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Sox9 Transactivation and Testicular Expression of a Novel Human Gene, *KIAA0800*

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Abstract The Sry and Sox9 sex-determination factors initiate and promote testis differentiation by gene transactivation through similar promoter elements. However, knowledge is limited concerning what genes are regulated by Sry/Sox9 in the testis. Identification and characterization of Sry/Sox9-regulated genes are critical for understanding sexual differentiation. We now demonstrate that a novel human gene, *KIAA0800*, is preferentially expressed in the testis and is transactivated by Sox9. The *KIAA0800* promoter is repressed by an upstream element involving a polyT track and two *Alu* repeats. Two specific Sox9-bindings sites have been identified in the *KIAA0800* promoter by using DNaseI footprinting assays and gel electrophoretic mobility shift assays. Sox9 transactivation of the *KIAA0800* promoter appears to be exerted mainly through the relief of promoter repression. Genes homologous to the human *KIAA0800* exist in organisms with differentiated sex tissues including mouse, *Drosophila*, and *C. elegans*, but not in unicellular organisms, including yeast and bacteria. Further, our recent sequence analysis shows that KIAA0800 protein is 97% identical between human and mouse. Thus, *KIAA0800* is a novel Sox9-activated gene that is evolutionarily conserved and potentially involved in sexual differentiation. J. Cell. Biochem. 86: 277–289, 2002.

Key words: differentiation; sex; *KIAA0800*; Sox9; transactivation

During embryogenesis, expression of *Sry* from the Y chromosome sets off a cascade of gene transcription events that lead to the development of the testis, whereas in the female, the ovary develops by default [Berta et al., 1990; Koopman et al., 1991; Capel, 1998; Haqq and Donahoe, 1998; Koopman, 1999; Swain and Lovell-Badge, 1999]. *Sry* expression in the mouse genital ridge is transient, detectable only between 10.5–12.5 days post coitum [Hacker et al., 1995]. *Sry* expression is immediately followed by the activation of the *Sry*-related gene, *Sox9*, which helps activate downstream genes in the testis differentiation pathway [Foster et al., 1994; Wagner et al., 1994; Kent et al., 1996; Morais da Silva et al., 1996]. Unlike *Sry*, *Sox9* expression is decreased at later stages of testis development, but persists throughout adulthood [Morais da Silva et al., 1996; de Santa Barbara et al., 2000; Frojdman et al., 2000; Hanley et al., 2000]. To date, only limited information is available on what genes are activated by *Sry*/*Sox9* in the testis. The gene for the anti-Mullerian hormone (*Amh*) or Mullerian inhibiting substance (*Mis*) has been well characterized for its synergistic activation in the testis by Sox9 and the steroidogenic factor Sf1 [Shen et al., 1994; De Santa Barbara et al., 1998; Arango et al., 1999]. *Sox9* is also preferentially expressed in chondrocytes and activates the expression of type II collagen [Bell et al., 1997; Lefebvre et al., 1997; Ng et al., 1997].

Sry and Sox9 interact with DNA through a conserved High Mobility Group (HMG) domain that is frequently mutated in sex-reversed XY females [Capel, 1998; Haqq and Donahoe, 1998; Koopman, 1999]. The HMG domains of Sry and Sox9 bind to similar DNA motifs. PCR-based selection has helped determination of the consensus Sry binding site with a core of AACAAT [Dubin and Ostrer, 1994; Harley et al., 1994]. Sox9 binds to the consensus *Sry* site as well as divergent motifs present in the well-

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characterized *Mis/Amh* promoter and the promoter for the type II collagen [Shen et al., 1994; Bell et al., 1997; De Santa Barbara et al., 1998; Mertin et al., 1999]. Unlike *Sox9*, which is highly conserved throughout evolution, *Sry* has diverged extensively in regions outside of the HMG domain [Whitfield et al., 1993; Koopman, 1999]. The human *Sry* does not cause sex reversal in transgenic XX female mice [Koopman et al., 1991], suggesting that *Sry* functions in a species-specific manner. Among the Sry proteins, only the mouse Sry has a transactivation domain and maintenance of this domain is essential for Sry sex-determination function [Dubin and Ostrer, 1994; Bowles et al., 1999]. In contrast, Sox9 of various species has both an N-terminal HMG domain and a C-terminal transactivation domain, and transactivates through specific promoter elements in cell lines [Lefebvre et al., 1997; De Santa Barbara et al., 1998; Kamachi et al., 1999]. In animals, tissue-specific Sox9 co-factors may cooperate with Sox9 to transactivate different sets of genes in the chondrocytes and in the testis [Kent et al., 1996; Morais da Silva et al., 1996].

The human *KIAA0800* cDNA was originally cloned as one of the large cDNAs from the human brain [Nagase et al., 2000] and as an interaction partner to the HIV-1 regulatory protein Vpr [Zhang et al., 2001]. By RT-PCR analysis, we discovered that *KIAA0800* is preferentially expressed in the testis [Zhang et al., 2001]. In this study, we have confirmed the initial observation by examining mRNA from multiple human tissues. We show that Sox9 specifically transactivates the *KIAA0800* promoter. Interestingly, the *KIAA0800* promoter is repressed by a negative regulatory element involving promoter-distal *Alu* repetitive sequences and a promoter-proximal poly-T track, suggesting that the *KIAA0800* promoter is tightly controlled. Despite the identification of specific Sox9 binding sites in the *KIAA0800* promoter, we have observed Sox9 targeting of the minimal *KIAA0800* promoter in the absence DNA binding. Our results indicate that Sox9 transactivation of the *KIAA0800* promoter may be exerted mainly through countering promoter repression.

MATERIALS AND METHODS

Cell Culture, DNA Transfection, and CAT Assay

There are 3 types of cells, Cos-7, 293 and NT2/D1 cells (ATCC) were propagated in DMEM supplemented with 10% fetal calf serum. DNA transfection was performed in 12-well plates using the Superfect reagent (Qiagen) following recommended conditions. Cos-7 cells were plated at 5×10^4 cells/well the day before transfection, whereas 293 cells were plated at 2×10^5 cells/well. Normally, 1.0 μg of the CAT plasmid and 0.5 μg of the activator plasmid or the pEGFP-N1 vector plasmid (ClonTech) were co-transfected, unless indicated otherwise. Two days after transfection, cell lysates were prepared (200 μl) and CAT assays performed under published conditions [Zhao and Giam, 1992]. All transfections and CAT assays were repeated at least two times to confirm the result.

Primer Extension

PolyA$^+$-mRNA was purified from 7×10^6 cells using the Micro mRNA Purification Kit (Amersham Pharmacia Biotech). The reverse primer for *KIAA0800* AGCTTACACAGCCTCAGGTCCCG(+106) was labeled with γ-^{32}p-ATP and T4 polynucleotide kinase (New England Biolabs), and 15 ng of the labeled primer was used for reverse transcription with 0.5 μg of polyA$^+$-mRNA and the M-MLV reverse transcriptase (Life Technologies) under recommended conditions. Products were analyzed on a 7% sequencing gel. Dideoxy sequencing reactions were carried out with the same primer and the pKIAA-CAT DNA (see next) using the Sequenase Kit (USB) under recommended conditions.

