specific gene *Nanog* encodes a homeodomain-bearing transcription factor required for maintaining the undifferentiated state of stem cells. However, the molecular mechanisms that regulate *Nanog* gene expression are largely unknown. To address this important issue, we used luciferase assays to monitor the relative activities of deletion fragments from the 5′-flanking region of the gene. An adjacent pair of highly conserved Octamer- and Sox-binding sites was found to be essential for activating pluripotential state-specific gene expression. Furthermore, the 5′-end fragment encompassing the Octamer/Sox element was sufficient for inducing the proper expression of a green fluorescent protein reporter gene even in human embryonic stem (ES) cells. The potential of OCT4 and SOX2 to bind to this element was verified by electrophoretic mobility shift assays with extracts from F9 embryonal carcinoma cells and embryonic germ cells derived from embryonic day 12.5 embryos. However, in ES cell extracts, a complex of OCT4 with an undefined factor preferentially bound to the Octamer/Sox element. Thus, *Nanog* transcription may be regulated through an interaction between *Oct4* and *Sox2* or a novel pluripotential cell-specific Sox element-binding factor which is prominent in ES cells.

Mammalian pluripotential stem cells, which are defined by their ability to differentiate into a variety of specialized cellular lineages, are found in both preimplantation embryos and many adult tissues. They can also be isolated and maintained in vitro as embryonal carcinoma (EC) cells, embryonic stem (ES) cells, and embryonic germ (EG) cells (30).

The pluripotential state of cells is maintained under the regulation of some key genes whose expression is specific to pluripotential cells. The *Oct4* gene, which is a member of the mammalian POU family of transcriptional factor genes, functions as a key regulator of the pluripotential state (16, 20). *Sox2*, known to act cooperatively at promoters with *Oct4*, activates transcription of the *Fgf4*, *Utf1*, *Sox2*, and *Fbx15* genes (17, 33, 34, 42). Furthermore, the genes transcribed in the trophoectodermal lineage, *Cdx-2* and *Hand-1*, are negatively regulated by *Oct4* (20).

Another key molecule involved in the signaling pathway for maintaining the capacity for the self-renewal and pluripotency of mouse ES cells is leukemia inhibitory factor (LIF) (26, 38). LIF directs the activation of transcription factor STAT3 by phosphorylation through binding to the heterodimer of the LIF receptor and gp130 (6). Recently, it was also shown that the LIF signal is not sufficient to support the self-renewal of mouse ES cells under culture conditions in the absence of serum and feeder cells. An additional signal provided by bone morphogenetic proteins is required and induces the activation of the inhibitor of differentiation (*Id*) genes, which repress differentiation into the neuroectodermal lineage (41). In addition to the roles of these genes, it was demonstrated that *Ezh2*, a mammalian homologue of the Polycomb-group gene *Enhancer of zeste* in *Drosophila* (12), forms a complex with *Eed* (embryonic ectoderm development). This complex plays an important role in maintaining the pluripotency of ES cells and blastocyst inner cell mass cells through histone H3 lysine 27 methylation-based repression of specific homeotic genes (4, 7). Null mutation of the *Oct4*, *Sox2*, or *Ezh2* gene results in early embryonic lethality (1, 16, 21); interestingly, however, loss of the *Bmp4*, *Lif*, *Lif* receptor, or *Stat3* gene induces no obvious defect, at least in mouse preimplantation development (28, 31, 36, 39). It is known that LIF is dispensable for supporting the self-renewal and pluripotency of monkey and human ES cells (32).

NANOG is a newly identified homeodomain-bearing protein that may act as a transcription factor and that is transcribed specifically in pluripotential cells in mouse preimplantation embryos, ES cells, and EG cells (3, 15, 35) and monkey and human ES cells (8, 9). A critical requirement for *Nanog* in the maintenance of pluripotency has been suggested by the loss of pluripotency in *Nanog*-deficient ES cells and in *Nanog*-null embryos shortly after implantation (15). In addition, *Nanog* overexpression leads to the clonal expansion of ES cells by bypassing regulation by LIF-STAT3 signaling and maintenance of OCT4 levels (3). Thus, *Nanog* is an important regulator of pluripotency and self-renewal of ES cells and early embryonic cells. However, it remains largely unknown how the pluripotential cell-specific expression of *Nanog* is controlled and how the other stem cell-specific genes are implicated in *Nanog* expression.

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To address the molecular mechanisms of pluripotential cell-specific expression, we investigated the regulatory elements that are involved in the control of *Nanog* transcription. We show that the undifferentiated state-specific expression of a green fluorescence protein (GFP) reporter gene in mouse ES cells can be induced by the addition of a 2.5-kb 5′-flanking region of *Nanog*, indicating that transcriptional *cis* regulatory elements exist in this region. Luciferase assays with deletion constructs of the 5′-flanking region revealed that the −332-bp fragment (−332 fragment) containing a pair of adjacent Octamer and Sox elements plays a crucial role in directing transcriptional up-regulation. Consistent with these results, we found that transcription was down-regulated by the introduction of sequence mutations in the Octamer and/or Sox elements. In nuclear extracts from F9 EC cells, specific binding of OCT4 to the Octamer element and of SOX2 to the Sox element was detected. Similar results were seen in EG cell extracts. In ES cell extracts, however, a complex of OCT4 and a novel pluripotential cell-specific Sox element-binding protein (PSBP) preferentially bound to the Octamer/Sox element. *Nanog* transcription is therefore regulated differently in ES, EG, and EC cells, and a novel factor (PSBP) may be involved in maintaining the ES cell-specific undifferentiated state.

MATERIALS AND METHODS

Cell culture and differentiation. Mouse R1 ES, TMA-58G EG, and F9 EC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 15% fetal bovine serum, 10^{-4} M 2-mercaptoethanol, and 1,000 U of recombinant LIF (Chemicon)/ml at 37°C in 5% CO_2. Human ES cell line KhES-1 was cultured in DMEM with 20% knockout serum replacement (Invitrogen), 2 mM L-glutamine, nonessential amino acids, and 10^{-4} M 2-mercaptoethanol, according to 2001 guidelines for the derivation and utilization of human ES cells (Ministry of Education, Culture, Sports, Science and Technology of Japan). Mouse and human ES cells were maintained on mouse primary embryonic fibroblast feeder cells inactivated with mitomycin C. Embryoid bodies (EBs) were formed by suspension culturing for 5 days. Chemical differentiation induction was performed with 10^{-6} M all-*trans*-retinoic acid (Sigma). NIH 3T3 and COS-1 cells were cultured in DMEM containing 10% fetal bovine serum. Differentiation experiments were conducted with medium in the absence of LIF.

Transgenic cell lines. The *LR/Nanog*-GFP transgene was constructed with a GFP-internal ribosome entry site (IRES)-*puro-pA* reporter cassette and with a 2.5-kb 5′-end genomic fragment and a 3.9-kb 3′-end genomic fragment of *Nanog* in vector pGEM-T Easy (Promega). After linearization, the transgene was electroporated into 10^7 R1 ES cells at 250 V and 500 μF with a Gene Pulser (Bio-Rad). Genomic DNA obtained from puromycin-resistant clones was screened by Southern blot hybridization analysis. A stable transformant ES cell line, −332-GFP TG, was obtained as a G418-resistant clone after cotransfection of vector p−332-pEGFP-N1 (Clontech) and vector pPgk-neo(TAKARA) into R1 ES cells.

Southern blot hybridization analysis. Genomic DNA was digested with restriction enzymes, electrophoresed through 1.0% agarose, and transferred to Hybond N$^+$ nylon membranes (Amersham) by alkali blotting. Membranes were hybridized at 42°C overnight with either a 5′ probe (500 bp) or a 3′ probe (750 bp) labeled with ^{32}P-dCTP by using a Megaprime DNA labeling system (Amersham). Membranes then were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min and with 0.1× SSC–0.1% SDS at 65°C for 15 min.

