SOX7 and GATA-4 Are Competitive Activators of Fgf-3 Transcription*

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Akira Murakami‡, Huqing Shen†, Sanami Ishida§, and Clive Dickson¶

From the ‡Department of Viral Oncology, Institute for Virus Research, Kyoto University, Shogoin, Sakyō-ku, Kyoto 606-8507, Japan and the ¶Cancer Research UK, London Research Institute, Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom

Fgf-3 is expressed in a dynamic and complex spatiotemporal pattern during mouse development. Previous studies identified GATA-4 as a transcription factor that binds the key regulatory element PS4A of the Fgf-3 promoter and stimulates transcription. Here we show that members of the SOX family of transcription factors also bind PS4A and differentially modulate transcription. At least five SOX genes, Sox2, Sox6, Sox7, Sox13, and Sox17, were expressed in F9 cells, and of these, Sox7 and Sox17 were dramatically induced in parallel with Fgf-3 following differentiation into parietal endoderm-like cells with retinoic acid and dibutyryl cAMP. Complexes could be detected on PS4A with SOX2, SOX7, and SOX17 by using nuclear extracts from differentiated F9 cells. However, only Sox7 expression markedly activated the Fgf3 promoter in these cells. By contrast, SOX2 was a poor activator of Fgf-3 transcription, and when Sox2 was co-expressed with Gata4, it negatively modulated the strong activation mediated by GATA-4. More detailed analyses showed that SOX7 competes with GATA-4 for PS4A occupancy and to activate the Fgf3 promoter. In situ hybridization analysis showed that Sox7 is co-expressed with Fgf3 and Gata4 in the parietal endoderm of E7.5 mouse embryos. In culture, GATA-A-deficient embryonal stem cells were shown to express Fgf-3 upon differentiation into embryoid bodies, although at lower levels than were found in wild type embryonic stem cells. This Fgf-3 expression was virtually abolished when Sox7 expression was suppressed by RNA interference. These results show that SOX7 is a potent activator of Fgf-3 transcription.

Fibroblast growth factors (Fgfs)† compose a large family of related proteins that can induce or confer a range of biological responses on cells such as proliferation, differentiation, survival, and motility (reviewed in Refs. 1–3). Mouse targeting studies have implicated Fgfs in diverse aspects of vertebrate development (reviewed in Refs. 4–7). Fgf-3 was identified as an oncogene in mouse mammary tumors (8) but later was found to be expressed at a variety of sites during embryogenesis, suggesting its potential involvement in a number of developmental processes (9–13). In the post-implantation conceptus, Fgf-3 is expressed in the parietal endoderm and in a number of embryonic tissues such as the primitive streak mesoderm, early hindbrain and forebrain, cerebellum, sensory cells of the inner ear, pharyngeal pouches, retina, tooth mesenchyme, and tail bud (9, 12, 13). Mice deficient for Fgf-3 show developmental abnormalities of the inner ear and partial fusion of the caudal vertebrae, indicating an essential role of Fgf-3 in the formation of these tissues (14).

To understand how the complex pattern of Fgf-3 transcription is regulated, we have been analyzing its promoter to identify the transcriptional regulators that control activity. F9 embryonal carcinoma cells can differentiate into several early cell lineages in cell culture (15). Treatment with retinoic acid (RA) and dibutyryl cAMP (Bt2cAMP) preferentially induces F9 cells to differentiate in parietal endoderm-like cells, which is accompanied by the induction of Fgf-3 (16, 17). By using differentiated F9 cells, we previously identified positive and negative regulatory elements within the 5′-proximal region of the Fgf-3 promoter, and we concluded that an element designated PS4A was essential for promoter activity (18, 19). Further analyses have demonstrated that the transcription factor, GATA-4, binds PS4A and was implicated as a positive regulator of Fgf-3 expression in differentiated F9 cells (20, 21). GATA-A has an important function during early cardiogenesis, because it regulates the expression of a number of cardiac structural genes (reviewed in Ref. 22). Moreover, targeted disruption of Gata4 results in defective visceral endoderm differentiation in vitro (22) and in vivo results in early embryonic lethality because of abnormalities in ventral heart tube formation (24, 25).