DNaseI Foot-Printing

The -469 to -172 region of the KIAA0800 promoter was cloned into the SacI site of pEGFP-N1 as a SacI fragment in forward and reverse orientations to generate pCMV-Ki(SS)-F and pCMV-Ki(SS)-R constructs, respectively. The BglII /Bam HI fragment of both constructs was dephosphorylated, purified from an agarose gel, and labeled with γ-^{32}P-ATP and T4 polynucleotide kinase (New England Biolabs). The labeled DNA fragment was cleaved with Pst I, and ~0.5 ng of the labeled DNA was incubated with purified Sox9-FH (see next) at room temperature for 30 min in 3 μl of a buffer containing 10 mM HEPES (pH 7.5), 50 mM KCl, 6% glycerol, 0.5 mM DTT, and 0.1 mM PMSF. The incubation mixture was then digested with 0.06 U of DNaseI (Promega) for 10 min at 25°C in a 5 μl volume in the presence of 5 mM $CaCl_2$. Samples were examined on a 6% sequencing gel. Dideoxy-sequencing reactions were carried out

using the Sequenase Kit (USB) with a primer derived from the pEGFP-N1 vector. The sequencing ladders thus carried 19 extra bases compared with the bands derived from the DNaseI-treated probe.

Sequences of PCR Primers

Primer no. 1 CACCTTGAATTCCCATGAATCTCCTGGACCCCTTCATGAAGATG,
Primer no. 2 ACATCAGAATTCACTCGAGAGGTCGAGTGAGCTGTGTGTAGAC,
Primer no. 3 ACACGTCTCGAGAACAGCCTGGCCAACACGGC,
Primer no. 4 AGTCACAAGCTTACACAGCCTCAGGTCCCGCACTC,
Primer no. 5 ACACTGCTCGAGCCACCAAGCACACTTGGAATGCGTGTGCAG,
Primer no. 6 GTTGGCCAGGCTGTTCTCGAGCTCCTGGGCTGAG,
Primer no. 7 ACAGCACTCGAGTGCCTTCTGATTTAATGAGTGCTG,
Primer no. 8 ACATGACTCGAGCAAGACCTAGCCTGTAAGAATCAATAGATAC,
Primer no. 9 ACACTGCTCGAGGTTGGGTTTCTGAAATGAAGCAGTGCCATC,
Primer no. 10 GGGGCTCTTCGACCCCGATCGTGCAGGCGTTCTCTTC,
Primer no. 11 CGCAAACCGCCTCTCCTTTATTTGGCC,
Primer no. 12 AATAAAGGAGAGGCGGTTTGCG,
Primer no. 13 TCTAAGGGAGTACTCCAATCGC,
Primer no. 14 GCGATTGGAGTACTCCCTTAGA.

Sox9 Constructs

The human *Sox9* open reading frame (ORF) (GenBank Accession Number NM_000346) was PCR-amplified with primer no. $1+2$ from testis-derived cDNA. The PCR DNA was inserted into pEGFP-N1 (ClonTech) at the EcoRI site to generate pCMV-Sox9. All PCR reactions were performed with the High Fidelity Polymerase (Roche) under suggested conditions, except for *Sox9* PCR when 6% DMSO was included. The *Sox9* ORF was completely sequence-confirmed and then transferred to pCMV-Vpr-T [Zhang et al., 2001] to generate pCMV-Sox9-FH. The pCMV-Sox9-FH DNA was digested with BglII/SacI to remove the 5′ region of the Sox9 ORF, and then ligated to a BglII-SacI linker, GATCTACCATGGCAGGATCCGAGCT, to generate pCMV-Sox9(ΔN)-FH. The BglII-Bam HI fragment of pCMV-Sox9-FH, which contains the complete *Sox9* ORF, was transferred to the Bam HI site of pBacPak8 vector (Clontech) to generate pBacPak8-Sox9-FH for baculovirus expression.

CAT Reporter Constructs

Primer no. $3+4$ were used to PCR-amplify the -716 to $+123$ region of the *KIAA0800* promoter from HeLa cell genomic DNA. The PCR DNA was used to replace the LTR-containing XhoI/HindIII fragment of pLTR-CAT [Zhang et al., 2001] to generate pΔA2ΔA3-CAT. The full-length promoter construct, pKIAA-CAT, was made by insertion at the XhoI site of pΔA2ΔA3-CAT of a PCR fragment amplified with primer no. $5+6$. The pΔA-CAT construct was prepared by replacing the XhoI/HindIII fragment of pLTR-CAT with a PCR DNA amplified with primer no. $7+4$. The PCR DNA amplified with primer no. $5+6$ was inserted at the XhoI site of pΔA-CAT to generate pΔA1-CAT. The pΔA2-CAT and pΔA3-CAT constructs were prepared by inserting the PCR DNA amplified with primer no. $5+8$, and the PCR DNA amplified with primer no. $9+6$, respectively, into the XhoI site of pΔA2A3-CAT. The PCR DNA amplified with primer no. $10+4$ was used to replace a SmaI/HindIII fragment of the pKIAA-CAT construct to generate pΔT-CAT. To construct pΔA/Sx(aM)-CAT that carries mutations in *Sox-a*, two PCR reactions were performed with primer no. $7+11$, and primer no. $4+12$, using pΔA-CAT as the template. The two PCR DNAs were used together as the template for a second round of PCR with primer no. $4+7$, and the final PCR DNA was used to replace the XhoI/HindIII fragment of pΔA-CAT. The construct with mutations in both *Sox-a* and *-b* motifs, pΔA/SxM-CAT, was generated similarly by using primer no. $7+13$ and the pΔA/Sx(aM)-CAT template, and primer no. $4+14$ and the pΔA-CAT template for the first round PCR. The "Minimal Promoter" construct, pMP-CAT, was prepared by digesting pΔT-CAT with XhoI and SmaI, treatment with Klenow, and religation. For all PCR-amplified promoter regions, automatic sequence analysis was performed over at least 90% of the amplified region to confirm the accuracy of the construct.

Sox9-FH Expression and Partial Purification From Insect Cells

Sox9-FH-expressing baculovirus was obtained with pBacPak8-Sox9-FH following recommended protocols (ClonTech). Sf21 cells (6×10^6) in a T-75 flask were infected with 6×10^6 PFU of Sox9-FH baculovirus and incubated for 36 h. Infected cells were harvested and lysed for 30 min at 4°C in a buffer containing 20 mM HEPES (pH 7.5), 500 mM NaCl, 10% glycerol, 0.5% NP-40, 0.5 mM DTT, and 0.2 mM PMSF. Cell lysate was bound to 100 μl of the Flag antibody beads (M2AG, Sigma), and washed with the cell lysis buffer. The bound Sox9-FH was eluted with 200 μl of the cell lysis buffer containing 100 mM NaCl, 0.1% NP-40, and 1 mg/ml of the Flag peptide.

Preparation of a Small Scale Nuclear Extract From Transfected 293 Cells

293 cells were plated in a 100-mm dish and transfected with pCMV-Sox9-FH or the pEGFP-N1 vector. Two days later, cells were collected and resuspended in 200 μl of a hypotonic buffer: 10 mM HEPES (pH 7.5), 5 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF. After incubation on ice for 10 min, cells were lyzed by passing through a 27G needle 10 times. Nuclei were pelleted by a 1 min centrifugation in a microcentrifuge at 1,800*g*. The nuclear pellet was washed once with the hypotonic buffer containing 20% glycerol, and extracted for 30 min at 4°C with the hypotonic buffer containing additional 0.5 M NaCl and 20% glycerol. The crude nuclear extract was clarified by centrifugation and dialyzed for 2 h against the hypotonic buffer containing additional 0.125 M NaCl and 20% glycerol.

