Oct4 and Sox2 Directly Regulate Expression of Another Pluripotency Transcription Factor, Zfp206, in Embryonic Stem Cells

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It is well known that Oct4 and Sox2 play an important role in the maintenance of embryonic stem cell pluripotency. These transcription factors bind to regulatory regions within hundreds of target genes to control their expression. Zfp206 is a recently characterized transcription factor that has a role in maintaining stem cell pluripotency. We have demonstrated here that Zfp206 is a direct downstream target of Oct4 and Sox2. Two composite sox-oct binding sites have been identified within the first intron of Zfp206. We have demonstrated binding of Oct4 and Sox2 to this region. In addition, we have shown that Oct4 or Sox2 alone can activate transcription via one of these sox-oct elements, although the presence of both Oct4 and Sox2 gave rise to a synergistic effect. These studies extend our understanding of the transcriptional network that operates to regulate the differentiation potential of embryonic stem cells.

Embryonic stem cells (ESCs)2 are derived from the inner cell mass of the blastocyst and exhibit both pluripotency and self-renewing capabilities. For proper developmental outcome, ESCs must tightly regulate their differentiation status, and through continuing study, the molecular basis of that regulation process is beginning to emerge. Systematic, genome-wide interrogations have identified hundreds of genes, including several transcription factors, which have expression patterns tightly correlated with ES cell differentiation (1–6). Two key transcription factors, Oct4 and Sox2, have been identified that are crucial for maintenance of the pluripotent state of ESCs (7, 8). ESCs lose the capacity to maintain pluripotency upon knockdown of expression of these transcription factors by RNA interference (9, 10). Gene knock-out studies confirm the importance of Oct4 and Sox2 for early embryonic development. It has been demonstrated by chromatin immunoprecipitation studies that Oct4 and Sox2 bind to a few thousand regulatory sites in the ES cell genome (11, 12). It is likely that many of these target genes play a role in modulating ES cell differentiation. Indeed, the transcription factor Nanog, an established regulator of pluripotency, is transcriptionally regulated directly by Oct4 and Sox2 (13).

Zfp206 is a transcription factor that is highly expressed in mouse and human ESCs and down-regulated upon differentiation (3, 14). Zfp206 contains a SCAN domain and 14 zinc-finger domains, which suggests that it may be a transcription factor that binds DNA directly. Zfp206 is expressed in the inner cell mass but not in trophectoderm, suggesting that it may play a role in establishing cell fate decisions regarding embryonic versus extraembryonic tissue (15). There is wide temporal and spatial distribution of RNA and protein in the early embryos, indicating that Zfp206 may regulate multiple cell fate decisions (14). Recent data have demonstrated that overexpression of Zfp206 promotes the formation of undifferentiated mouse ESC colonies in vitro (14). We have obtained similar results and further found that overexpression of Zfp206 renders ESCs resistant to retinoic acid-induced differentiation, whereas knocked down expression of Zfp206 made ESCs more easily differentiated by retinoic acid (data to be published elsewhere).3 The pattern of expression and functional impact of Zfp206 on ESC differentiation establishes that this transcription factor plays an important role in maintaining pluripotency.

Given that Zfp206 plays a key role in controlling ESC differentiation, it is important to understand how this transcription factor fits into the network of other transcription factors involved in maintaining pluripotency of ESCs, such as Oct4 and Sox2. Oct4 (also known as Oct3 and encoded by Pou5f1) is a POU domain-containing transcription factor that binds an octamer sequence, ATGCAAAT (12, 16). Sox2 is an HMG domain-containing transcription factor that binds to the consensus motif CATGGTT (17, 18). Oct4 and Sox2 were shown to reciprocally regulate Pou5f1 and Sox2 transcription via the Oct4-Sox2 complex in ESCs (16). In addition, Oct4 and Sox2 positively regulate Nanog, revealing that a tight transcriptional