Recently we identified another transcription factor, SOX6, as a PS4A-binding protein that negatively regulates Fgf-3 transcription (26). SOX6 belongs to a family of transcription factors related by homology within their DNA binding domains (HMG box) to the mammalian testis-determining factor SRY (reviewed in Ref. 27–29). SOX proteins are expressed in a variety of both embryonic and adult tissues and have been implicated in the determination of cell fate decisions (27–29). Cells frequently express more than one SOX gene, although it is not clear to what level they are functionally redundant (30–34).

In this study we have identified five SOX genes, Sox2, Sox6, Sox7, Sox13, and Sox17, that are expressed in differentiated F9 cells. An analysis of SOX protein binding to PS4A in the Fgf-3 promoter, and functional studies in F9 cells and embryonal stem (ES) cells, implicates SOX7 as an important positive regulator of Fgf-3 transcription. Most surprising, GATA-4 and SOX7 both bind PS4A but appear to act independently and...
exclusively to regulate positively Fgf-3 transcription. In situ hybridization analysis shows co-expression of Sox7 and Fgf-3 further supporting a role for SOX7 in the regulation of Fgf-3 during early embryogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Mouse embryonal carcinoma cell line F9 was cultured and induced to differentiate into parietal endoderm-like cells with RA (0.1 μM) and Bt-cAMP (1 mM) as described previously (35). Differentiation to visceral endoderm-like cells was achieved in suspension of cell aggregates with RA (0.1 μM) as described (36). Mouse ES cell lines, R1 (a gift from Dr. S. Yamada) and Gata4′−′ (a gift from Dr. D. B. Wilson), were maintained and differentiated in suspension culture as described previously (23).

