Characterization of Solt, a novel SoxLZ/Sox6 binding protein expressed in adult mouse testis

Atsushi Yamashita, Michihiko Ito*, Nobuhiko Takamatsu, Tadayoshi Shiba

Department of Biosciences, School of Science, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228 Japan

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Abstract SoxLZ/Sox6, a member of the Sox protein family, contains a leucine zipper motif in addition to an HMG box, which is its DNA binding domain. Here we have identified a novel SoxLZ/Sox6 binding protein, termed Solt, which we obtained independently using both a far-Western blot and a yeast two-hybrid screen. Like *SoxLZ/Sox6* mRNA, *Solt* mRNA was exclusively expressed in the testis in mouse. Solt contains an unusual leucine zipper, which bound to the leucine zipper region of SoxLZ/Sox6 in vitro. In transient transfection assays in CHO cells with SoxLZ/Sox6 containing the transactivational region of herpes simplex virus VP16, expression of a reporter gene that carries a *cis* binding region for Sox proteins was significantly enhanced by the co-expression of Solt and Ca^{2+}/calmodulin-dependent protein kinase IV.

Key words: Sox; Leucine zipper; HMG box; Ca^{2+}/calmodulin-dependent protein kinase IV; Mouse testis

1. Introduction

The Sox (Sry-type HMG box) protein family, which belongs to the HMG box superfamily of DNA binding proteins, is characterized by a high-mobility-group (HMG) domain with strong amino acid similarity (> 50%) to that of SRY (sex-determining region of Y) protein, the human testis-determining factor [1–5]. It is generally accepted that Sox proteins participate in transcriptional regulation, because the HMG domains can bind to specific DNA sequences and bend linear DNA [6]. Sox proteins have been found throughout the animal kingdom and are involved in the regulation of developmental processes such as sex determination, sex differentiation, neurogenesis, chondrogenesis, and thymocyte differentiation [1,7–10]. Some Sox proteins, including Sry, Sox5, SoxLZ/Sox6, Sox9, and Sox17, are expressed in the testis [2,7,11–17]. Sry and Sox9 expression is restricted to the somatic cells of the gonad. Sry is responsible for testis formation [7], and Sox9 is associated with sex reversals and is involved in Sertoli cell differentiation [13–16]. In contrast, Sox5, SoxLZ/Sox6, and Sox 17 are expressed in male germ cells and involved in the later stages of spermatogenesis [11,12,17]. We previously identified and analyzed SoxLZ/Sox6, which contains a leucine zipper in addition to an HMG box [12]. SoxLZ/Sox6 forms homodimers through its leucine zipper, which inhibits its ability to bind DNA through its HMG box. However, a mutant of SoxLZ/Sox6 that lacks the leucine zipper constitutively binds DNA through its HMG box [12]. We have also reported very similar results with rtSox23, which also contains a leucine zipper [18]. It is believed that the Sox proteins containing the leucine zipper motif may function as transcription factors by heterodimerizing with other proteins. We showed that the leucine zipper region of rtSox23 associates with nucleoporin p62 [18]. It has also been reported that the leucine zipper regions of L-Sox5 (the long variant of Sox5) and SoxLZ/Sox6 are essential for binding to each other [10]. As L-Sox5 is not expressed in the testis, the partner for SoxLZ/Sox6 in the testis has not been identified yet. Nonetheless, it is likely that SoxLZ/Sox6 could transactivate transcription by heterodimerizing with other proteins through its leucine zipper.

Here we report the cloning of a novel cDNA, termed *Solt*, from a mouse testis cDNA library. Its gene product, Solt, interacts with the leucine zipper region of SoxLZ/Sox6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that the expression of *Solt* mRNA is restricted to the testis. In transient transfection assays with SoxLZ/Sox6 containing the transactivation domain of herpes simplex virus VP16, the expression of a luciferase reporter gene under the control of a promoter containing a synthetic *cis* element that is bound by the HMG box of SoxLZ/Sox6 was poorly enhanced in the presence of Solt. However, co-expression of both Solt and a constitutively active form of Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), which is expressed in male germ cells [19], significantly enhanced the reporter gene expression.