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2. The abbreviations used are: ESC, embryonic stem cell; EMSA, electrophoretic mobility shift assay; RNAi, RNA interference; siRNA, small inhibitory RNA; ChIP, chromatin immunoprecipitation; shRNA, short hairpin RNA.
3. Z.-X. Wang and L. W. Stanton, unpublished data.
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ES Cell Culture—E14 mouse ESCs (American Type Culture Collection [ATCC]), either cultured on mouse primary embryonic fibroblast feeders or under feeder-free conditions, were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum (ESC-qualified; Invitrogen), 0.055 mM β-mercaptoethanol (Invitrogen), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 5000 units/ml penicillin/streptomycin, and 1000 units/ml of leukemia inhibitory factor (Chemicon) and maintained at 37 °C with 5% CO₂. The V6.4 (129S4/SvJae × C57BL/6j)F₁ ES cells cultured on mouse embryonic fibroblasts were maintained in Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum, 0.055 mM β-mercaptoethanol, 4 mM L-glutamine, 40 μg/ml gentamicin sulfate (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 1000 units/ml leukemia inhibitory factor.

Site-specific lacZ Knock-in Stable Cell Line—The Red/ET recombination method (Gene Bridges GmbH, Dresden, Germany) was employed to make the site-specific knock-in construct that contains 5.43 kb of left homologous arm and 10.374 kb of right homologous arm for the homologous recombination (20). Briefly, we inserted prokaryotic Pg2 promoter into the plasmid pTCT5.2 (20) to generate a plasmid pTCT5.2-Pg2 that contains both eukaryotic PGK and prokaryotic Pg2 promoters. The genomic DNA of Zfp206, which is 16,147 bp in length, was subcloned into a minimer vector by the Red/ET method using the bacterial artificial chromosome subcloning kit (Gene Bridges GmbH) from the BAC clone RP24–440G8 (containing 172 kb of genomic DNA). The cassette of PCR product from the pTCT5.2-Pg2 plasmid was inserted into this subcloning plasmid to generate a novel plasmid in which part of the second exon (328 bp in length) of Zfp206 containing the translation initiation site has been replaced. For homologous recombination, V6.4 ESCs were stably transfected with the construct that contains the lacZ (β-galactosidase) reporter gene using a standard protocol of electroporation (20). Individual colonies were picked after selection with 300 μg/ml G418 for 10 days with cells grown on neomycin-resistant mouse embryonic fibroblasts (DR4, ATCC). Southern blot was used to verify the site-specific integration into the genomic DNA. 20 μg of genomic DNA from different neomycin-resistant cell clones was digested by SpeI (New England Biolabs), loaded onto agarose gel, and transferred onto nylon membrane. Southern blot was conducted using PCR product localized on the upstream of the left homologous arm and was labeled using the PCR digoxigenin probe synthesis kit (Roche Applied Science) following the manufacturer’s instructions.

Promoter Reporter Constructs—We cloned the Zfp206 promoter (1468 bp in length) from the BAC clone (RP24–440G8) and the Nanog (417 bp) promoter regions from the genomic DNA into pGL4-basic vector (Promega). The beginning of the first intron (1062 bp) or the end of the first intron (938 bp) were cloned upstream of the Zfp206 promoter-luciferase or Nanog promoter-luciferase construct in the forward or reverse orientation. The Sox2, Oct4, and both the Oct4 and Sox2 binding site were modified using the QuickChange II site-directed mutagenesis kits (Stratagene) to incorporate 3-bp mutations, which were subsequently verified by sequencing.

RNA Interference (RNAi)—RNAi experiments were performed with Dharmacon siGENOME SMARTpool reagents against mouse Oct4, Sox2, or Nanog. The Dharmacon siCONTROL non-targeting small inhibitory RNA (siRNA) pool was used as a negative control. All duplexed RNA oligonucleotides were synthesized at Dharmacon as 21-mers with 3'-UU overhangs forming a 19-bp duplex core. Based on the manufacturer’s instructions, four duplex individual siRNAs were combined into a signal pool for one target gene. Mouse ESCs were transfected according to the manufacturer’s instructions. Briefly, E14 cells were transfected with gene-specific SMARTpool or the siCONTROL non-targeting siRNA pool three times at days 0, 2, and 4 using DharmFECT 2 transfection reagent (Dharmacon). The RNA was extracted at days 1, 3, and 5 for reverse transcription-PCR assay. Total RNA was isolated using TRIzol reagent (Invitrogen) and purified with the RNEasy kit (Qiagen). The first strand cDNA was synthesized using the high capacity cDNA archive kit (Applied Biosystems) with 1500 ng of total RNA, and quantitative PCR analyses were performed in real time using an ABI PRISM 7900 sequence detection system (Applied Biosystems). Expression was normalized to β-actin, and each sample was analyzed in duplicate or triplicate. A threshold cycle (Ct) of >33 indicates undetected transcripts. A 19-bp gene-specific RNAi sequence for Oct4 and Sox2 (13) was synthesized and cloned into pSUPER.puro vector (Oligoengine), which can express short hairpin RNAs (shRNAs) as described previously (22).