**Electrophoretic Mobility Shift Assay (EMSA)**—Experimental conditions and preparation of nuclear extracts from F9 cells have been described previously (20). Poly(dI)-poly(dC), poly(dI-dC)-poly(dI-dC), or poly(dG-dC)-poly(dG-dC) (Amersham Biosciences) was used as a non-specific competitor for the experiments shown in Figs. 1 and 2. Poly(dI-dC)-poly(dI-dC) was used for all other experiments. The antisera to SOX2 was kindly provided by Dr. Robin Lovell-Badge (National Institute for Medical Research, London, UK). The antisera to Gata4 was purchased from Santa Cruz Biotechnology. The antiserum to SOX7 was kindly provided by Dr. Robin Lovell-Badge (National Institute for Medical Research, London, UK). The antisera to Sox6 was kindly provided by Dr. Yoshiki Hiraoka (Keio University School of Medicine, Tokyo, Japan). In vitro transcribed and translated proteins were synthesized from cDNA encoding an N-terminal deletion mutant, containing proteins to DNA (Fig. 1) (42). Several complexes did not form on a probe containing two nucleotide substitutions (4AyM4) demonstrating sequence-specific binding. To determine which nucleotides of PS4A are required for formation of the complexes containing proteins to DNA (Fig. 1) (42). However, when poly(dG-dC)-poly(dG-dC) was used as the non-specific competitor in the EMSA, novel complexes were formed with undifferentiated F9 cells (lanes 1 and 4 in Fig. 1) (20). Subsequently, Sox6 was identified as another PS4A-binding protein through a yeast one-hybrid screen (26). Sox proteins were not detected in the original EMSA experiments because poly(dI)-poly(dC) was used as the non-specific DNA competitor, and this can interfere with the binding of HMG domain-containing proteins to DNA (Fig. 1) (42). Hence, the recognition sequence for the Sox7 HMG domain fragment obtained as described above as a probe. The cDNA fragment was sequenced on both strands and shown to contain the same coding sequence as published previously (26). The complexes did not form on a probe containing two nucleotide substitutions (4AyM4) demonstrating sequence-specific binding (Fig. 1, lanes 7 and 8). To determine which nucleotides of Sox4 are required for formation of the complexes containing proteins to DNA (Fig. 1) (42). However, when poly(dG-dC)-poly(dG-dC) was used as a non-specific competitor in the EMSA, novel complexes were formed with undifferentiated as well as differentiated F9 nuclear extracts (Fig. 1, lanes 6 and 7). Although part of the complex formed with differentiated cell extracts is attributable to G4-binding (compare lanes 4 and 6). The complexes did not form on a probe containing two nucleotide substitutions (4AyM4) demonstrating sequence-specific binding (Fig. 1, lanes 7 and 8). 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tal probe (Fig. 2C, left panel), and therefore it was not pursued further. When using differentiated F9 nuclear extracts, the analysis is complicated by the presence of GATA-4 binding (Fig. 2C, middle panel). Therefore, a competitor for GATA protein binding was added to eliminate the GATA-4 complex (Fig. 2C, bottom panel). Although the amount of the major complex decreased in the presence of the competitor (Fig. 2C, bottom panel, arrow), the complexes formed with PS4A were still detected (lane 1), indicating the presence of proteins other than GATA-4. In the presence of the competitor, complex formation was significantly suppressed by point mutations in probes 4A17C, 4A18G, 4A20T, and 4A21G (Fig. 2B, bottom panel, lanes 5–8) and reduced for probes 4A15A and 4A16G (lanes 3 and 4). Hence, the deduced recognition sequence for the PS4A-binding proteins is therefore (C)(T)ATTGT, the same as that determined using undifferentiated F9 nuclear extracts. Surprisingly, this sequence overlaps in part with one of the bipartite binding sites for GATA-4 identified by using differentiated F9 nuclear extracts (boxed in Fig. 2A) (21). Most interesting, a complex was formed with the 4A17C probe (Fig. 2B, lane 5, middle panel) that was not detected in the presence of the GATA competitor (lane 5, bottom panel). This complex was completely supershifted by an anti-GATA-4 serum (Fig. 2C, lane 5, middle panel).
right panel), confirming that it contains GATA-4. At least three non-GATA-4 protein-containing complexes were distinguish-
able using differentiated F9 nuclear extracts, as indicated in Fig. 2B, arrow and two asterisks, lanes 1, 2, and 9. Because these complexes all failed to form on the same point-substit-
tuted probes, the same sequence seemed to be recognized by the proteins forming these complexes. The sequence (C)(T)ATTGT or ACAATA(A/G) in the opposite orientation is similar to the consensus binding motif of SOX proteins (A/T)(A/T)CAA(A/T)G. This is consistent with the binding of SOX6 to PS4A (26) and suggests the proteins in the complexes are likely to be members of the SOX family.

Identification of SOX Genes Expressed in F9 Cells—SOX family proteins contain a highly homologous DNA binding do-
main, the so-called HMG box (27, 28). To identify the SOX proteins expressed in F9 cells, we performed low stringency PCR using a cDNA library from undifferentiated or differenti-
ated parietal endoderm-like F9 cells with degenerate primers based on homology to the HMG box as described previously (38). Products of the predicted size were subcloned and se-
quenced. As seen in Table I, Sox2 and Sox7 were the most frequently isolated clones and therefore likely to be the most abundant SOX family transcripts in undifferentiated and differenti-
ated F9 cells, respectively. In addition, clones for Sox6, Sox13, and Sox17 transcripts were also isolated. To assess the relative expression level of each SOX gene in F9 cells, the clones were used as probes in a Northern blot hybridization (Fig. 3). F9 cells can be induced with RA alone to differentiate into cells showing markers of visceral endoderm (VE-like), or RA and Bt,cAMP treatment produces cells showing markers of the parietal endoderm (PE-like). An analysis of SOX gene ex-
pression in these different cell types showed quite distinct patterns of expression. Untreated F9 cells primarily expressed Sox2 and Sox13, and after RA treatment the resulting VE-like cells continued to express these SOX genes. In addition, the expressions of Sox6, Sox7, and Sox17 were induced in the VE-like cells. In contrast, the PE-like cells expressed only Sox7 and Sox17 at a high level. Fgf-3 expression is associated with the parietal endoderm in vivo and was also induced in the PE-like cells in culture, although a small amount was seen in the cells treated with RA alone. The presence of some Sparc mRNA in the VE-like culture indicates generation of PE-like cells as a minor population even with RA treatment alone, and this could account for the low level of Fgf-3 expression in the VE-like culture.