Electrophoretic Mobility Shift Assay (EMSA)

All probes were labeled with γ-^{32}p-ATP and T4 polynucleotide kinase. Unless indicated otherwise, purified Sox9-FH (25 ng) or crude nuclear extracts (4 μg of total protein) were incubated with the probe for 30 min at room temperature in 8 μl of a buffer containing 25 mM HEPES (pH 7.5), 25 mM KCl, 0.5 mM EDTA, 0.5 mg/ml BSA, 10% glycerol, 0.25 mM DTT, and 0.05 mM PMSF. In addition, assays with crude nuclear extracts contained 0.5 μg of polydG:polydC (Roche). Protein–DNA complexes were resolved by electrophoresis on a 5% polyacrylamide gel using a Tris-glycine buffer. Gels were dried and analyzed on a phosphoimager.

RESULTS

KIAA0800 Is a Conserved Gene Preferentially Expressed in the Testis

With the KIAA0800 amino acid sequence, we performed a Blast search of databases and discovered *KIAA0800* homologues in species with differentiated sexual tissues, including *C. elegans*, *Drosophila*, and mouse (Table I), but not in unicellular organisms like yeast and *E. coli*. Recently, we have cloned the complete cDNA for the mouse *KIAA0800* homologue based on the highly homologous mouse EST sequences matching to the 5′ and the 3′ ends of the human *KIAA0800* cDNA. Complete sequence analysis of the mouse *KIAA0800* cDNA shows that mouse and human are 97% identical at the KIAA0800 protein level and 91% identical at the *KIAA0800* cDNA level (data not shown). KIAA0800 has no known functions, but conserved regions exist in the identified KIAA0800 proteins from the various species (data not shown), suggesting that KIAA0800 has a novel function conserved in higher eukaryotes.

To gain a better understanding of the *KIAA0800* expression profile in human tissues, we examined the human multiple tissue expression array (MTE array, ClonTech) by hybridization with a *KIAA0800* cDNA probe. The MTE

TABLE I. Amino Acid Sequences Homologous to KIAA0800

			Homology	
Species	Gene	Accession no.	No. of amino acids	Score
Human	*KIAA0800*	NP_055518.1	1,507	3,230
D. melanogaster	*CG10080*	AAF46740.1	1,566	222
C. elegans	*ZK1251.9*	CAA92505	1,211	157
A. thaliana	*F6E21.80*	T10670	1,846	106
Mouse	(EST)	AI119075	99[a]	—

[a]Sequence is matched to amino acids no. 1–99 of the human KIAA0800 [Zhang et al., 2001].
Sequence homology search was performed by Blast on the NCBI website.

array contains polyA$^+$ mRNA from multiple human tissues and various RNA$^-$ controls. As shown in Figure 1, *KIAA0800* expression is the highest in the testis (F8) with a basal level expression in all other tissues, including the placenta (B8), uterus (D8), prostate (E8), and ovary (G8) (Fig. 1, top panel). Quantitation of the signals in column 8 shows that *KIAA0800* expression in the testis (F8) is more than ninefold of the average of other tissues (Fig. 1, bottom panel). In contrast, the house-keeping gene *ubiquitin* is expressed relatively uniformly in all the tissues (Fig. 1, middle panel). Similar analysis of the Mouse Master RNA Blot (ClonTech) also shows a preferential testicular expression of the mouse *KIAA0800* homologue (data not shown).

To confirm the testis-specific expression of *KIAA0800*, we performed a Northern hybridization with polyA$^+$ mRNAs prepared from various human tissues (Fig. 2, top panel). As shown, *KIAA0800* is expressed at a high level in the testis (Fig. 2, lane 4), whereas its expression in other tissues, including prostate (Fig. 2, lane 3) and uterus (Fig. 2, lane 5), is only marginal. This analysis also suggests that the *KIAA0800* mRNA does not have variants derived from alternative splicing. Hybridization of the same blot with a *Sox9* probe shows that *Sox9* is co-expressed with *KIAA0800* in the testis (Fig. 2, middle panel). Control hybridization of the same mRNA blot with a β-actin probe reveals a more uniform pattern of expression (Fig. 2, bottom panel). The co-expression of *KIAA0800* with *Sox9* in the testis provides a biological basis for *Sox9* transactivation of *KIAA0800*.

Mapping KIAA0800 Transcriptional Start Site

By Blast database search, we have located *KIAA0800* on human chromosome number 3, and identified the *KIAA0800* promoter/enhancer, which contains three *Alu* repetitive sequences, in tandem and inverse orientations to each other (Fig. 3A). To determine the exact transcriptional start site, we prepared polyA$^+$ mRNA from the testicular cell line NT2/D1, and performed a primer extension analysis with a primer corresponding to a region close to the 5′ end of the reported *KIAA0800* cDNA. This analysis suggests that the transcriptional start site (position +1) is 71-bp upstream of the beginning of the reported *KIAA0800* cDNA (Fig. 3B, lane 1). Identical results were obtained with polyA$^+$ mRNA from 293 cells and HeLa

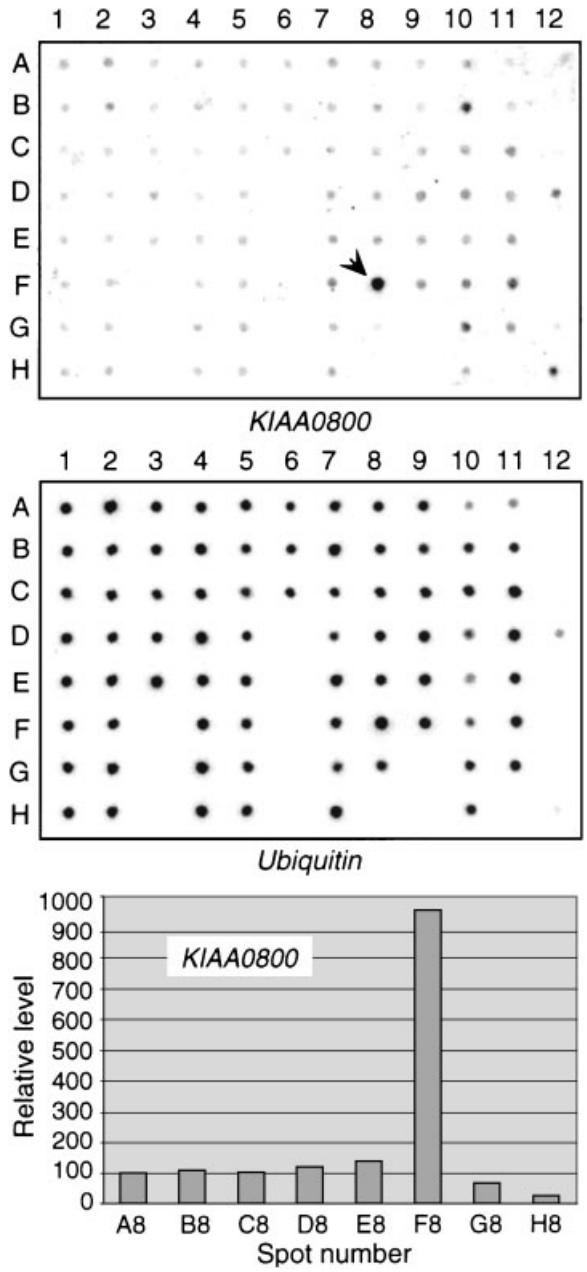

Fig. 1. Analysis of *KIAA0800* expression in 76 human tissues using the multiple tissue expression array (ClonTech). Both the *KIAA0800* and the ubiquitin cDNA probes were prepared with a nick-translation kit (Life Technologies) and α-^{32}p-dCTP. Hybridization conditions were as recommended (ClonTech), and the blots were analyzed by phospho-imager (Storm 840, Molecular Dynamics). Column 8: A, lung; B, placenta; C, bladder; D, uterus; E, prostate; F, testis; G, ovary; and H, blank control. Control nucleic acids in column 12 (A–H): yeast total RNA, yeast tRNA, *E. coli* rRNA, *E. coli* DNA, poly(rA), human C_0t-1 DNA, human DNA (0.1 μg), and human DNA (0.5 μg). All blank spots in columns 3, 6, 8, 9, and 11 contain no mRNA samples. Hybridization of both probes to *E. coli* DNA (D12) was due to the presence of vectors in the probes. Bottom: signals in column 8 (excluding F8) of the *KIAA0800* blot were averaged and the average was set to a relative level of 100.