Luciferase and lacZ Reporter Assays—200 ng of luciferase reporter plasmid, 1 μg of Oct4 or Sox2 shRNA in plasmid
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pSUPER.puro, and 20 ng of plasmid pRL-SV40 were co-transfected into E14 cells in a 24-well plate by Lipofectamine 2000 (Invitrogen) following the supplied protocol. Puromycin (1 μg/ml) was applied 24 h post-transfection. The pRL-SV40 plasmid served as an internal control for normalizing the transfection efficiency. After 48 h of puromycin selection, the E14 cells were lysed and luciferase activity determined with the Dual Luciferase system (Promega) using a Centro LB960 96-well luminometer (Berthold Technologies). Transfections were done in duplicate and on two independent occasions.

To quantitate β-galactosidase activity (lacZ expression) in V6.4 ESCs upon Sox2 and Oct4 knockdown, 1 μg of Sox2 or Oct4 shRNA in plasmid pSUPER.puro and 3 ng of plasmid pRL-SV40 were co-transfected into V6.4 heterozygous ESCs in a 96-well plate. Puromycin (1 μg/ml) was applied 24 h post-transfection. The 96-well plates were equilibrated to room temperature before adding Beta-Glo reagent (Promega) after 48 h of puromycin selection. 100 μl of reagent was added to the culture medium. The sample was mixed and incubated for 1 h at room temperature and measured using a luminometer. The V6.4 cells were lysed, and Renilla activity was determined with the Dual Luciferase system (Promega) at the same time.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from E14 mouse ESCs grown under feeder-free conditions using the method of Rodda et al. (13). Equal molar oligonucleotides labeled with Cy5 at the 5′ termini of both sense and antisense strands were annealed in 95°C water until cooling to room temperature. The double-stranded DNA was diluted to 1 μM for EMSA assay. EMSA was performed in 10-μl mixtures containing 20 mM HEPES, pH 7.9, 100 mM KCl, 1.66 mM dithiothreitol, 0.83 mM EDTA, 20% glycerol, 1 μl of Cy5-labeled double-stranded DNA (1 μM), 12–16 μg of nuclear extracts and 1 μl of poly(dG-dC) (2 μg/μl, Amer sham Biosciences), 1% protease inhibitor mixture (Roche Applied Science). Antibodies (Santa Cruz Biotechnology) were added after the initial incubation (25 min) for an additional 25 min as follows: 2 μl of anti-Oct4 (catalog number sc-9081) and 8 μl of anti-Sox2 (sc-17320) or 2 μl of anti-Oct1 (sc-232), 8 μl of anti-Oct6 (sc-11660), 8 μl of anti-Sox4 (sc-17326), and 2 μl of anti-JunB (sc-46x). For competitive studies, a specified 1 μl (25 μM) of unlabeled double-stranded competitor was added together with Cy5-labeled DNA.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays with E14 mouse ESCs were carried out as described previously (12, 13). Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and formaldehyde was inactivated by the addition of 125 mM glycine. Sonicated chromatin extracts containing DNA fragments with an average size of 500 bp were immunoprecipitated using Oct4 (Asn-19), Sox2 (Tyr-17), or glutathione S-transferase control polyclonal antibodies (Santa Cruz Biotechnology). For all ChIP experiments, quantitative PCR analyses were performed in real time using the ABI PRISM 7900 sequence detection system and 2X SYBR Green master mix (Applied Biosystems) as described previously (12, 13). Relative occupancy values were calculated by determining the apparent immunoprecipitate efficiency (ratios of the amount of immunoprecipitated DNA over that of the input sample) and then normalized to the level observed at the control region, which was defined as 1.0. Primer pairs were as follows: for Oct4/Sox2 binding site 1 (BS1) region 1, 5′-TGGGGGCCACATGTGGAATCG3′ and 5′-TGGAGGGAGAGGC CATTGTCAGCCT-3′; region 2, 5′-CTGTCTCTTGGAATGGCACA ACCCC-3′ and 5′-CGTGCTTGTGTGTGACTAGGCT-3′; region 3, 5′-CGGGCCAGATTTCTACAAAGGC-3′ and 5′-CCTACCCATGAATTTGCTGAC-3′; region 4, 5′-TGGGTAGAACCGAATATGG-3′ and 5′-TGCTTCTGCTGC TACTTCATCCC-3′; region 5, 5′-TGTTATGAAATGTCAGTCTGCGCA-3′ and 5′-GACATTTCTGCTGTCAAGAGACAGC-3′; and region 6, 5′-GTTCTCCACATGTGTTGATG-3′ and 5′-CAAGTACGGTTGTAGGGAAA-3′. For Oct4/Sox2 binding site 2 (BS2) region 7, 5′-GCTGTAGGCAAGCTAGCA- GTG-3′ and 5′-CAGGGATCCAGGTAGACAAACA-3′; region 8, 5′-GAATGTTGTGTGCTGATATGCCC-3′ and 5′-GAAAGCAACAGCCAGCAAGTAT-3′; region 9, 5′-ATCCCTGACCTGGAATTTCACTTG-3′ and 5′-AAATTTAGAAGCCAGTTGTGTGG-3′; region 10, 5′-ACTGTTGTGAGGGTGTTTCCCTG-3′ and 5′-TGGAGGGAGGCGCCACCT- CTA-3′; region 11, 5′-GTGGCCACACCTGCTTTCGAGCA-3′ and 5′-TTTCGTGTTGTCGAGACCGGTG-3′; region 12, 5′-GAAACCTGCAATTTCCACTGGC-3′ and 5′-GGCCGACTGAAAGATCACAATC-3′; and region 13, 5′-CTTGGGCTTCCAAATCTGCTGTTG-3′ and 5′-TGGGTGATGTGTTGAGA CACGT-3′.