Identification of SOX Protein Complexes Formed with PS4A—EMSA was used to examine the binding of SOX pro-
teins to the PS4A element. The ability of SOX2 to form com-
plexes with PS4A was demonstrated using undifferentiated F9 cell nuclear extracts and supershifting part of the complex with an anti-SOX2 serum (Fig. 4A, lane 2). As an additional control, in vitro transcribed/translated SOX2 was shown to bind PS4A and yielded a complex with a mobility indistinguishable from that formed with F9 nuclear extracts (Fig. 4B, lanes 1 and 2). We suspect that the incomplete supershift of the complex is because of a low antibody titer, although we cannot exclude the presence of other SOX proteins. When using differentiated F9 nuclear extracts, a minor complex with the same mobility as that attributed to SOX2 was detected (Fig. 4A, lane 3) and partially supershifted with the anti-SOX2 serum (lanes 4 and 6). This suggests that a small amount of SOX2 complex was also formed with differentiated F9 nuclear extracts and is consistent with the reduced level of Sox2 transcripts in differ-
entiated F9 cells (Fig. 3). The major complex formed with differentiated F9 nuclear extracts was slightly less mobile and significantly decreased by addition of either anti-SOX7 or anti-
GATA-4 serum (Fig. 4C, lanes 3–5). However, the complex was essentially lost when both antisera were added together (Fig. 4C, lane 6). Thus, the major complex derived from differenti-
ated F9 cell nuclear extracts with PS4A is likely to be a mixed population of complexes resulting from SOX7 or GATA-4 bound to the probe. The anti-SOX7 serum had no effect on the com-
plexes formed with undifferentiated F9 nuclear extracts (Fig. 4C, lanes 1 and 2). As a size control for the SOX7 complex, in vitro transcribed/translated SOX7 was shown to form a complex with the same mobility (Fig. 4C, compare lanes 3 and 7). A complex apparent as the upper band in Fig. 4C, lane 6, is probably SOX17 bound to PS4A, because in vitro transcribed/ translated SOX17 formed a complex with the same mobility (Fig. 4D, lanes 2 and 3). Hence, the EMSA results are consistent with the abundance of Sox2, Sox7, and Sox17 mRNAs in F9 cells (Fig. 3). Although in vitro transcribed/translated SOX6 and SOX13 bound PS4A (see Ref. 26; data not shown), complexes formed by these proteins were not detected with either differentiated or undifferentiated F9 nuclear extracts.

Analysis of SOX Gene Expression in F9 Cells—To test the

| Member of SOX family | No. of clones<sup>a</sup> | dF<sub>9</sub>| |
|----------------------|-----------------|---------|
| Sox2                 | 7               | 20      |
| Sox6                 | 0               | 2       |
| Sox7                 | 3               | 66      |
| Sox13                | 0               | 5       |
| Sox17                | 0               | 7       |
| Total                | 10              | 100     |

<sup>a</sup> PCR products were cloned into pCR4-TOPO and sequenced.<sup>b</sup> Undifferentiated F9 cells.