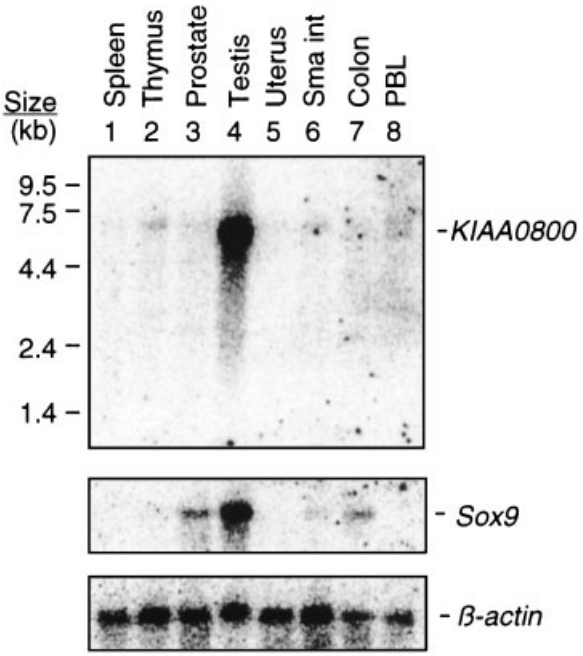

Fig. 2. Preferential testicular expression of *KIAA0800* and *Sox9*. Multiple Tissue Northern blot IV (ClonTech) was hybridized with a nick-translated cDNA probe under recommended conditions. The same blot was consecutively hybridized with *KIAA0800, Sox9,* and *β-actin* probes, with the previous probe stripped from the membrane before the next hybridization. After hybridization, the blot was subjected to phospho-image analysis. ''SmaInt'', small intestine; PBL, peripheral blood lymphocytes.

cells (data not shown). Several sequence features are highlighted in the sequence of the *KIAA0800* promoter (Fig. 3A). Among them are a poly-T track containing 36 T residues, named as *T36*, and two Sox9-binding sites identified by DNaseI footprinting and EMSA (see the next section).

Sox9 Specifically Activates the *KIAA0800* Promoter

The preferential expression of *KIAA0800* in the testis prompted us to examine the possibility that the *KIAA0800* promoter is transactivated by Sox9. To directly analyze regulation of the *KIAA0800* promoter by Sox9, we prepared various *KIAA0800* promoter-CAT reporter constructs and *Sox9* expression constructs (Fig. 4A). Promoter activities were analyzed by CAT assays with extracts prepared from 293 cells transfected with a reporter construct in the presence or absence of pCMV-Sox9 (Fig. 4B). As shown, Sox9 activated the *KIAA0800* promoter (nucleotide position -2850 to $+123$) by more than sevenfold (Fig. 4B, compare lane 1 and 2). Titration of pCMV-Sox9 resulted in a dose-dependent activation of the *KIAA0800* promoter (data not shown). A C-terminal Flag/6xHis-tagged Sox9, Sox9-FH, had comparable activity to the authentic Sox9 (Fig. 4B, lane 3). However, Sox9(ΔN)FH, an N-terminal deletion mutant of Sox9-FH lacking part of the HMG DNA binding domain (Fig. 4A), was defective in transactivation (Fig. 4B, lane 4). Sox9-FH had no detectable activity towards the long terminal repeat (LTR) promoter of the human immunodeficiency virus (Fig. 4B, lane 5 and 6), suggesting that Sox9 transactivation of the *KIAA0800* promoter was highly specific. Western blot analysis with the Flag antibody showed that Sox9-FH was expressed as expected (Fig. 4C, lane 3 and 6), whereas Sox9(ΔN)FH was partially degraded (Fig. 4C, lane 4).

Sox9 Transactivation of the *KIAA0800* Promoter Is Modulated by *T36* and the *Alu* Repeats

Since *Alu* elements have been noticed for their roles during transcriptional regulation [Britten, 1996], the three *Alu* elements in the *KIAA0800* promoter were deleted individually to examine effects on Sox9 transactivation (Fig. 5A). Deletion of *Alu-1* (pΔA1-CAT) did not affect Sox9-FH transactivation or basal promoter activity significantly (Fig. 5B, lane 3 and 4). In contrast, deletion of *Alu-2* (pΔA2-CAT; Fig. 5B, lane 5 and 6), *Alu-3* (pΔA3-CAT; Fig. 5B, lane 7 and 8), or *T36* (pΔT-CAT; Fig. 5B, lane 9 and 10) reduced the degree of Sox9-FH transactivation and raised the basal promoter activity dramatically. Western blot analysis showed that Sox9-FH was expressed to similar levels in even-numbered lanes as expected (Fig. 5B, middle panel). Thus, the KIAA0800 promoter appears to be repressed by the upstream element involving *T36*, *Alu-2*, and *Alu-3*, and Sox9 transactivation is most effective when the *KIAA0800* promoter is fully repressed.

Identification of Specific Sox9-Binding Sites Between *Alu-1* and *T36* in the *KIAA0800* Promoter

Despite the effects of *Alu-2* and *-3* on Sox9 transactivation of the *KIAA0800* promoter, we observed that Sox9 was capable of transactivating the *KIAA0800* promoter even when all three *Alu* repeats were removed (see the next section). We decided to examine potential Sox9 binding to the *KIAA0800* promoter lacking all three *Alu* repeats by using DNaseI footprinting assay.