2. Materials and methods

2.1. Plasmid construction

DNA fragments for plasmid constructs were generated by PCR amplification using the Expand high fidelity PCR system (Roche Diagnostics). The resulting plasmids were confirmed by sequence analysis. Using PCR, the coding sequence corresponding to the leucine zipper-containing region from amino acids 102 to 264 in mouse SoxLZ/Sox6 [12] was fused at the C-terminus with the phosphorylation site, RRASV, for the catalytic domain of the heart muscle kinase (HMK), and cloned in-frame into pET15b (Novagen) to express a hexahistidine (His)-tagged fusion protein in *Escherichia coli*.

To construct the plasmid pAS2-LZ as a bait for the yeast two-hybrid screen, the amplified PCR fragment encoding amino acids 102–264 of mouse SoxLZ/Sox6 was subcloned in-frame into the GAL4-DNA binding domain vector pAS2 (Clontech).

The plasmid pGEX-LZ was constructed by cloning the sequence

*Corresponding author. Fax: (81)-42-778 9408.
E-mail: ito@jet.sci.kitasato-u.ac.jp

Abbreviations: HMG, high mobility group; LZ, leucine zipper; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; GST, glutathione *S*-transferase; HMK, heart muscle kinase; RT-PCR, reverse transcriptase-polymerase chain reaction

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encoding the leucine zipper of mouse SoxLZ/Sox6 (amino acids 102–264) into pGEX-3X (Pharmacia) to produce a fusion protein consisting of glutathione *S*-transferase (GST) and the leucine zipper region of SoxLZ/Sox6 in *E. coli*.

The FLAG epitope-tagged plasmid pCMV/FLAG was made by the N-terminal addition of an oligonucleotide encoding the FLAG sequence MDYKDDDDK in pRc/CMV (Invitrogen). To produce proteins by an in vitro transcription/translation system with T7 RNA polymerase (Promega) and express them in mammalian cells, the coding sequences of SoxLZ/Sox6, Solt, and a constitutively active form of mouse CaMKIV that contained the N-terminal 313 amino acid residues [20] were cloned in-frame into pCMV/FLAG; the resulting constructs were named pCMV/FLAG-SoxLZ/Sox6, pCMV/FLAG-Solt, and pCMV/FLAG-CaMKIV(N), respectively. A series of constructs for deletion mutants of Solt, Solt (D 87–173), Solt (D 87–108), and Solt (D 145–173), lacking amino acids 87–173, 87–108, and 145–173, respectively, were made and inserted into pCMV/FLAG by assembling the PCR fragments.

The mammalian expression plasmids, pCMV/SoxLZ/Sox6-VP16, which encodes SoxLZ/Sox6 with the transactivation domain of herpes simplex virus VP16, and pCMV/SoxLZ/Sox6ΔLZ-VP16, which lacks the leucine zipper region of SoxLZ/Sox6-VP16 (amino acids 105–356), were described previously [12]. The luciferase reporter plasmid 4×SRY-luc was constructed by inserting the *Bgl*II–*Hin*dIII fragment containing four copies of an AACAAT sequence (SRY-type HMG domain binding *cis* element) and the TATA box from the adenovirus major late promoter [12] into the *Bgl*II–*Hin*dIII sites of the pGL3-basic vector (Promega), encoding firefly luciferase.

2.2. Preparation of RNA and construction of cDNA library

Poly(A)$^+$ RNAs from various tissues of 4-week-old C57BL/6 mice were prepared as described previously [12]. cDNA was synthesized using poly(A)$^+$ RNA from testis by directional random priming, according to the directional cDNA library manual (Novagen), and inserted into λSCREEN-1 (Novagen). This directional cDNA expression library was used for a far-Western screen. To obtain full-length cDNA clones, a mouse testis cDNA library in a pMOS*Slox* phage vector (Amersham) was constructed from testis poly(A)$^+$ RNA using oligo(dT) priming.