<sup>c</sup> Differentiated F9 cells generated by RA and Bt,cAMP treatment.
Differentiated F9 nuclear extract.

indicate the major complex formed using the differentiated F9 nuclear extract and its supershifted band formed upon the addition of anti-SOX7 serum, respectively. Lanes 5 and 6 are a longer exposure of lanes 3 and 4, respectively. B, complex formed with in vitro transcribed/translated SOX2 protein (iv–SOX2). C, effects of anti-SOX7 serum and anti-GATA-4 serum on the complexes formed with F9 nuclear extracts. The complex formed with in vitro transcribed/translated SOX7 (iv–SOX7) is shown for comparison. The arrow and arrowheads indicate the major complex formed using the differentiated F9 nuclear extract and its supershifted band formed upon the addition of anti-SOX7 serum, respectively. D, complex formed with in vitro transcribed/translated SOX17 (iv–SOX17). The arrow indicates the complex formed using the differentiated F9 nuclear extract.

ability of different SOX proteins to function at PS4A, individual SOX expression vectors were co-transfected into F9 cells with a PS4A-containing reporter, pPS4A×3tkLuc (Fig. 5B). An immunoblot analysis of SOX protein levels showed that although there was some variation in the total amount of protein, a similar level was expressed in both undifferentiated and differentiated F9 cells from the individual constructs (Fig. 5C). The activity of the reporter alone (columns labeled vector) was higher in differentiated compared with undifferentiated F9 cells, reflecting endogenous Fgf-3 transcription (see Fig. 3). Elevated expression of SOX2 showed 3.5- and 1.5-fold reporter activity in differentiated and undifferentiated F9 cells, respectively, indicating that SOX2 is not an effective activator of PS4A. This appears to be the reason why Fgf-3 transcription remains at a low level in undifferentiated F9 cells despite the high expression of Sox2 and the efficient binding of SOX2 to PS4A (Figs. 3 and 4A). On the other hand, enhanced expression of SOX7 caused a 10-fold increase of reporter activity in both undifferentiated and differentiated F9 cells, demonstrating that SOX7 is a potent activator of PS4A. As Sox7 expression is induced after F9 cell differentiation (Fig. 3), and SOX7 is the major protein bound to PS4A using differentiated F9 nuclear extracts (Fig. 4C), it is a prime candidate transactivator of endogenous Fgf-3 transcription. It is highly likely that Fgf-3 transcription is also modulated by other SOX proteins competing for binding, such as SOX17 that has low activation activity and SOX6 and SOX13 that repress transcription (Fig. 5). However, the contribution of these proteins to Fgf-3 transcription seems to be small, because they were minor components of the SOX proteins that bound to PS4A (Fig. 4).