RESULTS

Expression of Zfp206 Is Regulated by Oct4 and Sox2—A recent report (14) and our own data (to be published elsewhere)3 have identified Zfp206 as a transcription factor that plays a key role in maintaining the pluripotency of ESCs. The expression pattern of Zfp206 is quite similar to the patterns of expression for Nanog, Oct4, and Sox2 in ESCs; they are all expressed in undifferentiated cells, and that expression is quickly repressed upon differentiation. Because Nanog, Oct4, and Sox2 are key transcription factors in the maintenance of pluripotency in ESCs, we set out to determine whether they can regulate the expression of Zfp206. Therefore, we examined the expression of Zfp206 in response to the knockdown of Nanog, Oct4, and Sox2 expression by RNAi. We were able to achieve efficient and substantial knockdown of the target genes in ESCs by a repeated transfection on alternating days with siRNA. When mouse ESCs were transfected with gene-specific siRNAs against Nanog, Oct4, and Sox2, a 50% knockdown of expression was observed 1 day after transfection and a 70–90% reduction of the targeted gene was observed at day 5 (Fig. 1). These effects were gene-specific, as there was no knockdown of β-actin mRNA expression by these three siRNAs, and a non-targeting siRNA had no impact on the expression of Nanog, Oct4, and Sox2. The expression level of Zfp206 was examined in these transfected cells. Upon Oct4 or Sox2 knockdown (Fig. 1, A and C), a down-regulation of the Zfp206 transcript level to <30% of control levels was observed. In the Nanog RNAi cells, the expression level of Zfp206 was reduced to only 70%. These results suggest that Zfp206 is regulated by Oct4 and Sox2, perhaps directly or indirectly, as part of the regulatory network that controls ESC pluripotency.
Oct4 and Sox2 Regulate the Activity of Zfp206 in Vivo—To further examine the regulation of Zfp206 expression in ESCs, we knocked a lacZ reporter into the Zfp206 locus by homologous recombination. We constructed a targeting vector that contained a 5.5-kb genomic fragment upstream of the translational start site of the Zfp206 and a 10.4-kb genomic fragment downstream of the 3′/H11032 end of the second exon. The construct included the entire first exon and intron. The first 134 amino acids of the Zfp206 protein, which included the SCAN domain in the second exon, was replaced with a construct containing the internal ribosome entry site IRES-lacZ-neomycin-3′/H11032 UTR reporter and the selection cassette (supplemental Fig. 1A). The targeting construct was electroporated into V6.4 ESCs and 196 neomycin-resistant colonies were assessed for homologous recombination by Southern blotting. We identified four independent clones that had the correct restriction digest pattern (supplemental Fig. 1B). All four of the undifferentiated, heterozygous colonies were positive for lacZ, indicating appropriate expression of this reporter from the Zfp206 locus (Fig. 2A, upper panel). To determine whether down-regulation of lacZ expression was linked to endogenous Zfp206 expression, ESC clones were differentiated by retinoic acid. After five days of continuous retinoic acid treatment, the cell morphology changed significantly, and lacZ became undetectable (Fig. 2A, lower panel).