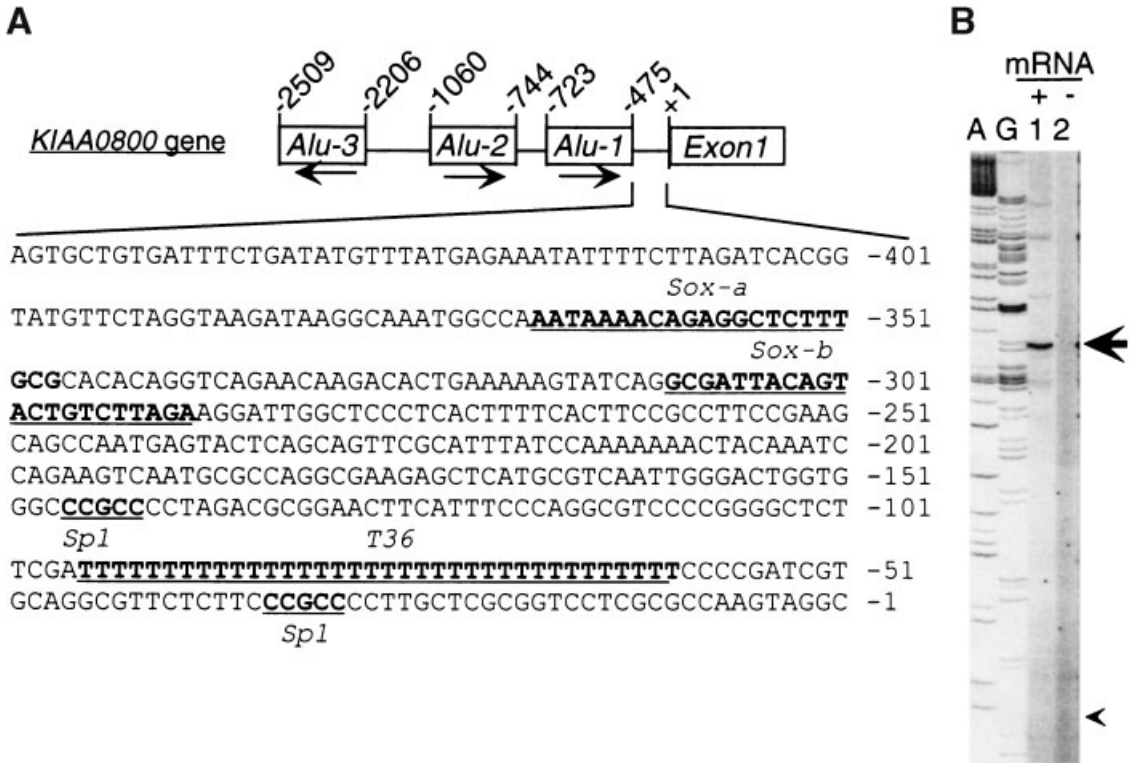

Fig. 3. *KIAA0800* promoter: sequence features and transcriptional start site. **A**: *KIAA0800* promoter sequence features. Promoter region from −1 to −2509 corresponds to nucleotide no. 307339–309847 of GenBank Accession Number NT_022494. *Alu* repetitive sequences were identified on the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu) by the method of ''RepeatMasker.'' The two Sp1 sites are revealed by database search and have not been characterized. **B**: Primer extension assay. ''**A**'' and ''**G**'' lane were dideoxy-sequencing reactions with the unlabeled primer. Thick arrow: Major product of the assay; thin arrow: position +72 corresponding to the 5′ end of the reported *KIAA0800* cDNA (GenBank Accession Number NM_014703).

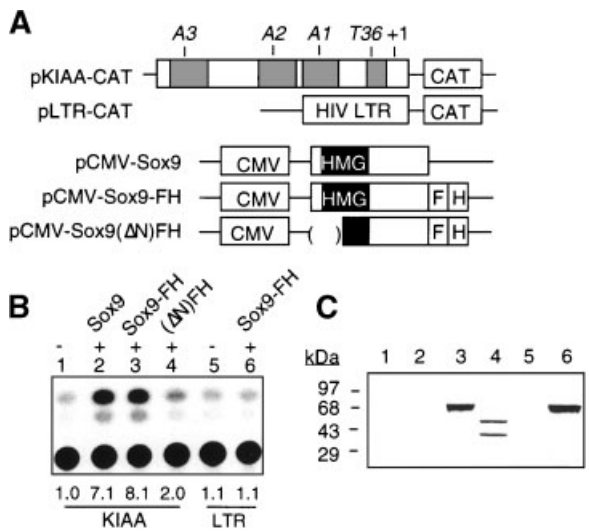

Fig. 4. Transactivation of *KIAA0800* promoter by Sox9. **A**: *KIAA0800* promoter and Sox9 constructs. *A1–A3*: *Alu-1* to *Alu-3*. HMG: high mobility group domain in Sox9.''FH'' tag for Sox9-FH and Sox9(ΔN)FH: Flag/Hexa-histidine tag [Zhao et al., 1994]. **B**: CAT assays with pKIAA-CAT and pLTR-CAT. 293 cells were transfected as described in ''Experimental Procedures.'' Vector DNA pEGFP-N1 was used as a control in lane 1 and 5. CAT assays were performed with 1 μl of cell lysate. Numbers below represent relative CAT activities (as a percent of acetylated chloramphenicol) as determined by phospho-image analysis. **C**: Western blot of the same cell lysates as in (B) with the Flag M2 antibody (Sigma) and alkaline phosphatase-conjugated anti-mouse IgG (Pierce). A 5–15% denaturing gel was used.

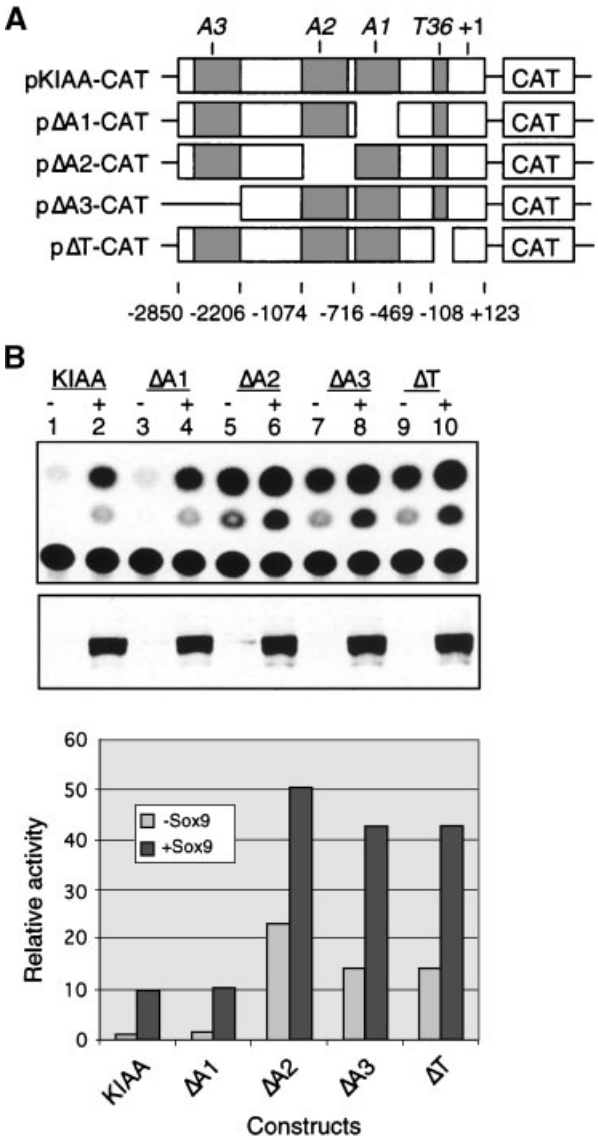

Fig. 5. Deletional analysis of the *KIAA0800* promoter for transactivation by Sox9. **A**: Promoter constructs. Nucleotide positions of the various deletion boundaries are indicated below. **B**: **Top panel**: CAT assays performed with various constructs of the *KIAA0800* promoter. Transfection and CAT assays were under the same conditions as in Figure 4 except with 0.6 μg of the pCMV-Sox9-FH (''+'') or the pEGFP-N1 vector as control (''−''). **Middle panel**: Western blot analysis of the cell lysates as in Figure 4. **Bottom panel**: Relative promoter activity as determined by phospho-imager analysis was plotted, with the activity of the wild-type pKIAA-CAT in the absence of Sox9 normalized to 1.

Sox9-FH was first expressed in insect cells by using the baculovirus expression vector, and purified by using a Flag monoclonal antibody to near homogeneity. With a radiolabeled probe corresponding to the − 108 to + 123 region of the *KIAA0800* promoter, we did not observe significant binding by Sox9 (data not shown). However, with a probe corresponding to the − 469 to − 172 region of the *KIAA0800* promoter, we detected Sox9 footprints in two regions, − 369 to − 348, and − 311 to − 290 (Fig. 6). These two foot-printed regions were observed whether the DNA probe was labeled on the top strand (Fig. 6B, lane 1–5) or on the bottom strand (Fig. 6B, lane 6–10). These two regions were named as Sox9-binding site *a* (*Sox-a*) and Sox9-binding site *b* (*Sox-b*), respectively. Interestingly, both Sox-9-binding sites have a region similar to the well-characterized core of the Sry binding site, AACAAT (Fig. 6A). In addition, the 3′ half of *Sox-a* is nearly identical to the Sox9 binding site present in the *Amh* promoter [Shen et al., 1994], suggesting that *Sox-a* may contain two binding sites for Sox9 (see the next section). For *Sox-b*, the cryptic Sry binding site ACAG is located within a perfect palindrome (see Discussion section).