Construction of reporter and expression vectors. Deletion fragments of the mouse *Nanog* promoter were PCR amplified from the mouse genome with a common antisense primer that spans an XhoI restriction site (+50 bp from the transcriptional start site, 5′-CTACTCGAGCGCAGCCTTCCCACAGAAA-3′) and various sense primers into which an XhoI restriction site was introduced (−2,342 bp, 5′-CTACTCGAGTGGTGTAAACAGTGGGTCTG-3′; −332 bp, 5′-CTACTCGAGATCGCCAGGGTCTGGA-3′; and −153 bp, 5′-CTACTCG AGCCTGCAGGTGGGATTAACT-3′). The PCR products were digested with XhoI and ligated into the XhoI site of pGL3-Basic (Promega) or cytomegalovirus promoterless vector pEGFP-N1 (Clontech) (−m332 or −m153). PCR fragments of the human *Nanog* promoter were amplified from human ES cell DNA

with a sense primer (−380 bp from the transcriptional start site) having the sequence 5′-GCTGGTTTCAAACTCCTGACTTC-3′ and an antisense primer (+24 bp) having the sequence 5′-TCCTGGAGTCTCTAGATT-3′ and ligated into vector pGEM-T Easy. NotI-NotI (−380 to +24 bp), NotI-PstI (−123 to +24 bp), and NotI-StyI (−101 to +24 bp) fragments were blunt ended and recloned into the SmaI site of cytomegalovirus promoterless vector pEGFP-N1. Oligonucleotide-directed mutations were introduced into the Octamer and/or Sox elements by PCR with primers having nucleotide replacements.

Oct4 and *Sox2* open reading frames (ORFs) amplified by reverse transcription-PCR with primers EcoRI-Oct4-ORF-F (5′-CCGAATTCGGATGGCTGGACA CCTGGCTTCAG-3′), BglII-Oct4-ORF-R (5′-AGAGATCTTTAACCCCAAA GCTCCAGGTTC-3′), EcoRI-Sox2-ORF-F (5′-CCGAATTCGGATGTATAA CATGATGGAGACGG-3′), and BglII-Sox2-ORF-R (5′-AGAGATCTTCACA TGTGCGACAGGGGCAGT-3′) were subcloned into vector pGEM-T Easy. EcoRI-BglII fragments of *Oct4* and *Sox2* were ligated into expression vectors pCMV-Myc and pCMV-HA (Promega), respectively.

For cotransfection reporter assays, three tandem repeats of the Octamer and Sox elements, which were produced by ligation of synthetic oligonucleotides (Nanog-O/S-F, 5′-GATCCTTACAGCTTCTTTTGCATTACAATGTCCATG GTGGA-3′; and Nanog-O/S-R, 5′-GATCTCCACCATGGACATTGTAATGC AAAAGAAGCTGTAAG-3′), were cloned into vector pTK-Luc (Clontech) to produce pTAL-Luc.

Transient expression assays were performed with Lipofectamine 2000 (Invitrogen). Samples were analyzed 2 days after transfection.

Luciferase reporter assays. ES (5.0×10^5), F9 (5.0×10^5), and NIH 3T3 (2.5×10^5) cells were incubated in six-well tissue culture plates for 24 h. Each reporter construct (1.25 pmol) was cotransfected with vector phRL-TK (0.125 pmol) (Promega) as an internal control by using Lipofectamine 2000. Cell extracts were prepared 48 h after transfection, and luciferase activities were evaluated by using a dual-luciferase assay system (Promega). The luciferase activity of each construct was calculated relative to that of control vector pGL3-Basic. All transfection experiments were repeated in triplicate. For cotransfection reporter assays, construct 3×Oct/Sox-pTK-Luc (0.06 pmol) was cotransfected with vectors pCMV-Myc-*Oct4* (0.6 pmol), pCMV-HA-*Sox2* (0.6 pmol), and phRL-TK (0.006 pmol) into NIH 3T3 cells. The promoter activities are reported as means ± standard errors.

Western blot hybridization analysis. Cell extracts (20 μg/lane) were separated through 12% polyacrylamide by SDS-polyacrylamide gel electrophoresis and transferred to Protran nitrocellulose membranes (Schleicher & Schuell). After blocking was done with 3% skim milk in phosphate-buffered saline for 1 h, the membranes were incubated with anti-NANOG (1:1,000 dilution) (9), anti-OCT4 (1:500) (Santa Cruz), anti-SOX2 (1:500) (Chemicon), anti-GFP (1:1,000) (Clontech), anti-Myc (1:1,000) (Covance), anti-hemagglutinin (HA) (1:1,000) (Covance), anti-histone H3 (1:3,000) (AbCam), or anti-β-actin (1:3,000) (AbCam) antibody overnight at 4°C. After washing was done with 0.1% Triton X-100 in phosphate-buffered saline, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3,000) (Amersham) for 90 min. Bands were detected with an ECL Western blotting detection kit (Amersham).