**GATA-4 and SOX Are Independent Activators of the Fgf-3 Promoter**—As both SOX proteins and GATA-4 bind to the PS4A element (Fig. 4), it is possible that they could form a higher order complex on PS4A to cooperatively activate the Fgf-3 promoter. To clarify the relationship between GATA-4 and SOX protein binding, we performed binding competition experiments. The complexes formed on PS4A with F9 nuclear extracts, or with in vitro translated factors, were specifically competed with DNA containing the SOX (HMG) or GATA-binding site (Fig. 6A). When differentiated F9 nuclear extract was used, addition of the SOX competitor (HMG) allowed formation of the complex attributed to GATA-4 binding (Fig. 6, lane 2), whereas the GATA competitor allowed the formation of complexes attributed to the binding of the SOX proteins (lane 3). Furthermore, in vitro transcribed/translated SOX7 and GATA-4/3575N (which forms a faster migrating complex than SOX7 because of the lack of the N-terminal 75 amino acids) each bound to PS4A in the absence of the other (Fig. 6, lanes 5 and 6). Thus, GATA-4 and SOX proteins bind PS4A independently of each other. In addition, we examined whether GATA-4 and SOX7 are associated with each other when both proteins bind PS4A. Under conditions of limiting PS4A DNA, labeled GATA-4 and SOX7 proteins were added to a differentiated F9 cell lysate made from cells expressing tagged GATA-4 and SOX7. The PS4A probe was isolated through a biotinylated tag, and the bound GATA-4 and SOX7 proteins were detected by fluorography (Fig. 6B). To determine whether both proteins bind to the same PS4A site, the bound proteins were immunoprecipitated using antibodies to the FLAG and His tags on SOX7 and GATA-4, respectively (Fig. 6B). Immunoprecipitation of GATA-4 did not precipitate SOX7 and vice versa. Therefore, we conclude that GATA-4 and SOX7 bind exclusively to PS4A to form complexes. As their recognition sequences overlap, it is likely that they bind PS4A competitively. To test this prediction, we performed a reporter assay in which GATA-4 and the SOX proteins were co-expressed with an Fgf-3 reporter plasmid, pFgf-3/Luc, which contained a 1.7-kb fragment of the Fgf-3 promoter region (Fig. 7). Expression of the individual SOX protein resulted in Fgf-3 promoter stimulation similar to that seen using the synthetic promoter containing PS4A sites.
Figs. 5B and 7), with SOX7 showing the strongest activation. Expression of GATA-4 alone caused a stronger stimulation than that caused by any SOX protein, suggesting that GATA-4 has a greater trans-activation ability. When GATA-4 was co-expressed with each SOX protein, an activity intermediate between those induced by the individually expressed GATA-4 and SOX proteins was obtained. These results are consistent with the independent and exclusive binding of SOX and GATA-4 to the Fgf-3 promoter (Fig. 6). When SOX7 and SOX17 were expressed with GATA-4, the activity of the Fgf-3 promoter was approximately equal to the average of the individual SOX and GATA-4 activation levels, indicative of independent but exclusive transcription activators. In contrast, co-expression with the other SOX proteins, SOX2, SOX6 and SOX13, resulted in a considerable suppression of activity compared with GATA-4 alone, consistent with them being poor activators but able to displace GATA-4 on PS4A.

To assess the relative potency of GATA-4 or SOX as an endogenous trans-activator of Fgf-3 transcription, we constructed variants of pFgf-3/Luc that retained only a GATA or SOX site or neither binding site (Fig. 8A). These constructs or the parental vectors were transfected into F9 cells and stable clones selected. As seen in Fig. 8B, the 4A20T mutation that affected both binding sites greatly decreased the complexes on PS4A, and the cells containing the cognate reporter construct showed only a basal level of promoter activity (Fig. 8C). Introduction of a mutation to abolish GATA binding (4AzM1 mutation) allowed binding of the SOX proteins only, as confirmed by the lack of GATA4 complex formation in the presence of the HMG competitor. The mutated SOX site (4A17G mutation)
allowed formation of a GATA-4 complex that was competed by the GATA competitor (Fig. 8). F9 cells containing the reporter harboring a GATA site mutation showed only a slightly lower activity than cells expressing parental reporter, whereas cells with the reporter containing the SOX binding mutation showed substantially higher activity (Fig. 8). These results demonstrate that in differentiated F9 cells, both the endogenous SOX proteins and GATA-4 could stimulate the Fgf-3 promoter independently of the other. In the absence of SOX protein binding activity, GATA-4 appears the more potent activator. However, this may in part be due to a lack of a negative effect of SOX2 binding as mentioned above, and the effect could also negatively affect the activity of SOX7 in the absence of GATA-4 binding.

Fig. 7. Effects of co-expressed SOX proteins and GATA-4 on the Fgf-3 promoter. A, schematic representation of the reporter plasmid (20) used in B. Only the major transcription start site, P3, is indicated. B, reporter assay of pFgf-3/Luc co-transfected with effector plasmids for the expression of SOX and/or GATA-4 proteins. Undifferentiated F9 cells were transfected with the reporter together with the indicated effector plasmids. Luciferase activities relative to the value obtained with the vector alone are shown with standard error bars. C, Western blotting analysis of SOX and GATA-4 proteins expressed in the transfected cells. GATA-4 and each SOX protein were immunoprecipitated with an anti-His tag and an anti-FLAG antibodies, respectively, from the cell lysate and detected using the same antibody after SDS-PAGE and Western blotting. Asterisks indicate full-length products of SOX proteins. Nonspecific bands indicated by arrows are looked on as a control for protein loading.