FIGURE 1. Oct4 and Sox2 can regulate Zfp206 expression. Changes in endogenous gene expression levels of Oct4, Nanog, and Sox2 following genespecific Oct4 (A), Nanog (B), and Sox2 (C) RNA interference and corresponding changes in endogenous Zfp206 gene levels. cDNAs were prepared from the RNAi knockdown ESCs and analyzed using real-time PCR. The levels of the transcripts were normalized against values derived from E14 cells (no siRNA has transfection reagent) at each time point.

Oct4 and Sox2 Regulate the Activity of Zfp206 in Vivo—To further examine the regulation of Zfp206 expression in ESCs, we knocked a lacZ reporter into the Zfp206 locus by homologous recombination. We constructed a targeting vector that contained a 5.5-kb genomic fragment upstream of the translational start site of the Zfp206 and a 10.4-kb genomic fragment downstream of the 3′ end of the second exon. The construct included the entire first exon and intron. The first 134 amino acids of the Zfp206 protein, which included the SCAN domain in the second exon, was replaced with a construct containing the internal ribosome entry site IRES-lacZ-neomycin-3′ UTR reporter and the selection cassette (supplemental Fig. 1A). The targeting construct was electroporated into V6.4 ESCs and 196 neomycin-resistant colonies were assessed for homologous recombination by Southern blotting. We identified four independent clones that had the correct restriction digest pattern (supplemental Fig. 1B). All four of the undifferentiated, heterozygous colonies were positive for lacZ, indicating appropriate expression of this reporter from the Zfp206 locus (Fig. 2A, upper panel). To determine whether down-regulation of lacZ expression was linked to endogenous Zfp206 expression, ESC clones were differentiated by retinoic acid. After five days of continuous retinoic acid treatment, the cell morphology changed significantly, and lacZ became undetectable (Fig. 2A, lower panel).
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Identification of Two Composite oct-sox Elements within the First Intron on the Zfp206 Gene—We sought to determine whether Zfp206 is a direct target of regulation by Oct4 and Sox2. Previous work from our institute had comprehensively mapped Oct4 (12) and Sox2 (2) binding sites in mouse ESCs by a new ChIP strategy called ChIP-PET (23). A search of our ChIP-PET data base revealed two regions within the first intron of Zfp206 where both Oct4 and Sox2 occupancy had been mapped. We called these binding site 1 (BS1) and binding site 2 (BS2), which map at the 5’ and 3’ end, respectively, of intron 1 (supplemental Fig. 2). There were multiple, independent paired-end tags (PETs) for both sites, which provides confidence that they are real. Co-localization of Oct4 and Sox2 binding at BS1 and BS2 further confirms their validity, because these two transcription factors bind DNA as a heterodimer (20). Closer inspection of the sequence within BS1 and BS2 revealed the presence of consensus binding motifs for Oct4 and Sox2.

To further confirm that Sox2 and Oct4 do indeed directly bind to BS1 and BS2 within the first intron of Zfp206 in ESCs, ChIP-PET PCR was carried out with Sox2 and Oct4 antibodies. The ChIP material was amplified by PCR with 13 pairs of primers for regions within the promoter, exon 1, intron 1, exon 2, and intron 2 of Zfp206 (Fig. 3A). One pair of primers was specific for BS1 and another for BS2, regions 3 and 10, respectively (Fig. 3A). DNA fragments from region 3 containing the composite oct-sox element of BS1 were enriched up to 12- and 6-fold with the Sox2 and Oct4 antibodies respectively (Fig. 3B). Likewise, for region 10 (BS2), there were 7- and 3-fold enrichments with Sox2 and Oct4 antibodies, respectively (Fig. 3C). The neighboring regions on both sides of the oct-sox composite elements were not significantly enriched, showing the specificity of the binding. An irrelevant antibody against gluthionine S-transferase provided no enrichments. The ChIP-PCR data confirm the ChIP-PET results and indicate in vivo occupancy of Oct4 and Sox2 at two sites in intron 1 of Zfp206 in undifferentiated mouse ESCs.