Electrophoretic mobility shift assays (EMSAs). Expression vectors pCMV-Myc-*Oct4* and pCMV-HA-*Sox2* were transfected into COS-1 cells by using Lipofectoamine 2000. Whole-cell lysates were collected 30 h after transfection (11). Nuclear extracts of F9 EC, TMA-58G EG, and R1 ES cells were prepared as reported previously (22). Double-stranded synthetic oligonucleotide probes were labeled with ^{32}P-dCTP. Whole-cell extracts (10 μg) and nuclear extracts (10 μg) were preincubated for 10 min on ice in the presence of 2 μg of poly(dG-dC) (Amersham) in 20 μl of reaction buffer (10 mM Tris-HCl [pH 7.5], 1 mM MgCl₂, 0.5 mM dithiothreitol, 10% glycerol) and then incubated with 0.1 ng of radiolabeled probes for 30 min. Competition or supershift assays were performed by adding 1- to 300-fold excess cold competitors or 1 μg of rabbit polyclonal anti-OCT4, goat polyclonal anti-SOX2, or rabbit polyclonal anti-SOX2 antibody (each from Santa Cruz) prior to treatment with radiolabeled probes. Probe DNA-protein complexes were separated by electrophoresis at 150 V through 4% polyacrylamide in 0.25× Tris-borate-EDTA buffer at 4°C for 135 min and visualized by autoradiography.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation was performed as described previously (10). Protein and DNA were cross-linked by incubation in 1% formaldehyde. The chromatin then was sonicated to an average DNA fragment length of 200 to 1,000 bp. Soluble chromatin reacted with or without 2 μg of rabbit polyclonal anti-OCT4 or goat polyclonal anti-SOX2 antibody (each from Santa Cruz) was purified and collected in elution buffer (0.1 M NaHCO₃, 1% SDS). Cross-linking then was reversed with elution buffer containing RNase A (0.03 mg/ml) and NaCl (0.3 M) by incubation for 4 h at 65°C. Supernatant obtained without antibody was used as an input control.
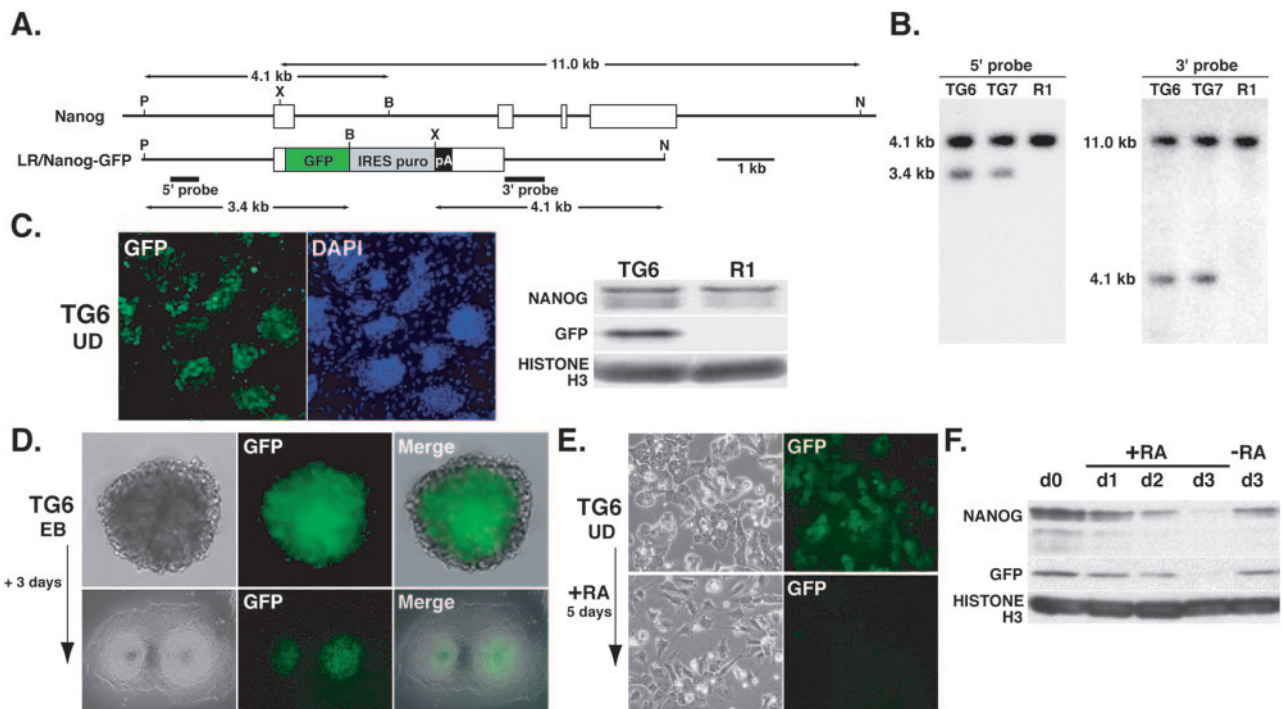
FIG. 1. Undifferentiated state-specific expression of the *LR*/*Nanog*-GFP transgene. (A) Structure of the *LR*/*Nanog*-GFP transgene containing 5′- and 3′-flanking regions. P, PvuII; X, XbaI; B, BglII; N, NcoI. (B) Southern blot hybridization analysis of TG6 and TG7 transgenic cell lines and the parental R1 ES cell line. The transgene-specific 3.4-kb PvuII-BglII fragment and the 4.1-kb XbaI-NcoI fragment were detected with 5′ and 3′ probes, respectively. (C) Expression of GFP in undifferentiated (UD) TG6 ES cells. GFP expression was visualized by fluorescence microscopy and Western blot hybridization analysis with anti-GFP antibody. Histone H3 was used as a control. (D) Expression of GFP restricted to undifferentiated ES cells located in the middle of 5-day-old EBs and in the center of colonies 3 days after culturing of 5-day-old EBs. (E) Down-regulation of GFP expression by in vitro differentiation with retinoic acid (RA) treatment for 5 days. (F) Western blot hybridization analysis of GFP and endogenous NANOG during RA-induced cell differentiation. Histone H3 was used as a control.

Following treatment with proteinase K for 1 h at 45°C, the DNA was purified and analyzed by PCR with the following primers: Nanog-O/S-ChIP-F, 5′-GTCTTT AGATCAGAGGATGCCCC-3′; Nanog-O/S-ChIP-R, 5′-CTACCCACCCCCT ATTCTCCCA-3′; Fgf4-O/S-ChIP-F, 5′-AGACTTCTGAGCAACCTCCCGA A-3′; and Fgf4-O/S-ChIP-R, 5′-CAACTGTCTTCTCCCCAACACTCT-3′.

RESULTS

Pluripotential state-specific expression of the *LR*/*Nanog*-GFP transgene. To explore the function of *Nanog* in regulating the stability of pluripotency and self-renewal of cells, we constructed a vector (*LR*/*Nanog*-GFP) with a 2.5-kb genomic fragment upstream of the 5′ end of the *Nanog* ORF and a 3.9-kb genomic fragment downstream of the 3′ end of the ORF (Fig. 1A). The full ORF of *Nanog* was replaced with a GFP-IRES-*puro-pA* reporter and selection cassette. By electroporation of the construct into R1 ES cells, 9 out of 11 GFP-positive clones examined were isolated as clones with random integration of the transgene(s). Southern blot hybridization analysis indicated that two clones (TG6 and TG7) carried intact 5′- and 3′-end genomic fragments. The 5′-end transgenic fragment was detected as a 3.4-kb band and the endogenous fragment was detected as a 4.1-kb band in PvuII-BglII-digested genomic DNA with a 5′-end-specific probe. The 3′-end transgenic fragment was detected as a 4.1-kb band and the endogenous fragment was detected as an 11.0-kb band in XbaI-NcoI-digested genomic DNA with a 3′-end-specific probe (Fig. 1B). The

transgene-specific bands were less intense than the endogenous bands in both cases, indicating that both clones contained a single integrated copy of the transgene. All of the undifferentiated TG6 and TG7 colonies were positive for GFP, as verified by both fluorescence microscopy and Western blot hybridization analysis (Fig. 1C).

To address whether the down-regulation of transgene expression would occur upon cell differentiation, as seen at the endogenous locus, GFP expression in 5-day-old EBs and in a 3-day-old culture of 5-day-old EBs without LIF or feeder cells was analyzed (Fig. 1D). GFP expression was not detectable in endodermal cells on the EB surface or in differentiated cells at the periphery of the adhesive colonies. To determine whether the down-regulation of GFP expression was linked to that of endogenous NANOG expression, ES clones were differentiated by chemical induction with retinoic acid (RA). After 5 days of continuous RA treatment, the cell phenotype changed significantly and GFP became undetectable (Fig. 1E). Western blot hybridization analysis revealed that decreasing GFP levels correlated well with decreasing NANOG levels after RA treatment (Fig. 1F). These data demonstrated that regulatory elements required for pluripotential state-specific regulation of *Nanog* gene expression are located on the transgene.

Octamer and Sox elements are required for *Nanog* expression. To further characterize the region required for *Nanog* expression, two additional GFP reporter transgenes (carrying a

−2342 or −332 5′-end genomic fragment but no 3′-end fragment) were constructed and transfected into ES cells. In addition, the constructs were transfected into NIH 3T3 fibroblast cells, which do not express endogenous *Nanog*. A pCMV-GFP construct was used in parallel to control for transfection efficiency. With both experimental constructs, GFP was highly expressed in ES cells but not in fibroblasts (Fig. 2A), while the control construct was highly expressed in both cell types (data not shown). These results suggested that regulatory elements essential for pluripotential state-specific expression are located in the 382-bp region immediately upstream of *Nanog*. To confirm this notion, stably transformed −332-GFP TG ES cells were generated and differentiated in vitro through RA treatment for 5 days. GFP expression that was observed in undifferentiated −332-GFP TG ES cells was completely suppressed on differentiation.