2.3. Far-Western blot screen

The His-tagged leucine zipper fusion protein with the HMK phosphorylation site was expressed in *E. coli* BL21 (DE3) pLysS, purified with nickel–resin (Invitrogen), and phosphorylated in vitro with [γ-^{32}P]ATP, using the catalytic subunit of HMK (Sigma), as described previously [18]. The directional mouse testis cDNA expression library in λSCREEN-1 was plated and treated with isopropyl-β-D-thiogalactopyranoside, according to the instructions in the directional cDNA library manual (Novagen). Filters were preblocked for 1 h at 4°C in a blocking buffer (20 mM HEPES [pH 7.9], 75 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100) containing 5% non-fat milk, then incubated for 18 h at 4°C in a blocking buffer containing 1% non-fat milk and the ^{32}P-labeled fusion protein at 1–5×10^5 cpm/ml. The filters were washed five times with TBST buffer (10 mM Tris–HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) and exposed to X-ray film. To convert positive phages to plasmids, *E. coli* BM25.8 cells expressing a P1 cre recombinase were infected with positive phages and plated on LB plates supplemented with ampicillin.

2.4. Yeast two-hybrid screen

A yeast two-hybrid screen was carried out using the Matchmaker two-hybrid system (Clontech). Yeast strain CG1945 was cotransformed with pAS2-LZ and a mouse testis cDNA library in the GAL4 transcriptional activation domain vector pGAD10 (Clontech). Transformants were selected on plates with 10 mM 3-aminotriazole that lacked of tryptophan, leucine, and histidine. All of the transformants were tested for β-galactosidase activity using a filter-lift assay.

2.5. Screening the mouse cDNA library

The partial Solt cDNA was labeled by random priming with [α-^{32}P]dCTP (Amersham) and used to screen the testis cDNA library in the pMOS*Slox* phage vector. Hybridization was performed as described previously [12]. The sequence of the full-length Solt cDNA was determined on both strands.

2.6. RT-PCR and Southern blot analysis

cDNA synthesis was performed using 1 μg of poly(A)$^+$ RNA and random hexamer primers of the Superscript choice system (Life Technologies). Two percent of the resulting cDNA was used for PCR reactions, which were performed for 15 cycles with 30 s at 95°C, 1 min at 57°C, and 1 min at 72°C. The specific primers for *Solt* were as follows: forward, 5′-TAGGAGCATGCTACCGTGTT-3′; reverse, 5′-CCGTACTGTGTGAAAACAGG-3′. To monitor RNA input and the efficiency of reverse transcription, all PCR reactions were conducted in the presence of specific primers for mouse β-actin: forward, 5′-GAGCAAGAGAGGTATCCTGA-3′; reverse, 5′-GTACCACCAGACAACACTGT-3′. RT-PCR products were fractionated on duplicate 2.5% agarose gels. One gel was stained with ethidium bromide. The other was subjected to Southern blot analysis with a radiolabeled probe for *Solt* [12].

2.7. In vitro protein–protein interaction assay

GST and GST-LZ fusion protein were expressed in *E. coli* and affinity purified using glutathione beads (Amersham Pharmacia Biotech). In vitro transcription/translation was performed using the TNT T7-coupled reticulocyte system (Promega) in the presence of [^{35}S]methionine. GST alone or GST-LZ immobilized on beads was mixed with in vitro translated ^{35}S-labeled Solt and its deletion mutant proteins for 2 h at 4°C in 100 μl of the binding buffer (20 mM Tris–HCl [pH 7.9], 2.5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.1% NP-40, 10% glycerol). The beads were washed five times with the binding buffer and were boiled in SDS sample buffer. The final products were fractionated on SDS–polyacrylamide gels and detected by autoradiography.