Fig. 8. Activity of the Fgf-3 promoter containing mutations affecting either the SOX or GATA-binding site. A, nucleotide sequences of PS4A and its substituted derivatives used as probes in B or introduced into pFgf-3/Luc to form the mutated reporters in C. Asterisks indicate substituted nucleotides. The recognition sequences of GATA-4 and SOX proteins are boxed with solid and dashed lines, respectively. B, EMSA using the indicated probes and a nuclear extract from differentiated (d) (with RA and Bt2cAMP) F9 cells in the presence or absence of a competitor. HMG and GATA competitors were described in the legend of Fig. 7. C, reporter assay of pFgf-3/Luc derivatives containing the mutations in PS4A as illustrated in A. Each reporter plasmid was transfected into F9 cells, and the resulting colonies of stable transfectants were pooled. Each pool contained more than 500 transfected clones to minimize positional effects. Undifferentiated cells of all these pools showed only a basal level of luciferase activity (data not shown), and activities in the cells after the induction of differentiation with RA and Bt2cAMP were measured and normalized to plasmid copy number as determined by Southern blot hybridization. Values relative to that of pFgf-3/Luc are shown with standard error bars.

Co-expression of Sox7, Gata4, and Fgf-3 in the Parietal Endoderm of Mouse Embryos—Because the differentiated F9 cells generated by retinoic acid and dibutylryl cAMP treatment show properties of parietal endoderm (35), expression of Fgf-3, Sox7, and Gata4 was analyzed at embryonic day (E)7.5 in mouse embryos by in situ hybridization (Fig. 9). Consistent with previous reports (9, 12), Fgf-3 is expressed in the same cells as those expressing Sparc, a marker of the parietal endoderm (43), and in cells at the embryonic/extraembryonic border, which correspond to the embryonic mesoderm migrating through the primitive streak. Gata4 expression was also detected in the parietal endoderm and embryonic mesoderm, and additionally in the visceral endoderm as reported (44, 45). Most interesting, the expression pattern of Sox7 is quite similar to that of Gata4, showing expression in the parietal and visceral endoderm and the embryonic mesoderm (Fig. 9), Thus, Sox7 and Gata4 are co-expressed with Fgf-3 in both the parietal endoderm and embryonic mesoderm.

Expression of Fgf-3 in GATA-4-deficient ES Cells—In addition to F9 cells, ES cells also express Fgf-3 following differen-
tiation (12). As seen in Fig. 10A, expression of Fgf-3 was induced as early as 3 days by culturing the ES cells (R1) as embryoid bodies (EB). Similar to F9 cells, an induction of Gata4 and Sox7 expression was seen in EBs at day 3 suggesting their involvement in Fgf-3 expression, although their expression was sustained beyond that of Fgf-3. Lineage markers for the prim-itive endoderm and early mesoderm, Hnf3β/H9252 and T (Brachyury) respectively, began to appear at day 3, consistent with the co-expression of Fgf-3, Gata4, and Sox7. In EBs from GATA-4-deficient ES cells, Fgf-3 was expressed at a much lower level and at later times of differentiation than in R1 cells. Although this may indicate that GATA-4 is a major activator of Fgf-3 expression, the lower Sox7 expression in the Gata4 null cells complicates the interpretation.

The mutant EBs are defective in endoderm differentiation (23) and show a lower and delayed mesoderm induction (Fig. 10A). This suggests an alternative explanation for the low level and delay in Sox7 and Fgf-3 expression. To assess directly the contribution of SOX7 to Fgf-3 expression in the absence of GATA-4, Sox7 expression was suppressed by the method of RNA interference (41) (Fig. 10B). Gata4−/− ES cells were transfected with the vector pKS/mH1-Sox7 together with a hygromycin selectable marker, and several drug-resistant clones were selected. After the induction of differentiation, analysis of six independent clones showed three with a substantial loss of Sox7 mRNA. These three clones showed a similar loss of Fgf-3 mRNA. The other three clones showing no significant loss of Sox7 mRNA showed no significant loss of Fgf-3 expression.