To evaluate the level of transcriptional activity, reporter gene expression was monitored by luciferase assays with deletion fragments of the 5′-flanking region in R1 ES, F9 EC, and NIH 3T3 cells (Fig. 2B). Consistent with the results of the GFP reporter assays in ES cells, the −332 fragment induced high-level luciferase expression in both ES and EC cells. While the slight decrease in activity seen with the −2342 and −332 fragments was statistically significant ($P < 0.05$), a much more notable decrease was observed with the −153 fragment, resulting in approximately 15% activity in comparison to that obtained with the −2342 fragment. This level of activity was similar in all cell lines tested, suggesting that this activity was nonpluripotential state-specific basal promoter activity. These results suggested that a key *cis*-acting pluripotency-specific element(s) lies within the region between positions −332 and −154.

Sequence analysis demonstrated the presence of conserved Octamer (TTTTGCAT) and Sox (TACAATG) elements between positions −166 and −180 (Fig. 2C). To examine the functions of these elements, triple point mutations were introduced by replacement of DNA residues in each element (Fig. 2D). Luciferase assays with −332 fragments carrying mutated elements clearly showed a dramatic reduction in luciferase activities in all cases, to levels similar to that found with the −153 fragment (Fig. 2E). Thus, factors that interact with the Octamer and Sox elements are likely to play a crucial role in regulating the expression of *Nanog* in a pluripotential state-specific manner.

Octamer and Sox elements regulate expression in human ES cells. *Nanog* has also been identified in humans and monkeys, in which specific expression in the nuclei of undifferentiated ES cells has been shown by immunocytochemical analysis with anti-NANOG antibody (9). Comparative DNA sequence analysis of the region spanning from −300 bp to the translational start site revealed a high degree of conservation between humans and monkeys (93.2%) but not between humans and mice (54.1%) or between monkeys and mice (51.7%). However, the Octamer and Sox elements present in both humans and monkeys showed 100% identity to the mouse sequence (Fig. 3A).

To address whether the Octamer and Sox elements are required as *cis* regulatory elements for *Nanog* transcription in humans, human −h380, −h123, and −h101 fragments were linked to GFP and transfected into mouse and human ES cells

(Fig. 3B). The −h380 and −h123 fragments produced relatively high-level expression in both mouse and human ES cells, while the −h101 fragment, in which the Octamer and Sox elements were missing, promoted very low-level expression. For human ES cells, these findings were confirmed by Western blot hybridization analysis (Fig. 3C). Similarly, the mouse −m332 fragment promoted high-level expression in human ES cells, while the −m153 fragment induced low-level expression (Fig. 3D). A similar considerable reduction in GFP expression was also seen with −m332 fragments carrying mutations in the Octamer and Sox elements (Fig. 2D) (data not shown). These results suggested that the Octamer and Sox elements play a similar role in regulating *Nanog* transcription in humans and mice.

OCT4 and SOX2 bind to the Octamer and Sox elements. OCT4 and SOX2 are expressed in pluripotential cells and are well characterized as factors that bind to the Octamer and Sox elements, respectively. Indeed, it has been shown that they bind to these elements in regions proximal to the pluripotential cell-specific *Fgf4*, *Utf1*, *Sox2*, and *Fbx15* genes, where they act synergistically to activate transcription (17, 33, 34, 42). We therefore investigated the abilities of OCT4 and SOX2 to bind to the *Nanog* Octamer and Sox elements by EMSAs. Exogenous Myc-tagged *Oct4* (Myc-*Oct4*) and HA-tagged *Sox2* (HA-*Sox2*) were transfected into COS-1 cells, in which endogenous OCT4 and SOX2 are repressed. Western blot hybridization analyses with antibodies against OCT4, SOX2, Myc, and HA showed that exogenous OCT4 and SOX2 were abundant in whole extracts from COS-1 cells carrying the transgene(s) (Fig. 4A).

DNA fragments containing the Octamer and Sox elements of *Nanog* or *Fgf4* were synthesized and radiolabeled for use as specific EMSA probes (Fig. 4B). Following incubation of the Fgf4 probe with COS-1 cell extracts, Myc-OCT4, HA-SOX2, and Myc-OCT4/HA-SOX2 complexes were clearly detectable, as previously reported (42). Incubation of the Nanog probe with COS-1 cell extracts also resulted in clearly detectable binding of Myc-OCT4 and Myc-OCT4/HA-SOX2 complexes (Fig. 4C). Importantly, the affinity of binding of SOX2 alone to the *Nanog* Sox element was relatively weak, whereas SOX2 in combination with OCT4 resulted in the formation of a much stronger ternary protein-DNA complex. The specificity of OCT4 and/or SOX2 binding was confirmed by supershift analyses with anti-Myc and anti-HA antibodies (unpublished data).

To examine whether OCT4 and SOX2 activate *Nanog* expression by binding to their respective elements, reporter assays were performed with NIH 3T3 cells and with a pTK-Luc construct into which three tandem repeats of the Octamer/Sox element had been introduced (Fig. 4D). Cotransfection of this construct with Myc-*Oct4* and HA-*Sox2* led to an ∼3.5-fold increase in luciferase activity. Taken together, our data clearly show that the *Nanog* Octamer and Sox elements are able to recruit OCT4 and SOX2, respectively, leading to the up-regulation of *Nanog* gene expression.