2.8. Immunofluorescence

CHO cells were transiently transfected with pCMV/FLAG-SoxLZ/Sox6 or pCMV/FLAG-Solt using the LT-1 reagent (Mirus). After 36 h, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, permeabilized with 0.15% Triton X-100 in PBS for 30 min, and blocked with 5% non-fat milk in PBS for 2 h. They were then treated with the monoclonal antibody M5 against the FLAG epitope (Kodak) at a dilution of 1:100 with PBS for 1 h. After washing the cells four times with PBS, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) was added at a dilution of 1:200. Nuclei of cells were stained with Hoechst 33258 (Molecular Probes, Inc.) at a final concentration of 3 μg/ml.

2.9. Luciferase reporter assay

The 4×SRY-luc reporter and the mammalian expression vectors (see Fig. 4) were transiently transfected into CHO cells using the LT-1 transfection reagent. After 48 h, the cells were harvested and their luciferase activities were measured using the dual-luciferase reporter assay system (Promega). All transfections were standardized by cotransfecting with pRL-TK (Promega) expressing *Renilla* luciferase as an internal control.

3. Results and discussion

3.1. Isolation of a novel protein, Solt, that interacts with the leucine zipper region of SoxLZ/Sox6

To clarify the function of the SoxLZ/Sox6 leucine zipper motif during spermatogenesis, we tried to identify binding proteins with a leucine zipper region from a mouse testis cDNA expression library. Two kinds of screening methods, the far-Western blot and the yeast two-hybrid screen, were performed independently, because proteins that could be selected under both in vitro and in vivo (within yeast cells) conditions were expected to bind SoxLZ/Sox6 directly in cells. An expression cDNA library made from mouse testis was screened using a recombinant protein containing the leucine zipper region of SoxLZ/Sox6 as a probe (see Section 2). After the third screening, 27 positive clones were selected from a total of 2×10^6 plaques. Based on their sequences, we classified the clones into three separate groups. For the yeast two-hybrid screen, we used a mouse testis cDNA library (Clon-

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CCTGCTCTATGTTTGACGATTAGCATATAGTCTATGAGCCTTTGCTATTTCTGGATCTTT   60
ATATTTCTGTATAGGTCTAGAATTTCCTCATCAAGTGCGACAGAGTTTTTGTCGCCTTTA  120
AGATGTTAATTAAGATAAAAGAGGACAATTTGGGGGGAGTTGACATCTTCGCGTTATTGA  180
CACCTTTAACCTAGGAGCATGCTACCGTGTTCTGTCTATTCCAACAACAAAGCTGCATCC  240
                  M  L  P  C  S  V  Y  S  N  N  K  A  A  S    14

TGTACCACTTCTCCGTTGGTAATTTACAGTAGTTGCAGTAAGGTGAAGAAAACTATAAAG  300
 C  T  T  S  P  L  V  I  Y  S  S  C  S  K  V  K  K  T  I  K    34

GATATGTCAGAGAATAAACAAGAAGTACATCCAGATACTATTACAGATGTGGAAGCTGTT  360
 D  M  S  E  N  K  Q  E  V  H  P  D  T  I  T  D  V  E  A  V    54

ATAGATACAGAGGAAGAACTTATTAAAGAATGTGAAGAAATGTGGAAAGACATGGAAGAT  420
 I  D  T  E  E  E  L  I  K  E  C  E  E  M  W  K  D  M  E  D    74

TGTCAGAATAAATTATCACTTATTGGAACTGAGACACTCACGAATGCAGATGCTCAGCTC  480
 C  Q  N  K  L  S  L  I  G  T  E  T [L] T  N  A  D  A  Q [L]   94

TCATTACTTATTATGCAAATGAAATGTTTGACTGCTGAACTTGGTCAATGGAAGAAAAGA  540
 S  L  L  I  M  Q [M] K  C  L  T  A  E [L] G  Q  W  K  K  R   114

AAACCCGAAATAATTCCCCTAAATGAAGACGTTTTGTTAACATTAGGAAAAGAAGAGTTC  600
 K  P  E  I  I  P  L  N  E  D  V  L  L  T  L  G  K  E  E  F   134

CAAAAATTGAGATGTGATCTTGAAATGGTACTATCTACCATTCAATCAAAGAATGAAAAG  660
 Q  K  L  R  C  D  L  E  M  V [L] S  T  I  Q  S  K [N] E  K   154