Sox9 Binds to *Sox-a* and *-b* Specifically

To determine if Sox9 binds to *Sox-a* and *-b* specifically, EMSA assays were carried out with purified Sox9-FH and double-stranded oligo probes corresponding to *Sox-a* or *Sox-b* (Fig. 7A). Mutants of *Sox-a* and *-b*, *Sox-aM* and *-bM*, carried changes at suspected critical positions. In the case of *Sox-bM*, mutations were designed to maintain the palindromic structure. As shown in Figure 7B, Sox9-FH bound efficiently to *Sox-a* to yield two bands (Fig. 7B, lane 2), which could be due to double Sox9-FH binding to the *Sox-a* probe. This binding was specific, since excess unlabeled *Sox-a* efficiently inhibited Sox9-FH binding to the radiolabeled probe (Fig. 7B, lane 3–5), while *Sox-aM* inhibited this binding to a much lesser extent (Fig. 7B, lane 6–8).

We also examined if Sox9 expressed in cells was capable of binding to *Sox-a*. 293 cells were transfected with pCMV-Sox9-FH or the pEGFP-N1 vector control. A small-scale nuclear extract was prepared from the transfected cells and used for EMSA assays. With the *Sox-a* probe, while both nuclear extracts generated some probe-binding activities, the extract containing Sox9-FH yielded a novel band (Fig. 7B, lane 10). This band was specific for *Sox-a*, since inclusion of a 30-fold excess of unlabeled *Sox-a* inhibited the formation of this band (Fig. 7B, lane 11), while a same quantity of unlabeled *Sox-aM* failed to inhibit this band (Fig. 7B,

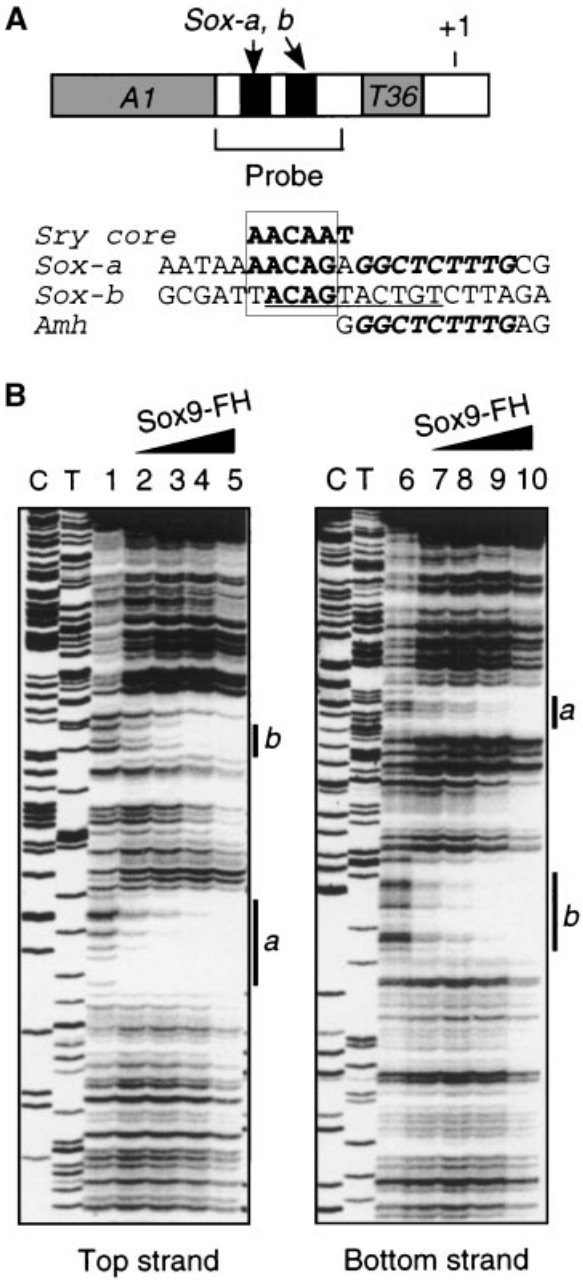

Fig. 6. Identification of Sox9 binding sites by DNaseI foot print analysis. **A**: Relative position of the probe used for DNaseI foot print assay, and the sequences of the revealed Sox9-binding sites in comparison to the core of the Sry binding site (*Sry*) and the Sox9 site in the *Amh* promoter (*Amh*). Identical sequences in *Sox-a* and the *Amh* Sox9 site are italicized. Underlined sequence in *Sox-b* is palindromic. **B**: Phospho-image analysis of DNaseI footprint. **Lane 2–5** or **7–10** contained 6, 12.5, 25, 50 ng of Sox9-FH, respectively. ''C'' and ''T'' lane are manual sequencing ladders (see Materials and Methods section). The top strand of the *KIAA0800* promoter fragment (lane 1–5), or the bottom strand (lane 6–10) was labeled with ^{32}P. ''*a*'' and ''*b*'' indicate the regions protected by Sox9-FH.

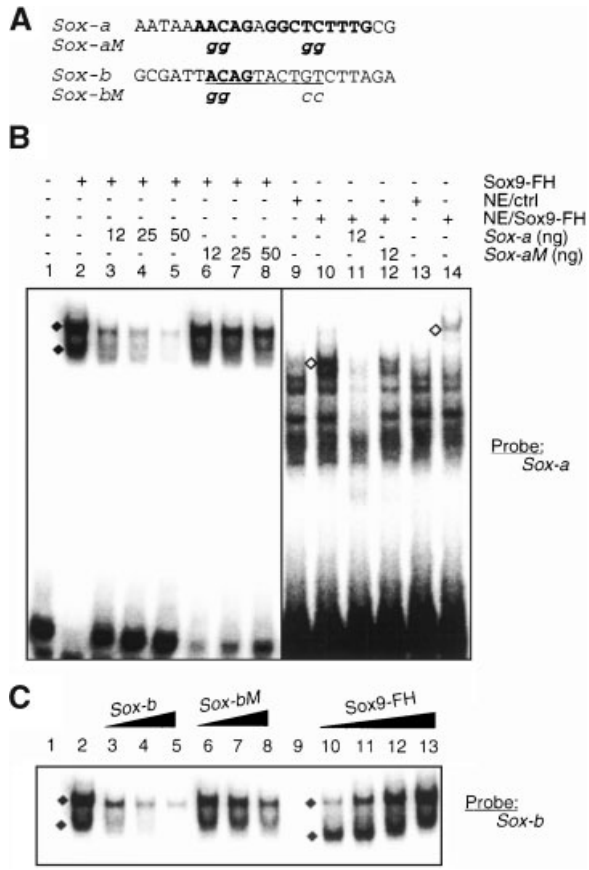

Fig. 7. Specific binding of Sox9 to *Sox-a* and *-b*. **A**: Sequences of the wild-type *Sox-a* and *-b* used as a probe for EMSA, and their mutants *Sox-aM* and *-bM*. **B**: EMSA assay with *Sox-a* probe and purified Sox9-FH (**lane 2–8**) or crude nuclear extracts from transfected 293 cells (**lane 9–14**). Unlabeled double-stranded oligos were used at different quantities as indicated. Flag antibody (2 μg) was included in lane 13 and 14. DNA–protein complexes formed by the purified Sox9-FH are indicated by filled diamonds, while the complexes formed by Sox9-FH in the nuclear extract is indicated by open diamonds. Lane 9–14 are shown at a fivefold higher sensitivity than lane 1–8. **C**: EMSA assay with the *Sox-b* probe and purified Sox9-FH. Conditions for lane 1–8 were the same as lane 1–8 of (B), except with different competitor oligos as indicated. Lane 10 was identical to lane 1 containing only the probe. Lane 10–13 contained 3, 6, 12, and 25 ng of Sox9-FH, respectively.

lane 12). Incubation of the Sox9-FH-containing extract with the Flag monoclonal antibody resulted in a mobility supershift of this band (Fig. 7B, lane 14). In contrast, the antibody did not induce any changes to the assay with the control nuclear extract (Fig. 7B, lane 13). These results suggest that Sox9-FH in the transfected cells is capable of specific binding to *Sox-a*.