Binding of OCT4 and SOX2 to Octamer and Sox elements in EC cell extracts. F9 cells are EC cells that have defective pluripotency and that were derived from embryonic day 6.0 (E6.0) embryos through carcinogenesis (27). As in R1 ES cells, OCT4, SOX2, and NANOG are expressed in the nuclei of F9 EC cells, as shown by Western blotting hybridization analyses
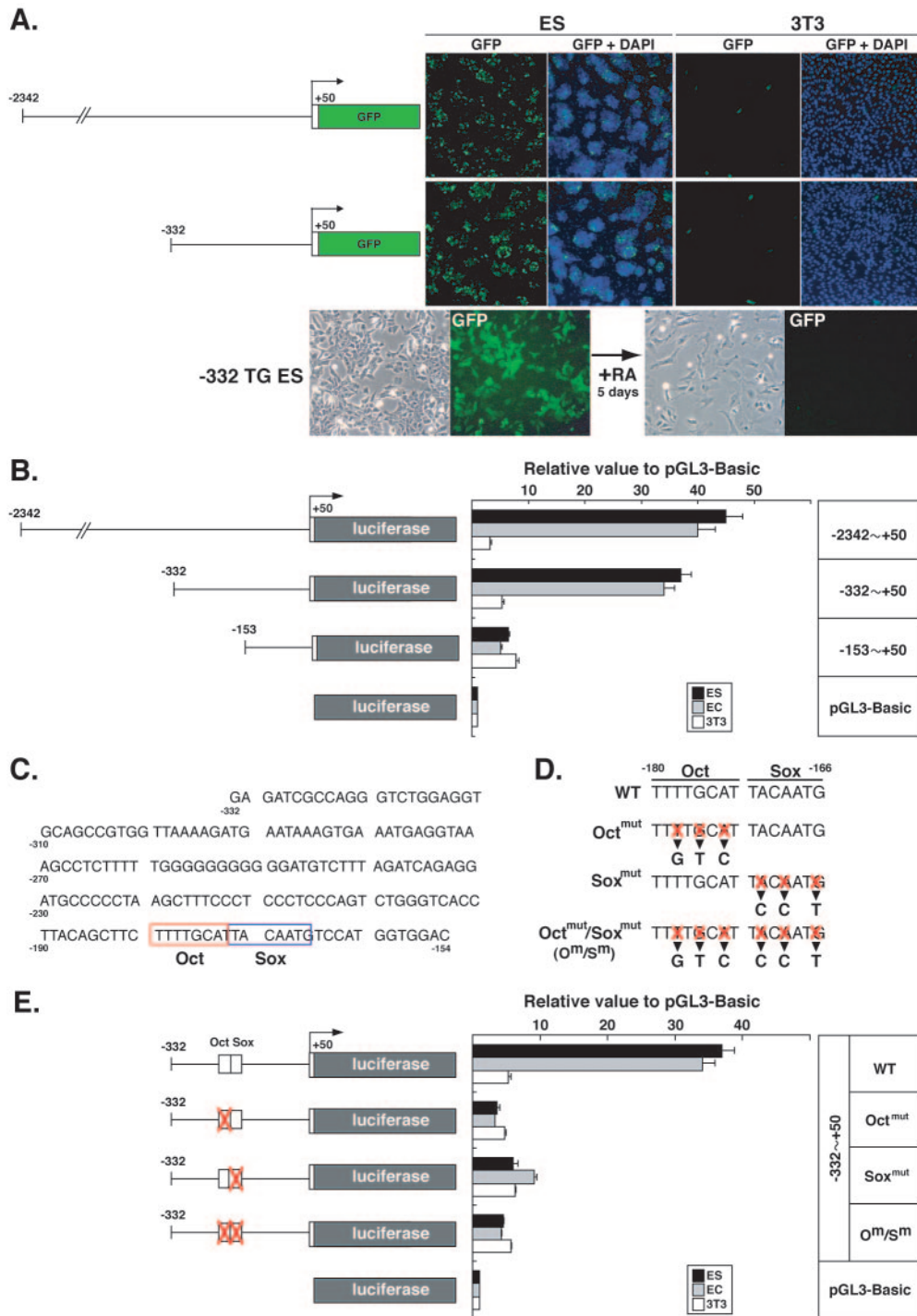
FIG. 2. Octamer and Sox elements are required for *Nanog* expression. (A) Transient expression of GFP transgenes with −2342 or −332 5′-end fragments in R1 ES and NIH 3T3 cells. Transcriptional down-regulation of GFP was detected in differentiated −332-GFP TG ES (−332 TG ES) cells by treatment with retinoic acid (RA). (B) Luciferase assays with deletion constructs in R1 ES, F9 EC, and NIH 3T3 cells. Luciferase activities are shown relative to those of pGL3-Basic. Bars represent the means ± standard errors of three independent experiments. (C) DNA sequence of the mouse 5′-flanking region between positions −332 and −154. Octamer (Oct) and Sox elements are outlined in red and blue, respectively. (D) Sequence mutations introduced into Octamer and/or Sox elements. (E) Luciferase assays with the −332 5′-end fragment with or without mutations in Octamer and/or Sox elements in R1 ES, F9 EC, and NIH 3T3 cells. Luciferase activities are shown relative to those of pGL3-Basic. Bars represent the means ± standard errors of three independent experiments.
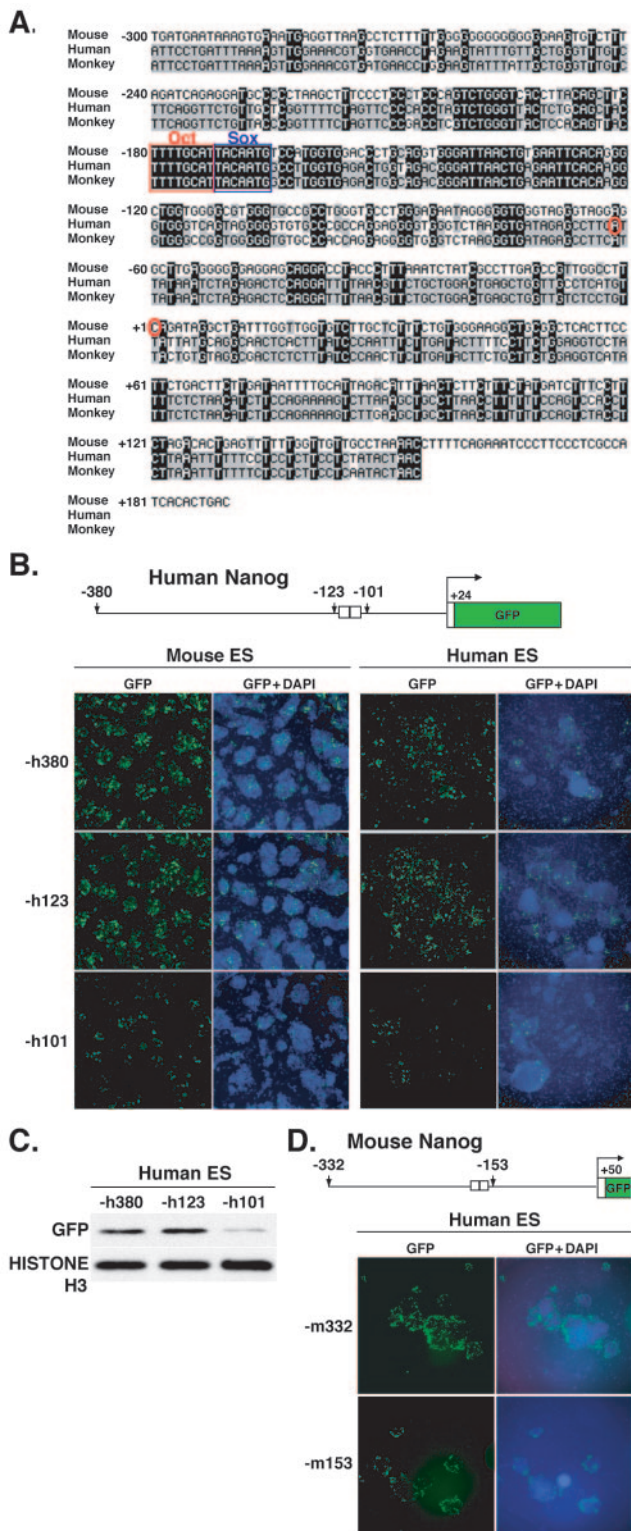
FIG. 3. Expression of the human *Nanog* reporter gene in mouse and human ES cells. (A) Comparative DNA sequence analysis of the *Nanog* 5′-flanking regions of mice, monkeys, and humans. Octamer (Oct) and Sox elements are outlined in red and blue, respectively. Red circles show putative transcriptional start sites. Identical nucleotides are highlighted in black. (B) Transient GFP expression under the regulation of the human *Nanog* promoter in mouse and human ES cells. The −h380 and −h123 5′-end fragments contain both Octamer

(Fig. 5A). To examine the abilities of endogenous OCT4 and SOX2 to bind to the *Nanog* Octamer and Sox elements, nuclear extracts from F9 cells were prepared for EMSAs. Incubation with the radiolabeled Nanog probe resulted in a prominent band at the position of the OCT4/SOX2 complex, while an independent SOX2-specific band was barely detectable (Fig. 5B). The complex mobilities correlated well with those found when COS-1 cell extracts containing exogenous Myc-OCT4 and HA-SOX2 were used.

To further investigate the factors binding to the Octamer and Sox elements, binding competition assays were performed with unlabeled Nanog probe or Nanog probe mutated in the Octamer element (Oct^{mut}), the Sox element (Sox^{mut}), or both (Fig. 5C). Depletion of a factor(s) binding to the Sox element by the Oct^{mut} competitor probe resulted in a decrease in OCT4/SOX2 complex binding and an increase in OCT4 binding that correlated well with increasing competitor probe concentration. Depletion of a factor(s) binding to the Octamer element by the Sox^{mut} competitor probe also caused a decrease in OCT4/SOX2 complex binding but no reciprocal increase in OCT4 binding. It should be noted that the binding of SOX2 alone was barely detectable due to a low affinity of binding to the *Nanog* Sox element (Fig. 4C). As expected, the Oct^{mut}/Sox^{mut} competitor probe had little effect, while the unlabeled Nanog probe induced a decrease in the binding of OCT4 and the OCT4/SOX2 complex. The binding of OCT4 and the OCT4/SOX2 complex was also verified by competition assays with depletion by the unlabeled Fgf4 probe. These data demonstrated that endogenous OCT4 and OCT4/SOX2 complex are able to bind to the *Nanog* Octamer and Sox elements in F9 cells.