TTAAAAGAAGACTTAGAAAGGGAGCAACAGTGGTTGGATGAACAGCAGCAAATACTGGAC  720
 L  K  E  D [L] E  R  E  Q  Q  W [L] D  E  Q  Q  Q  I [L] D   174

ACTCTTAACGTATTAAACAGTGATGTGGAAAATCAGGTGGTAACATTAACTGAGTCAAGA  780
 T  L  N  V  L  N  S  D  V  E  N  Q  V  V  T  L  T  E  S  R   194

ATCTTTAATGAACTGACAACTAAGATCCGTGGGATAAAGGAATTCAAGGAGAAGCTCTTG  840
 I  F  N  E  L  T  T  K  I  R  G  I  K  E  F  K  E  K  L  L   214

CTTACCTTGGGTGCTTTTTTAGACAATCATTTTCCTCTGCCTGAAGCAAGTACTCCAAAG  900
 L  T  L  G  A  F  L  D  N  H  F  P  L  P  E  A  S  T  P  K   234

AAAAGGAAAAACATTCAAGACTCGAATGCACAGCTGATAACACTGAATGAAATATTAGAG  960
 K  R  K  N  I  Q  D  S  N  A  Q  L  I  T  L  N  E  I  L  E   254

ATGCTTATAAATAGAATGTTTGATGTTCCACATGATCCATATGTTAAAATTCGTGATTCC 1020
 M  L  I  N  R  M  F  D  V  P  H  D  P  Y  V  K  I  R  D  S   274

TTTTGGCCACCATATATTGAGCTACTTCTGCGTTACGGAATTGCTTTGAGACATCCAGAA 1080
 F  W  P  P  Y  I  E  L  L  L  R  Y  G  I  A  L  R  H  P  E   294

GATCCATCCCAAATAAGGTTAGAAGCTTTCCATCAGTAAAATGATGACCTGTTTTCACAC 1140
 D  P  S  Q  I  R  L  E  A  F  H  Q  *                       306

AGTACGGAATCTTTACACCCATGGATAAAGAAAACACAATTTTACTGTTTAAATAAAGAA 1200
TATTGTTTGCAAATCAAAAAAAAAAAAAAAAAA                            1233
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Fig. 1. Nucleotide and amino acid sequences of Solt. The amino acids in two putative unusual leucine zippers that contain four and five heptad repeats, respectively, are denoted by boxes.

tech) to identify proteins that interact with the leucine zipper region of SoxLZ/Sox6. Nine positive clones were isolated by screening about 2×10^6 yeast transformants. Five of the clones contained overlapping cDNA segments and shared the sequence of one of the three groups obtained from the far-Western blot screen. The sequences of the remaining clones had no significant homology with those obtained from the far-Western blot screen. Therefore, we analyzed the common clone obtained by the two methods further. Using this clone as a probe, a full-length cDNA clone was isolated from a mouse testis cDNA library. This cDNA (DDBJ/EMBL/GenBank accession number AB043687) is 1233 nucleotides long and contains an open reading frame encoding a polypeptide of 306 amino acid residues (Fig. 1). We named this polypeptide Solt (SoxLZ/Sox6 leucine zipper binding protein in testis). The Solt sequence did not share strong homology with any database entry.

The deduced amino acid sequence of Solt contained two putative unusual leucine zippers (amino acids 87–108 and 145–173) (Fig. 1). The sequences encoding these leucine zippers were shared by all of the *Solt*-derived cDNA clones obtained by the two screening methods. To determine whether they were both required for binding to the leucine zipper region of SoxLZ/Sox6, a series of Solt deletion mutants translated in vitro was tested for binding with GST alone or a fusion protein consisting of GST and the leucine zipper region of SoxLZ/Sox6 (GST-LZ) (Fig. 2). The GST pull-down assay showed that the deletion mutant lacking the N-terminal leucine zipper bound to the leucine zipper region of SoxLZ/Sox6, while the deletion of the C-terminal leucine zipper of Solt completely eliminated the binding ability of the SoxLZ/Sox6. These results indicate that the unusual leucine zipper at the C-terminus of Solt is essential for the interaction with SoxLZ/Sox6.