We carried out similar experiments with the *Sox-b* probe and discovered that the purified

Sox9-FH bound to *Sox-b* probe specifically (Fig. 7C, lane 2–8), in a similar way as Sox9-FH binding to the *Sox-a* probe. Excess unlabeled *Sox-b* during the assay effectively inhibited Sox9-FH binding to the probe (Fig. 7C, lane 2–5), while excess *Sox-bM* inhibited Sox9-FH binding to a much lesser degree (Fig. 7C, lane 6–8). Titration of Sox9-FH during the assay suggested that Sox9-FH was capable of binding to two sites in the *Sox-b* probe (Fig. 7C, lane 10–13). As the concentration of Sox9-FH was increased, the position of the lower band was moved up. Titration of Sox9-FH during EMSA with the *Sox-a* probe revealed an identical pattern of binding (data not shown). With the crude nuclear extracts prepared from transfected 293 cells, however, a strong *Sox-b*-binding activity was detected that was not due to Sox9 (data not shown). The abundant cellular *Sox-b*-binding activity appeared to be specific also for the palindromic sequence in *Sox-b* (data not shown).

Sox9 Transactivation of the Minimal *KIAA0800* Promoter in the Absence of Sox9 Binding Sites

During our analysis of the *KIAA0800* promoter, we observed that the promoter remained to be significantly transactivated by Sox9 even when all the three *Alu*-repeats were removed (Fig. 8A, pΔA-CAT and Fig. 8B, lane 3 and 4). To examine whether *Sox-a* and *-b* are important for Sox9 transactivation, we prepared a mutant of pΔA-CAT by incorporating *Sox-aM* and *-bM* (see Fig. 7A) into pΔA-CAT (Fig. 8A). Results from transfection and CAT assays showed that the basal promoter activity was increased (Fig. 8B, lane 5, upper panel) and Sox9 transactivation remained significant (Fig. 8B, lane 6). The "Minimal Promoter" construct, pMP-CAT, was then constructed that contained the -60 to $+123$ region of the *KIAA0800* promoter. Within this minimal promoter, an Sp1 binding site has been identified (see Fig. 3A). This promoter construct has a higher basal activity than pΔA-CAT and Sox9 transactivation was lower than for pΔA-CAT (Fig. 3A, lane 7 and 8). Nevertheless, Sox9 transactivation remained significant on pMP-CAT, suggesting that Sox9 transactivation of the *KIAA0800* promoter could be exerted partly through direct targeting of the minimal promoter which contains no favorable Sox9 binding sites (see Discussion section).

Promoter Repression and Sox9 Transactivation of the *KIAA0800* Promoter Are Affected by Cell-Types

To examine if cell lines affect Sox9 transactivation, we also carried out transfection of various constructs into Cos-7 cells. Results from CAT assays are generally consistent with the results from 293 cells (Fig. 8B, lower panel). However, with the full-length promoter, the degree of Sox9 transactivation is lower in Cos-7 cells than in 293 cells (Fig. 8B, compare lane 1 and 2 of the two panels). Assays with other deletion mutants of pKIAA-CAT showed that the *KIAA0800* promoter was repressed to a lower degree. Deletion of *Alu-2*, *-3*, or *T36* from the pKIAA-CAT constructs enhanced basal promoter activity in Cos-7 cells only by less than twofold (data not shown) compared to more than 10-fold in 293 cells (Fig. 5).

DISCUSSION

In this report, we describe the preferential testicular expression of the novel human gene, *KIAA0800* and its transactivation by Sox9. The testis-specific expression of *KIAA0800* is shown by dot-blot of a multiple tissue expression array (Fig. 1) and confirmed by a Northern blot analysis of multiple tissue mRNAs (Fig. 2). The testicular co-expression of *KIAA0800* and *Sox9* (Fig. 2) provides a biological basis for *Sox9* transactivation of *KIAA0800*. The *KIAA0800* promoter/enhancer contains several elements that repress *KIAA0800* promoter activity and contributes to *Sox9* transactivation, including *Alu-2*, *-3*, and *T36* (Fig. 5). Promoter repression may help ensure that *KIAA0800* is expressed only when it is needed. Past studies have identified transcriptional repressors that function through binding to DNA, either directly or indirectly, and through interaction with the basal transcription machinery (for review, see Maldonado et al. [1999]). A nuclear factor has been identified, which binds to the *T36* element in the *KIAA0800* promoter, as well as other oligo-dA:oligo-dT stretches in *Alu-2* and *-3* (data not shown). Whether this factor and additional factors contribute to *KIAA0800* promoter repression remains to be characterized. When *Alu-2* and *-3*, both of which are required for promoter repression, were together inserted upstream of the HIV LTR promoter/enhancer, they failed to repress this promoter/enhancer (data not shown). Thus, upstream elements in