Sox2 and Oct4 Bind Directly to BS1 and BS2—Compared with previously identified composite sox-oct elements from different mouse target genes (supplemental Fig. 3) that are known to bind Sox2 and Oct4, the sox and oct elements within BS1 (Fig. 4A) are in reverse orientation with respect to each other, whereas the orientation of the sox and oct elements within BS2 (Fig. 4B) is similar to those of the other target genes. However, in both BS1 and BS2, the oct and sox elements are separated by 11 bp; this is in contrast to the enhancer regions of Fgf4, Utf1, Pou5f1, Nanog, Fbx15, and Sox2 (supplemental Fig. 3), in which the sox and oct elements are <3 bp apart (24–30).

To confirm our results and more precisely map the Oct4 and Sox2 binding sites within BS1 and BS2, we performed EMSAs. Duplexed oligonucleotide probes (39 bp) encompassing the oct-sox element of either BS1 or BS2 were combined with nuclear extracts from mouse ESCs. Three major protein-DNA complexes were observed using the BS1 probe (Fig. 4C, lane 1), and four protein-DNA complexes were found with the BS2 probe (Fig. 4D, lane 1). The addition of antibodies specific for either Oct4 (Fig. 4, C and D, lanes 5) or Sox2 (Fig. 4, C and D, lanes 8) resulted in supershifts of the Oct4 or Sox2 band, respectively. The extra band observed with BS2 (Fig. 4D, lane 1)
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The sox-oct Composite Element within BS1 of Zfp206 Conferred Activation on Different Promoters—

We have shown that Oct4 and Sox2 are able to bind directly to BS1 and BS2 of Zfp206. We were interested to determine whether these DNA-protein interactions have a functional role in regulating the expression of Zfp206. To answer this question, we tested whether the BS1 and BS2 regions would have an effect on two separate promoters, Zfp206 and Nanog. Luciferase reporter constructs were therefore generated. A 1062-bp genomic sequence of Zfp206, which contains the BS1, and another 938-bp genomic fragment of Zfp206, which contains the BS2 were isolated. These two genomic fragments were individually cloned into either a Zfp206 or Nanog promoter-driven luciferase reporter plasmid (Fig. 5A). BS1 was able to activate the expression of the reporter gene up to 8-fold when the Zfp206 promoter was used and ~13-fold when driven by a Nanog promoter (Fig. 5B). When the sox element within BS1 was mutated (BS1mS), this activation dropped to almost half of that for wild-type BS1 for both promoters. Upon mutation of the oct element within BS1 (BS1mO), the level of activation decreased to about one-quarter that of the wild-type BS1 for both promoters. When both sox and oct elements were mutated in BS1 (BS1mSmO), the activation was completely abolished, suggesting that the activation observed for BS1 was entirely due to the sox-oct element within BS1 and that this activation effect was not promoter-specific.

is likely due to binding of an Oct4-Sox2 heterodimer, because this band was supershifted by both Oct4 and Sox2 antibody (Fig. 4D, lanes 5 and 8). An additional band is likely due to binding of Oct1, as it was supershifted by an Oct1 antibody (Fig. 4, C and D, lanes 6). No supershifts were observed with antibodies to Oct6 (Fig. 4, C and D, lanes 7), JunB (Fig. 4, C and D, lanes 9), or Sox4 (Fig. 4, C and D, lanes 10), indicating specificity of our EMSA assay. The addition of a 25-fold excess of wild-type unlabeled probe successfully competed for binding to the Oct4-Sox2 complex (Fig. 4, C and D, lanes 2). However, when probe that had the oct binding site mutated was used for competition, the Oct4 complex remained, whereas the Sox2 complex was competed away (Fig. 4, C and D, lanes 3). Conversely, when probe that had the sox binding site mutated was used for competition, the Sox2 complex remained, whereas the Oct4 complex was competed (Fig. 4, C and D, lanes 4), indicating the DNA binding specificity of the Oct4-Sox2 complex. The above results confirm our ChIP results and further refine the binding sites for Oct4 and Sox2 within the first intron of Zfp206. In addition, an Oct4-Sox2 heterodimer was able to bind within BS2, despite the large spacer region between the oct and sox element, implying that a spacer size of 11 bp was still able to accommodate binding of an Oct4-Sox2 heterodimer.