3.2. The testis-specific expression of the Solt mRNA

The expression pattern of the *Solt* gene was examined by Northern blot analysis of poly(A)$^+$ RNA prepared from several tissues from adult mouse. A single band of approximately 1.3 kb was detected at a very low level only in testis (data not shown). We next carried out an RT-PCR analysis using a pair of primers designed to amplify a 960-bp fragment of the *Solt* sequence, using mRNAs from several tissues. Southern blot analysis of the PCR products with the *Solt* cDNA as a probe revealed signals of the expected size only in the testis sample (Fig. 3A). This adult expression pattern for *Solt* mRNA is the same as that for *SoxLZ/Sox6* mRNA.

3.3. The subcellular localization of overexpressed SoxLZ/Sox6 or Solt in mammalian cells

To examine the subcellular localization of SoxLZ/Sox6 and

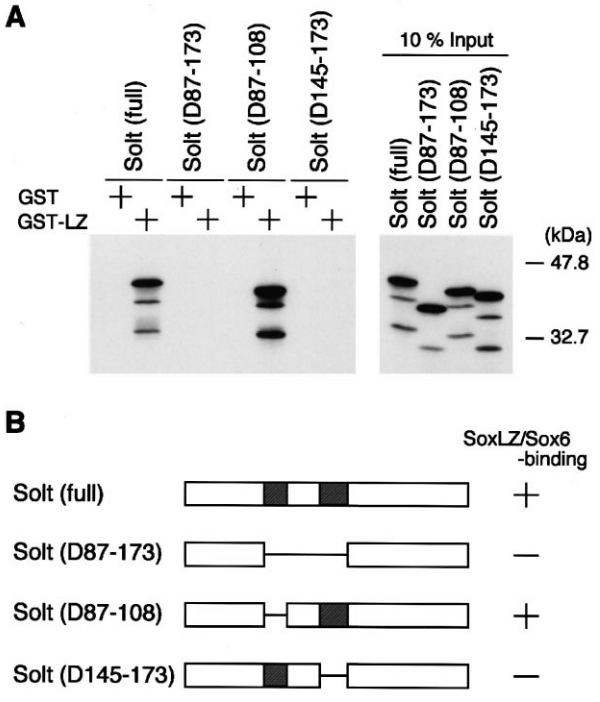

Fig. 2. Binding of Solt to the leucine zipper region of SoxLZ/Sox6 in vitro. A: Mapping of the SoxLZ/Sox6 binding region on Solt. In vitro translated ^{35}S-labeled Solt and its mutant proteins were incubated with 1 μg of either GST or GST-LZ, immobilized on glutathione beads. The protein complexes were washed extensively, and the bound proteins were analyzed by SDS–PAGE and autoradiography (left column). Ten percent of the ^{35}S-labeled proteins used in the binding reactions were loaded (right column). B: Schematic drawing of various Solt deletion mutants. The two putative unusual leucine zippers within Solt are indicated by shadowed boxes.

Solt, we performed an immunocytochemical study using transiently expressed N-terminally FLAG epitope-tagged SoxLZ/Sox6 or Solt in CHO cells. Each protein was detected using the anti-FLAG antibody and an FITC-labeled secondary antibody. In both cases, the immunofluorescence signal of SoxLZ/Sox6 or Solt was detected in nuclei, which were also visualized by Hoechst 33258 (Fig. 3B), suggesting that Solt and SoxLZ/Sox6 might be localized in nuclei in vivo.