DISCUSSION

In this study we have shown that members of the SOX family of transcription factors can bind and activate the Fgf-3 promoter. This activation is competitive with the previously identified transactivator GATA-4 that has an overlapping DNA binding footprint on the promoter (21). Although not as strong as GATA-4, SOX7 is the strongest of the SOX proteins tested.
that activate Fgf-3 transcription. Moreover, its expression correlates with Fgf-3 induction in F9 and ES cells. In GATA-4-deficient ES cells, suppression of Sox7 mRNA by RNAi shows a strong concordant loss of Fgf-3 expression, suggesting that Sox7 serves as a trans-activator of Fgf-3 transcription.

At least five SOX genes, Sox2, Sox6, Sox7, Sox13, and Sox17, are expressed in differentiated F9 cells (Table I and Fig. 3), and Sox2, Sox7, and Sox17 proteins present in differentiated F9 nuclear extracts were shown to bind PS4A (Fig. 4). In addition, Sox7 and to a lesser extent Sox17 activate the Fgf-3 promoter when expressed in F9 cells, whereas Sox6 and Sox13 repress promoter activity (Figs. 5 and 7). Moreover, in the presence of GATA-4, SOX2 appears to compete for binding and in this context acts as a negative modulator of Fgf-3 expression (Fig. 7B). Expression of multiple SOX genes is not exceptional. For example, Sox1, Sox2, and Sox3 are expressed in the developing nervous system (28), and Sox5, Sox6, and Sox9 are expressed in chondrocytes (30). Although the functional significance of multiple SOX gene expression in a single tissue is unclear, the different functional activities of these proteins, as shown here, could provide fine-tuning of gene expression through the same regulatory element.

Of the three SOX proteins that activated the Fgf-3 promoter, Sox7 was the strongest; Sox7 showed a much weaker activity, and Sox2 gave only a marginal positive response (Figs. 5 and 7). As there is little difference in binding efficiency of these three SOX proteins to PS4A (data not shown), the activating ability of Sox7 would appear to be inherent or mediated through an interaction with other factors or co-activators. As Sox7 is the most abundantly expressed SOX gene in differentiated F9 cells, and its expression is induced in parallel with that of Fgf-3 (Table I and Fig. 3), it seems likely that Sox7 is the main activator of Fgf-3 transcription in these cells. This is further supported by the knockdown experiments in Gata4−/− ES cells as indicated above. To date, there is little information concerning the sites of expression and target genes for Sox7. Here we were able to show Sox7 expression in F9 cells, ES cells, and at embryonic endodermal sites, suggesting a significant role for this transcription factor in early mouse development (Figs. 3, 9, 10, and 11).

SOX proteins can associate with other factors that bind adjacent to target promoters, often resulting in complexes that cooperatively regulate the target genes in a cell type-specific manner (reviewed in Refs. 46 and 47). Although both Sox7 and GATA-4 bind the PS4A element in the Fgf-3 promoter, their binding is competitive rather than cooperative (Fig. 6). The canonical GATA motif in PS4A is not sufficient for GATA-4 binding but requires a second binding site (21) that mediates the cooperative mechanism described previously (46, 47). It is surprising that in the absence of cooperativity, Sox7 and Gata4 are frequently expressed in the same cells or tissues expressing Fgf-3, e.g. differentiated F9 and ES cells and the parietal endoderm at E7.5 (Figs. 3, 9, and 10). It is not certain whether both Sox7 and GATA-4 contribute to the activation of Fgf-3 transcription in these cells. In binding experiments using Sox7 and GATA-4 made in vitro, both bind PS4A to a similar amount (Fig. 6), suggesting a similar affinity of each protein for PS4A. Therefore, it may be possible that both proteins are involved in the regulation of Fgf-3 expression in a cell or tissue-dependent manner.

In contrast to the parietal endoderm, Fgf-3 is not signific-
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