While we were unable to demonstrate specificity for SOX2 in these initial experiments, further analysis by supershift assays clearly demonstrated that both OCT4 and SOX2 are involved, since the inclusion of anti-OCT4 and anti-SOX2 antibodies resulted in reduced complex mobility (supershift), while the inclusion of rabbit and goat control IgGs had no effect (Fig. 5D). Thus, endogenous OCT4 and SOX2 form ternary protein-DNA complexes with the *Nanog* Octamer and Sox elements in F9 EC cell extracts.

We next examined the in vivo potential for the binding of OCT4 and SOX2 to the *Nanog* elements in ES and EC cells by chromatin immunoprecipitation assays with anti-OCT4 and anti-SOX2 antibodies (Fig. 5E). Exogenously expressed OCT4 and SOX2 bound to the Octamer and Sox elements, respectively, in both R1 ES and F9 EC cells, as seen for the *Fgf4* Octamer and Sox elements.

A novel factor binds to Octamer and Sox elements in ES cell extracts. R1 ES cells, TMA-58G EG cells, and F9 EC cells share important properties, which include a robust capacity for self-renewal and expression of NANOG, OCT4, and SOX2 (Fig. 5A). However, full pluripotency is retained only by ES

and Sox elements, while the −h101 5′-end fragment does not. (C) Western blot hybridization analysis of GFP expression in human ES cells. Histone H3 was used as a control. (D) Transient GFP expression under the regulation of the mouse *Nanog* promoter in human ES cells. The −m332 5′-end fragment contains the Octamer and Sox elements, while the −m153 5′-end fragment does not.
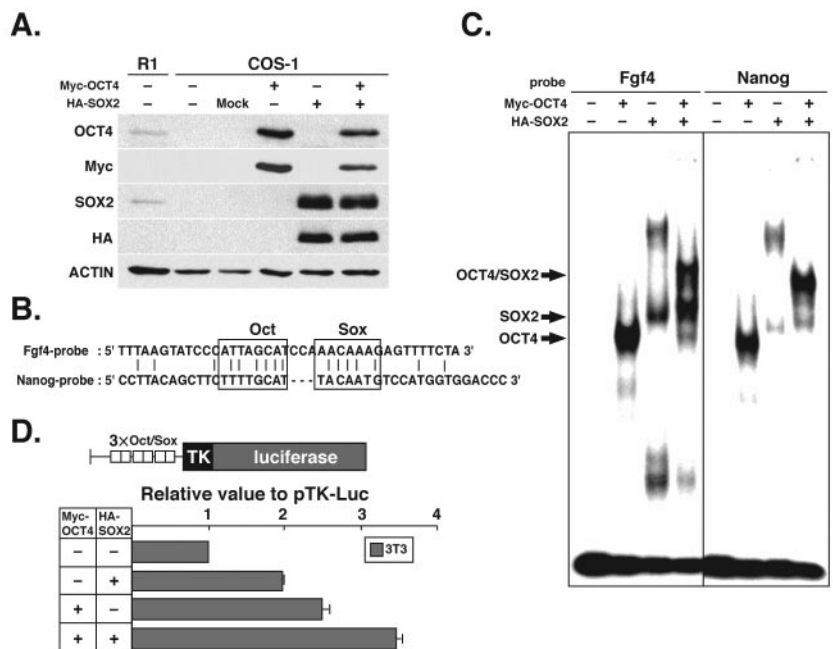
FIG. 4. Binding of exogenous OCT4 and SOX2 to Octamer and Sox elements in COS-1 cells. (A) Western blot hybridization analysis of exogenous Myc-tagged OCT4 and HA-tagged SOX2 expression with anti-OCT4, anti-SOX2, anti-Myc, and anti-HA antibodies. Actin was used as a control. (B) DNA sequences of Nanog and Fgf4 probes. Octamer (Oct) and Sox elements are outlined. (C) EMSA with Nanog and Fgf4 probes and COS-1 cells. Bands of the OCT4-DNA, SOX2-DNA, and OCT4/SOX2-DNA complexes are indicated. (D) Cotransfection reporter assays with Oct4 and Sox2 expression constructs in NIH 3T3 cells. Bars represent the means ± standard errors of three independent experiments.

cells. Notably, R1 ES cells were isolated from normal inner cell mass cells of blastocyst-stage E3.5 embryos, while F9 EC cells were derived from E6.0 embryos through carcinogenesis and TMA-58G EG cells were derived from homing primordial germ cells in genital ridges of E12.5 embryos. Thus, some of the factors involved in maintaining pluripotency in ES cells may not be present in EC or EG cells. We therefore repeated the EMSA, binding competition, and supershift assays with nuclear extracts from R1 ES cells and TMA-58G EG cells. Strikingly, the major complex that formed when ES cell extracts were used was clearly distinct from the OCT4/SOX2 complex detected in EC cell extracts or in EG cell extracts (Fig. 6A). The same complex was detected in ES cell extracts with both the Nanog and the Fgf4 probes. A decrease in the band intensity of the ES cell-specific major complex in binding competition assays with the Oct^{mut} or Sox^{mut} competitor probe demonstrated that the ES cell major complex required both the Octamer and the Sox elements for stable binding (Fig. 6B). This finding was confirmed by a lack of competition when the Oct^{mut}/Sox^{mut} competitor probe was used. Supershift assays revealed that ES cell major complex mobility was reduced following incubation with anti-OCT4 antibody whereas, in contrast to the findings for F9 cell nuclear extracts (Fig. 5D), anti-SOX2 antibody had little effect on complex mobility (Fig. 6C). This finding was confirmed with a second anti-SOX2 antibody. These data showed that OCT4 is an essential component of the ES cell major complex but that SOX2 is not. Thus, another as-yet-undefined component (PSBP) preferentially associates with OCT4 to form an ES cell-specific complex on the Octamer and Sox elements in the *Nanog* promoter region.

As EMSAs carried out with the Fgf4 probe gave results similar to those obtained with the Nanog probe, it is evident that the ES cell-specific OCT4/PSBP complex is not specific for the *Nanog* sequence (Fig. 6A). This conclusion was confirmed by preincubation with anti-OCT4 antibody, which resulted in a loss of the OCT4/PSBP complex, whereas preincubation with anti-SOX2 antibody had no effect (unpublished data). Therefore, in ES cells, some genes that have Octamer and Sox elements as *cis* regulatory elements may be regulated in part by the synergistic action of OCT4 and PSBP in preference to OCT4 and SOX2.

DISCUSSION

The pluripotential state-specific gene *Nanog* is transcribed under the control of a regulatory region that lies within 332 bp upstream of the transcriptional start site. This region contains Octamer and Sox elements, which are highly conserved among the 5′-flanking regions of the mouse, monkey, and human *Nanog* genes. Indeed, the mouse and human Octamer and Sox elements were sufficient for up-regulating GFP reporter gene activity in both mouse and human ES cells. In nuclear extracts of F9 EC cells, OCT4 and SOX2, which are coexpressed in undifferentiated stem cells, were capable of binding to the Octamer and Sox elements, respectively. Interestingly, in nuclear extracts of ES cells, OCT4 dominantly bound to the Octamer element, while an undefined factor (PSBP) preferentially bound to the Sox element (Fig. 6D). Thus, *Nanog* expression may be dominantly controlled by an interaction between OCT4 and PSBP in ES cells.