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To determine whether BS1 could function as an enhancer by reversing the orientation of BS1 in the reporter construct. It was observed that BS1 in reverse orientation was able to activate reporter gene expression and that mutation of either the sox, oct, or both elements decreased this activation (Fig. 5C). This showed that both orientations of BS1, with respect to the promoter, are functional and suggests that BS1 may function as an enhancer.

Oct4 and Sox2 Are Required for the Activation of Zfp206—To determine in ESCs whether the activation conferred by the sox-oct composite element within BS1 of Zfp206 is due to Oct4 and Sox2 proteins, we carried out luciferase assays using the constructs in Fig. 5A, but this time with knocked down expression of either Oct4 or Sox2 by RNAi. Endogenous Oct4 and Sox2 mRNA levels in ESCs were measured by real-time PCR after transfection with the respective RNAI constructs containing a puromycin resistance gene. Transfection of ESCs with shRNAs targeting Sox2, Oct4, or both Oct4 and Sox2 resulted in a decrease of Oct4 transcript level by 30, 40, and 60%, respectively (data not shown), whereas the Sox2 transcript level was reduced by 60, 30, and 50%, respectively (data not shown). The activation mediated by BS1 on the Zfp206 promoter was abolished upon knockdown of Oct4, Sox2, or both Oct4 and Sox2. Similar results were observed with BS1 in the reverse orientation (Fig. 6A). The same effect was also observed when the Zfp206 promoter was replaced with the Nanog promoter (Fig. 6A). All of these results suggest that, in ESCs, the activation conferred by the sox-oct composite element within BS1 of Zfp206 is dependent on the presence of Sox2 and Oct4 proteins.

To determine whether there is an absolute requirement of both the sox and oct elements within BS1 and Oct4 and Sox2 proteins for transcriptional activation effect, we transfected ES cells with different constructs in which BS1 had been mutated at either the sox or oct or both elements in combination with different RNAI plasmids. Although mutation of the sox element (BS1mS) reduced the reporter gene level to about a quarter compared with that of the wild type BS1 (BS1), knocking down Oct4, Sox2, or both Oct4 and Sox2 was able to reduce the activation mediated by BS1mS further (Fig. 6B). Similarly, mutation of the oct element (BS1mO) also decreased the activation of BS1 to the same level as BS1mS, and knocking down Oct4, Sox2, or both Oct4 and Sox2 reduced the activation mediated by BS1mO even further. On the contrary, mutations of both the oct and sox element (BS1mOMS) totally abolished the activation mediated by BS1, and knocking down Oct4, Sox2, or both Oct4 and Sox2 did not further reduce the reporter gene level. Similar results were obtained for BS1 in the reverse orientation. Collectively, these results demonstrate that Oct4 or Sox2 individually activate expression Zfp206 at the BS1 site, but in combination, they act synergistically to enhance expression.

Similar experiments were carried out with BS2, but no activation of the reporter gene was observed. Mutation of either sox or oct or both elements within BS2 did not result in any significant difference in reporter gene expression. This was somewhat surprising given that we have demonstrated that Oct4 and Sox2 are bound to these sites. It is possible that occupancy at the BS2 site requires co-regulatory factors and that Oct4 and Sox2 occupancy at this site is not sufficient to regulate Zfp206 expression.
DISCUSSION

Zfp206 has been identified as a gene that is highly expressed in undifferentiated ESCs and down-regulated upon differentiation (3, 14). It was also shown that Zfp206 is expressed in the pluripotent cells of the inner cell mass of the blastocyst (15). This pattern of expression is similar to that observed for pluripotency-regulating transcription factors such as Oct4, Sox2, and Nanog. The linkage between Zfp206 expression and differentiation status in ESCs and embryonic development suggested that this transcription factor may play a role in regulating pluripotency. It has now been established that Zfp206 does play a role in maintaining ES cell pluripotency, as overexpression renders ESCs resistant to differentiation, whereas knockdown of Zfp206 expression causes ESCs to spontaneously differentiate and be more sensitive to induced differentiation (14). Thus, Zfp206 expression causes ESCs to spontaneously differentiate and be more sensitive to induced differentiation (14).