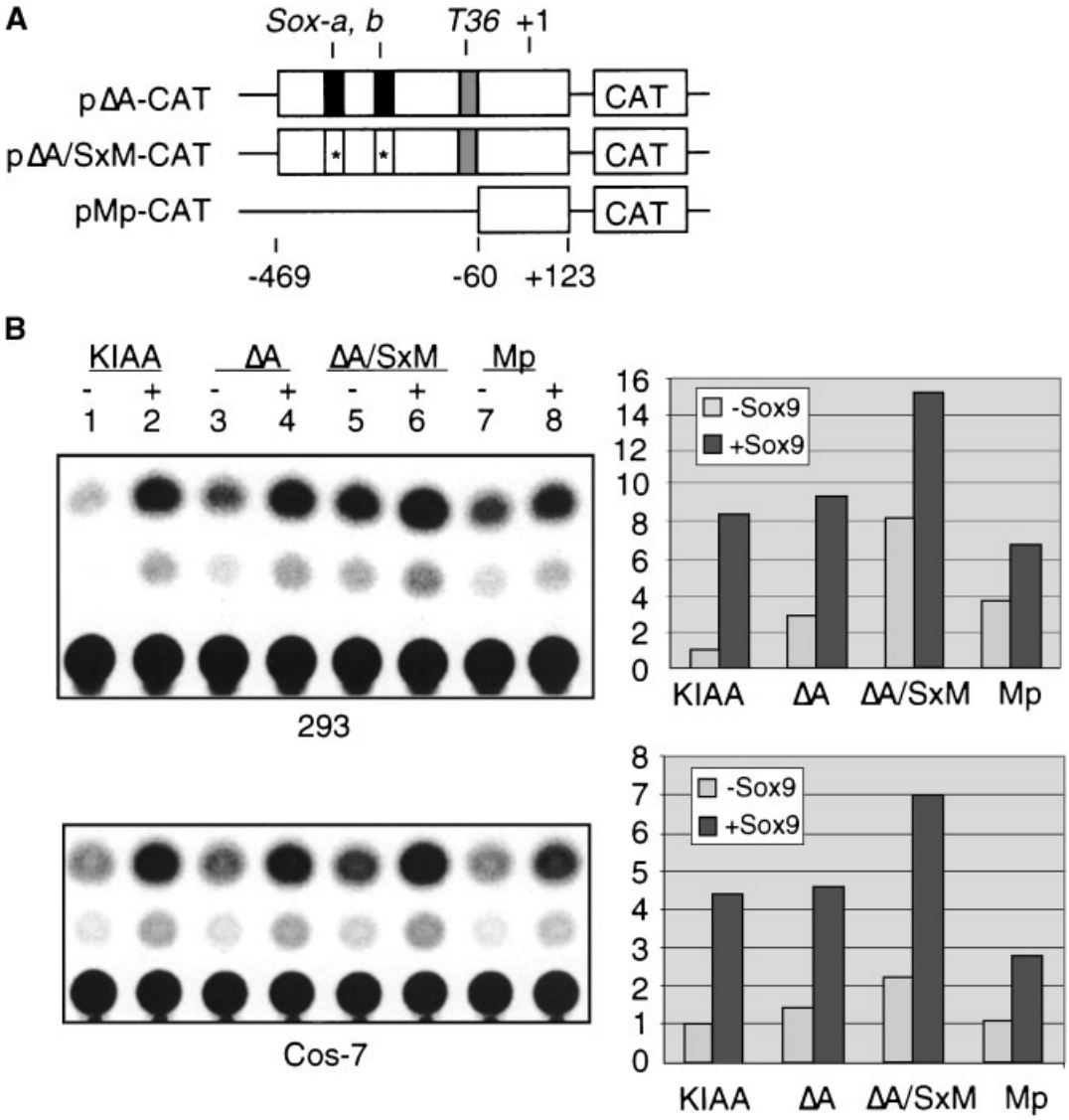

Fig. 8. Mutational analysis of *KIAA0800* promoter sequences close to the transcription start site. **A**: Mutant promoter constructs. pΔA-CAT carries a deletion of all three *Alu* repeats, pΔA/SxM-CAT is based on pΔA-CAT and carries mutations in *Sox-a* and *-b* motifs (same as shown in Fig. 7A). pMP-CAT refers to the minimal promoter. **B**: Transfection into 293 cells (**upper panel**) or Cos-7 cells (**lower panel**) and CAT assays. Relative CAT activities are plotted on the right.

the *KIAA0800* promoter may interact specifically with the basal promoter of *KIAA0800* to confer transcriptional repression. It is possible that specific DNA binding proteins function together with these repressive promoter elements to assure a tight repression of the *KIAA0800* promoter.

Sox9 transactivation is specifically observed with the *KIAA0800* promoter, and not with the HIV-1 LTR promoter/enhancer (Fig. 4). Promoter deletion analysis suggests that multiple sequence elements affect the *KIAA0800* promoter activity and Sox9 transactivation (Fig. 5). In general, maximal promoter repression is correlated with maximal Sox9 transactivation. This observation implies that Sox9 may enhance *KIAA0800* promoter activity by countering promoter repression. Previous studies have established that Sox9 has a strong transactivation domain and transactivates through binding to specific promoter elements [Lefebvre et al., 1997; De Santa Barbara et al., 1998; Kamachi et al., 1999]. The relatively inefficient Sox9 transactivation in cell lines has been attributed to a low DNA binding affinity of Sox9, which may be enhanced by tissue-specific co-factors

[Kamachi et al., 1999]. Recently, a post-transcriptional mechanism of Sox9-enhanced gene expression has been proposed [Ohe et al., 2002]. We have identified and characterized two specific Sox9 bindings sites within the 400-bp region upstream of the transcription start site for *KIAA0800*. Interestingly, both Sox9 binding sites appear to be a composite of two Sox9 binding sites. *Sox-a* contains a site similar to the consensus Sox9/Sry site AACAAT, as well as a site resembling the Sox9 site in the *Amh* promoter (Fig. 7). The core of *Sox-b* is a perfect palindromic sequence ACAGTACTGT, which is not known to be recognized by any other biochemically characterized transcription factor. Titration of Sox9 during EMSA suggests that Sox9 is capable of binding twice to both *Sox-a* and *-b* (Fig. 7). However, currently it is unclear if Sox9 is capable of forming a dimer in the absence of DNA binding.

Despite the specific recognition of both *Sox-a* and *-b* by Sox9, their roles during Sox9 transactivation in the cell lines appear to be small (Fig. 8). Sox9 significantly transactivates the minimal promoter in the absence of *Sox-a* and *-b* (Fig. 8). Our results are consistent with a model that Sox9 activates *KIAA0800* transcription by targeting the minimal promoter, whereas the upstream sequences repress the minimal promoter (Fig. 9). These two effects on the *KIAA0800* promoter may be competitive; however, whether the same component of the transcription factor assembly on the minimal promoter is targeted by promoter repression and Sox9 transactivation remains to be elucidated. Although in cell lines, *Sox-a* plays only a limited role for Sox9 transactivation, it may be a more active participant in the testis when Sox9 co-factors are available. Since *Sox-b* is bound efficiently by another nuclear factor, its effect during Sox9 transactivation may be less prominent. If Sox9 overcomes promoter repression by directly targeting the basal transcriptional machinery or by binding to *Sox-a* or *Sox-b*, positive transcription factors bound to the upstream sequences may be rendered more active. Alternatively, Sox9 may interact directly with the repressor and prevent its binding to the *KIAA0800* promoter.

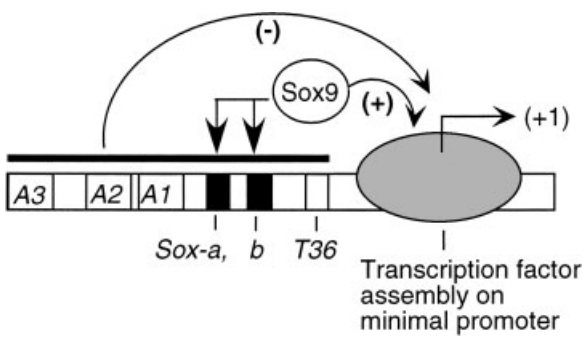

Fig. 9. Model for Sox9 transactivation of the *KIAA0800* promoter (see text for details). (+1): Transcription start site. (+) and (–) symbols refer to positive and negative effects on promoter activity, respectively. ''Transcription factor assembly on minimal promoter'' may contain the basal transcription machinery as well as transcription factors potentially bound to the vicinity of the transcription start site.

Our results support the hypothesis that *KIAA0800* is activated by Sox9 in the testis, and repressed in other tissues by an upstream element involving *T36*, *Alu-2*, and *Alu-3*. Sox9 transactivation is the most effective when the *KIAA0800* promoter is repressed. Considering the timing of expression of *Sry* and *Sox9*, we hypothesize that *KIAA0800* expression is activated by *Sox9* in vivo during early testis differentiation, and then maintained by *Sox9* and additional transcription factors in adulthood. Since, *Sox9* is expressed in pre-Sertoli/Sertoli cells, it is possible that *KIAA0800* is also expressed in these cells. However, this possibility does not preclude *KIAA0800* expression in other types of cells through activation by other transcription factors, such as Sf1, and post-Sox9 effectors. In our analysis of the 3-kb *KIAA0800* promoter, we have identified multiple control elements, including *Alu-1*, which enhances *KIAA0800* promoter activity when promoter repression is relieved (data not shown). It is possible that these various promoter control elements function coordinately to produce a tissue- and cell type-specific pattern of *KIAA0800* expression in a manner dependent on the developmental stage.

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