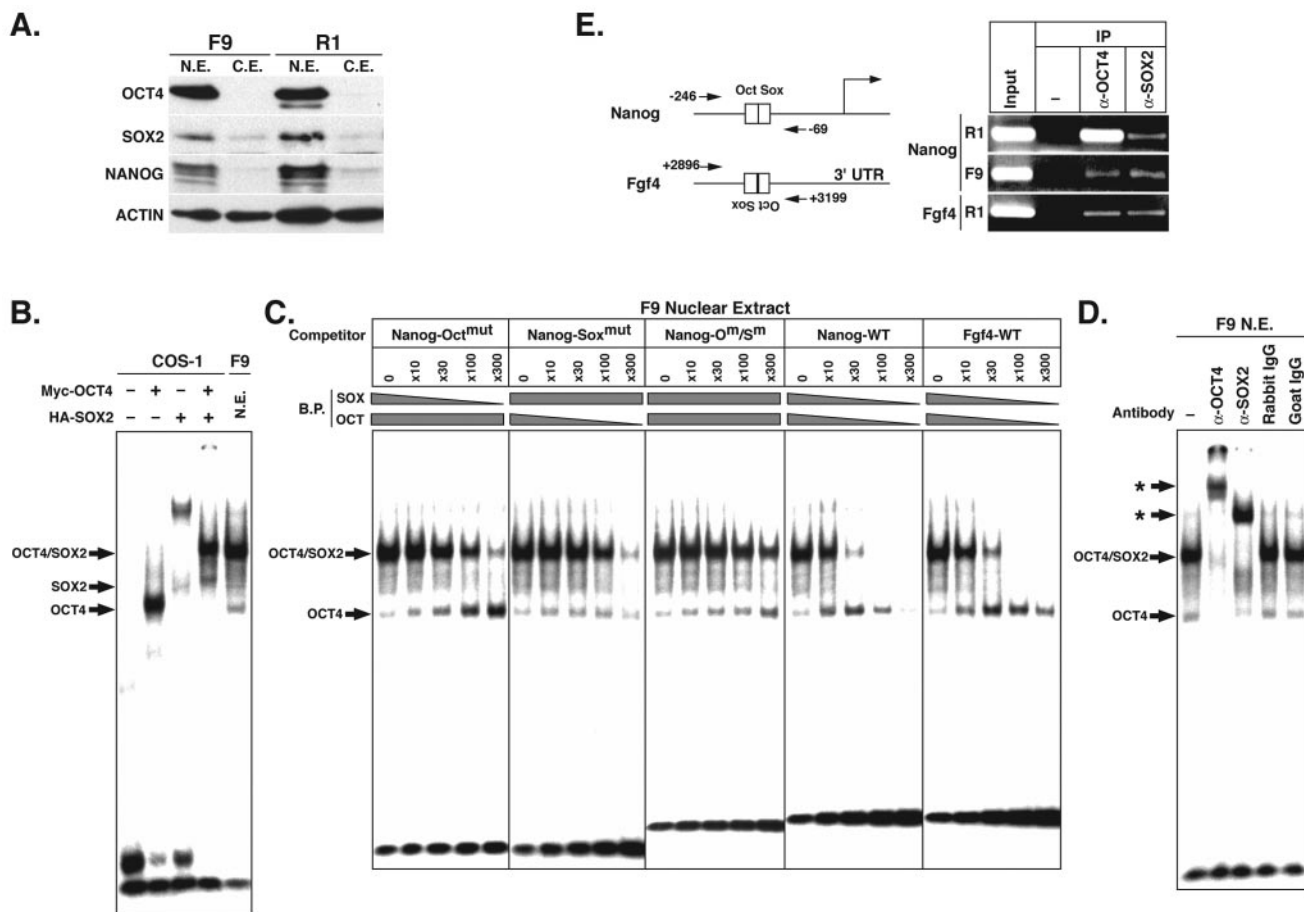It is evident that the Octamer and Sox elements are required

FIG. 5. Binding of endogenous OCT4 and SOX2 to Octamer and Sox elements in F9 EC cells. (A) Western blot hybridization analysis of endogenous OCT4, SOX2, and NANOG in nuclear extracts (N.E.) and cytoplasmic extracts (C.E.) of F9 EC and R1 ES cells. (B) EMSA with the Nanog probe and nuclear extracts of COS-1 and F9 EC cells. Bands of the OCT4-DNA, SOX2-DNA, and OCT4/SOX2-DNA complexes are indicated. (C) Competition assays with unlabeled probes with or without mutations in Octamer and/or Sox elements. Relative amounts of binding proteins (B.P.) are indicated by grey bars. Bands of the OCT4-DNA and OCT4/SOX2-DNA complexes are indicated. Oct^{mut} (O^m), Nanog probe with triple mutations in the Octamer element; Sox^{mut} (S^m), Nanog probe with triple mutations in the Sox element. (D) Supershift assay with anti-OCT4 or anti-SOX2 antibody and F9 EC cell nuclear extracts. Bands of the OCT4-DNA and OCT4/SOX2-DNA complexes are indicated by arrows, and supershifted bands are indicated by asterisks. Rabbit IgG and goat IgG were used as controls. (E) Chromatin immunoprecipitation assay demonstrating the in vivo potential of OCT4 and SOX2 to bind to *Nanog* and *Fgf4* Octamer and Sox elements, respectively.

for the up-regulation of mouse and human *Nanog* transcription and that factors that bind to these elements act to promote *Nanog* transcription through synergistic molecular interactions. Adjacent Octamer and Sox elements have been identified as *cis* regulatory elements in the *Fgf4*, *Sox2*, *Utf1*, and *Fbx15* genes, which are expressed in EC and ES cells and during embryogenesis (17, 33, 34, 42). *Sox2* is a member of the Sox (SRY-related HMG box) family that bears DNA-binding HMG domains and that is implicated in transcriptional regulation. The gene is expressed in pluripotential embryonic cells and neuronal cells (1). In pluripotential embryonic cells, expression is governed by at least two regulatory regions, the 5′-flanking region containing the CCAAT box and the 3′-flanking region containing the Octamer and Sox elements (34, 37). *Fgf4* is a member of the fibroblast growth factor family that is expressed in blastocyst inner cell mass cells and in developing embryos, as well as in ES and EC cells (19). Both Octamer and Sox elements are located in the intragenic 3′ untranslated

region, while a GT-box motif is located in the 3′-flanking region. Both regions are required for mediating optimal transcriptional *Fgf4* activation (13, 14). Thus, the expression of some pluripotential embryonic cell-specific genes appears to require the action of Octamer and Sox elements in combination with other gene-specific *cis* regulatory elements. For *Nanog*, luciferase assays with ES cell extracts demonstrated that activity controlled by the −2342 5′-end genomic fragment was about 15% higher than that controlled by the −332 5′-end genomic fragment (Fig. 2B); this finding implied that an unidentified *cis* regulatory element(s) lying in the region from position −332 to position −2342 may function, in combination with the Octamer and Sox elements, in enhancing and determining the specificity of *Nanog* expression.