In the study presented here, we have shown that expression of Zfp206 is positively regulated by Oct4 and Sox2 in mouse ESCs. Upon knockdown of Oct4 or Sox2 by RNA interference, Zfp206 level decreased (Fig. 1). Chromatin immunoprecipitation experiments had identified binding sites for Oct4 and Sox2 in Zfp206 (11, 12), which indicated that it may be a direct target of regulation by these established pluripotency transcription factors. This prompted us to further analyze the Zfp206 gene for functionally important Oct4 and Sox2 binding sites. The previous ChIP studies had identified two Oct4 and two Sox2 binding sites in the first intron of Zfp206. Both of these Oct4 binding sites are in close proximity to the Sox2 binding sites, thus forming two composite oct-sox elements, one located near the start of intron 1 on Zfp206 and the other one near the end of the same intron, BS1 and BS2, respectively. ChIP-PCR with Oct4 or Sox2 antibodies confirmed that Oct4 and Sox2 are indeed bound at both BS1 and BS2 in mouse ESCs (Fig. 3).

We tested BS1 and BS2 further by EMSA to precisely map the Oct4 and Sox2 binding sites. Our results show that both Oct4 and Sox2 bind at BS1 and BS2 (Fig. 4). Previous reports have shown that Oct4 and Sox2 can form a heterodimer that interacts with DNA at closely linked binding sites. We found that only BS2 bound Oct4 and Sox2 as a heterodimer. Composite oct-sox elements have been identified and found to be functional in the enhancer region of a number of different genes such as Fgf4, Utxf1, Fbx15, and Sox2. In these instances, the sox and oct elements are separated by 3 bp or less. In contrast, the sox and oct element in both BS1 and BS2 are separated by 11 bp. Thus, 11 bp of separation between the Oct4 and Sox2 binding sites does not preclude heterodimer binding. It is not clear why a heterodimer was not detected at BS1; it may be related to the fact that the sox and oct elements are in a different orientation compared with those in the BS2 element and all of the other known composite sox-oct elements.

We then went on to determine whether there were any functional differences between BS1 and BS2. A series of luciferase assays confirmed that the sox-oct element within BS1 is able to confer transcriptional activation, regardless of the promoters used. Moreover, the transcriptional activation is dependent on Oct4 and Sox2 proteins; either Oct4 or Sox2 can confer activation via BS1, although a combination of these two proteins result in synergistic activation. This suggests that a tight Sox2-Oct4 heterodimer complex is not the only way that these two transcription factors transcriptionally activate genes in pluripotent cells. We also found that BS1 works in both orientations, suggesting that it operates as an enhancer to regulate Zfp206 expression in response to Oct4 and Sox2 binding (Figs. 5 and 6). This was further confirmed by an in vivo experiment that showed that a regulatory region of Zfp206 containing BS1 is able to control the expression of an exogenous gene (Fig. 2) in undifferentiated ESC and its differentiated derivatives. Surprisingly, we could not demonstrate any transcriptional effect mediated by BS2, even though this site binds Oct4 and Sox2 heterodimers. One possibility is that BS2 cannot activate the Zfp206 promoter alone. Perhaps it is functional only in cooperation with other transcription factor binding sites. Another possibility is that Oct4 and Sox2 binding at BS2 could modulate the occupancy at nearby BS1, which in turn controls the Zfp206 promoter activity. Additional experiments are required to test this hypothesis.

The results presented here have expanded upon our understanding of the transcriptional network that is operative in
Zfp206 Is Regulated by Oct4 and Sox2

ESCs to control differentiation. We have found that Zfp206 is directly regulated by Oct4 and Sox2. Oct4 and Sox2 are often described as master regulators of ES cell pluripotency, although the key downstream effectors of their action have not been established. It now appears that Zfp206 is one of downstream effectors of Oct4 and Sox2 regulation. It will be of interest to define direct targets of Zfp206, which is expressed primarily in early embryonic development. It is not clear whether Zfp206 is a transcriptional activator or a repressor. In either case, identification of Zfp206 targets will improve our understanding of the molecular basis of early embryonic development and ESC differentiation.

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