There is significant evidence that *Nanog* plays a key role in maintaining the pluripotency of ES cells and embryonic cells. *Nanog*-deficient ES and embryonic cells show a complete loss of pluripotency (9, 15), whereas *Nanog* overexpression results
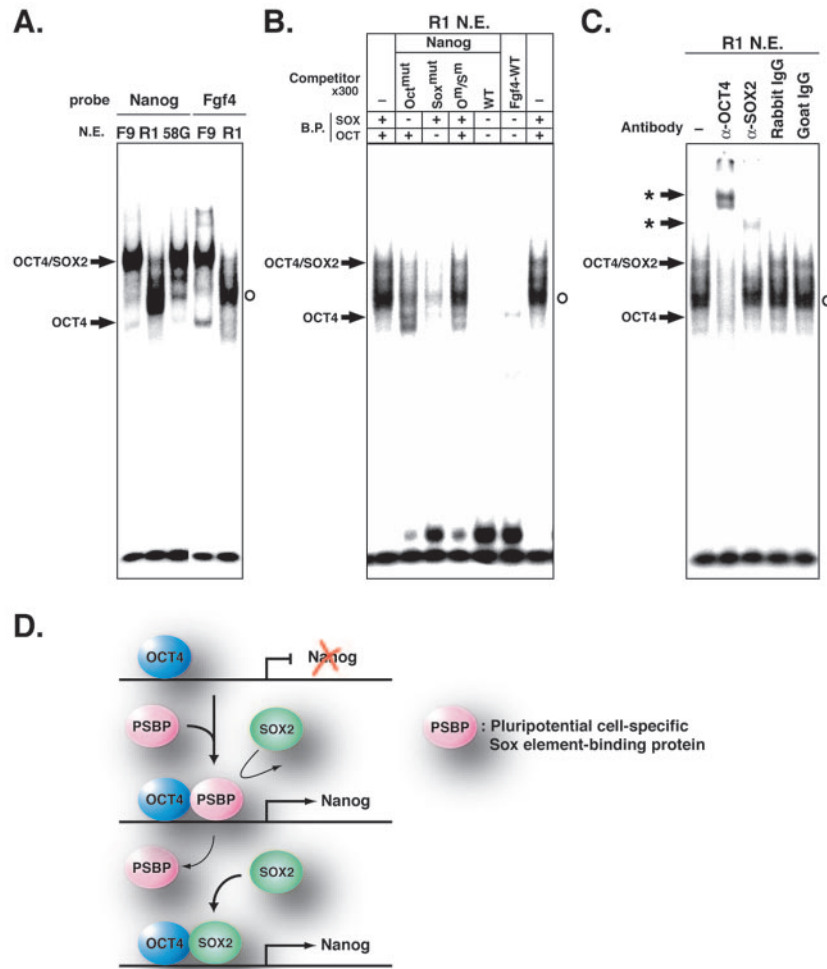
FIG. 6. Binding of endogenous OCT4 and PSBP to Octamer and Sox elements in R1 ES cells. Bands of the OCT4-DNA and OCT4/SOX2-DNA complexes are indicated by arrows, bands of the OCT4/PSBP-DNA complex are indicated by open circles, and supershifted bands are indicated by asterisks. (A) EMSA with Nanog and Fgf4 probes and nuclear extracts of F9 EC, TMA-58G EG, and R1 ES cells. (B) Competition assay with unlabeled probes with or without mutations in Octamer and/or Sox elements. (C) Supershift assay with anti-OCT4 or anti-SOX2 antibody and R1 ES cell nuclear extracts. Rabbit IgG and goat IgG were used as controls. (D) Schematic model for transcriptional regulation of *Nanog* in ES cells. In R1 ES cells but not in F9 EC cells, the OCT4/PSBP complex dominantly up-regulates *Nanog* transcription by binding to the Octamer/Sox element. PSBP binds to the Sox element with a greater affinity than SOX2.

in the clonal expansion of ES cells via the bypassing of regulation by LIF-STAT3 signaling and maintenance of OCT4 levels (3). Therefore, it is important to understand how the expression of *Nanog* acts in harmony with the expression of other embryonic factors through molecular communications in the stem cell-specific regulatory network. Our data clearly show that the ternary protein-DNA complexes of OCT4/PBSP and Octamer and Sox elements efficiently formed even in the presence of SOX2. The data also suggest that this complex formation is essential for the activation of *Nanog* transcription in ES cells. During embryonic development, mouse *Nanog* RNA and protein expression can be detected from the morula stage to the epiblast stage of E7.5 embryos (8, 9, 15). The pattern of expression of *Oct4* and *Sox2* is temporally similar. Interestingly, in E7.5 embryos, *Nanog* expression is spatially enhanced in the caudal region (primitive streak region) of the epiblast (8, 9), whereas *Sox2* expression is restricted to the presumptive

neuroectoderm in the anterior and is excluded from the posterior (primitive streak region) (1). These data suggest that the OCT4/PSBP complex may activate *Nanog* expression in vivo. In primordial germ cells of E7.5 embryos, *Oct4* and *PGC7/ Stella* are expressed, whereas *Nanog* is repressed (9, 23, 40). However, *Nanog* is expressed in primordial germ cells at E11.5 and in EG cells derived from E12.5 embryos (3, 9). Our EMSA data (Fig. 6A) suggest that OCT4 and SOX2 may function in this up-regulation of *Nanog* transcription, although the profile of expression of SOX2 in germ cells is not fully understood. Thus, even though the control of *Nanog* expression is closely linked to the *Oct4* regulatory network, *Oct4* expression alone is insufficient for inducing *Nanog* expression. We suggest that the expression of *Nanog* is tightly regulated by competing coactivators (SOX2 and PSBP) in different cell types, which have different affinities for the *Nanog* Sox element sequence.

To understand the relationship between *Nanog* and *Oct4*,

Nanog transcription in *Oct4*-deficient embryos was analyzed (3). mRNA in situ hybridization analysis demonstrated that *Nanog* expression was maintained in the blastocyst inner cell mass cells, suggesting that other pluripotential cell-specific factors may contribute to alternative transcriptional regulatory mechanisms. It has been shown that OCT1 and OCT6 are expressed in pluripotential embryonic cells (24, 29) and have the capacity to bind to Octamer elements in the *Fgf4*, *Sox2*, *Utf1*, and *Rex-1* (*Zfp-42*) promoter regions (2, 5, 17, 34). Thus, instead of OCT4, OCT1 or OCT6 may participate to form the DNA-protein complex with SOX2 or PSBP on the *Nanog* Octamer and Sox elements. In fact, it has been reported that OCT6 but not OCT1 can form a complex with SOX2 on the Octamer and Sox elements in *Sox2* (34). However, the affinity of OCT1 and OCT6 for binding to the *Nanog* Octamer element (TTTTGCAT) is low relative to that for binding to the consensus Octamer element (ATTAGCAT) (18). It remains to be explored whether other pluripotential genes contribute to the up-regulation of *Nanog* activity through interactions with the Octamer and Sox elements and whether *Nanog* expression in *Oct4*-null mutants is quantitatively equivalent to that in normal embryos.

ES cells promise to serve as an unlimited cell source of therapeutic materials for use in regenerative medicine. In clinical applications, it would be crucial to monitor the undifferentiated state of ES cells through numerous cell divisions and to selectively eliminate populations of spontaneously differentiated cells in cultures. Furthermore, following tissue-specific cell differentiation induction, a tool for the selective elimination of pluripotential ES cells is desirable to avoid contamination with a potential source for generating malignant tumors in vivo. For such purposes, the *Nanog* minimum promoter encompassing the Octamer and Sox elements may be a suitable tool for positive and negative selection of undifferentiated stem cells. Furthermore, genetically engineered human stem cells containing the herpes simplex virus thymidine kinase gene have been generated for selective elimination of undifferentiated ES cells with ganciclovir after in vitro and in vivo differentiation (25). In this context, the human *Nanog* promoter is an ideal candidate element for regulating the expression of a pluripotential cell-specific suicide gene. Further understanding of the mechanisms that regulate *Nanog* gene expression will also contribute to the field of stem cell engineering and its application to regenerative medicine.

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