3.4. Restoration of the transcriptional activity of SoxLZ/Sox6 by Solt and Ca^{2+}/calmodulin-dependent protein kinase IV

Our previous study showed that the leucine zipper-mediated homodimerization of SoxLZ/Sox6 represses its ability to transactivate transcription, because the homodimerization prevents DNA binding by the HMG box [12]. To determine the role of Solt on transcriptional regulation of SoxLZ/Sox6, we made use of a luciferase-expressing reporter construct with a synthetic promoter bearing four copies of the AACAAT motif, a *cis* element that is bound by the SRY-type HMG box, upstream of the TATA box [12]. We examined the influence of Solt in cotransfection experiments where the expression vector pCMV/SoxLZ/Sox6-VP16, encoding SoxLZ/Sox6 fused to the transactivational region of herpes simplex virus VP16 at the C-terminus, or pCMV/SoxLZ/Sox6ΔLZ-VP16, which lacks the sequence encoding the leucine zipper region [12], were transiently expressed in CHO cells with the reporter gene in the presence or absence of the Solt expression vector. We found that the luciferase activity was enhanced by the deletion of the leucine zipper of SoxLZ/Sox6, but inhibited by full-length SoxLZ/Sox6 (Fig. 4, lanes 2 and 6), which agreed with the results of the CAT reporter assay that we reported previously [12]. We were surprised to find that Solt had only a tiny effect in releasing the transcriptional inhibition seen with full-length SoxLZ/Sox6 (Fig. 4, lane 3). However, the transactivation of several leucine zipper-containing transcription factors is known to be regulated by phosphorylation. For example, the phosphorylation of a serine residue within the leucine zipper of CCAAT/enhancer binding protein β (C/EBPβ) by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is necessary for its transactivation [21]. Another CaM kinase member, CaMKIV, also enhances C/EBPβ-dependent reporter gene expression [22]. The CaMKIV transcript is expressed within germ cells of the testis and its expression levels are known to increase substantially during the progress of meiosis [19], which is similar to the expression pattern of *SoxLZ/Sox6* mRNA. Furthermore, nine putative phosphorylation sites for CaMK, Arg-X-X-Ser/Thr, were found in the amino acid sequence of SoxLZ/Sox6, while the

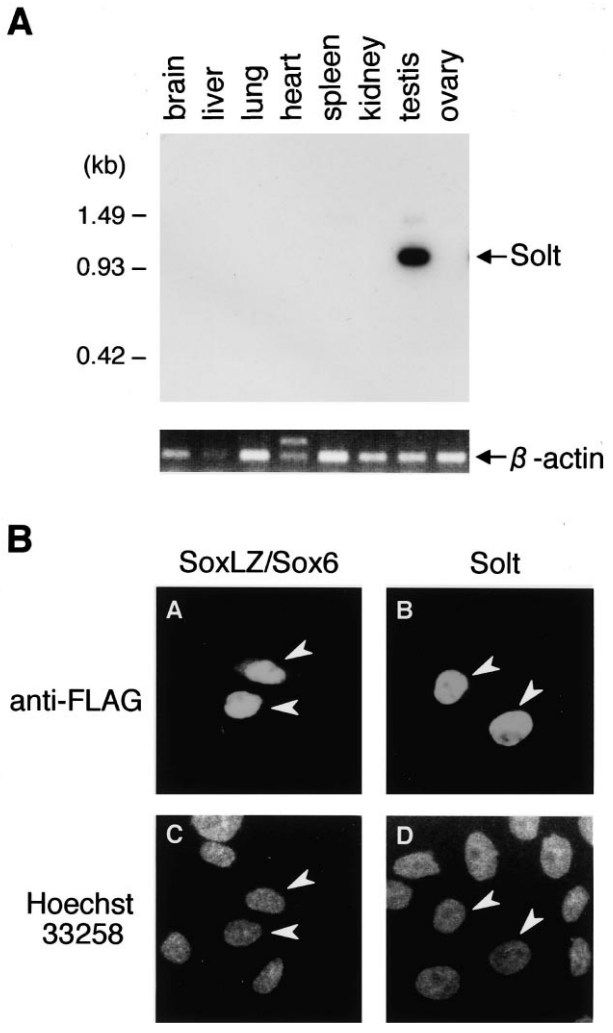

Fig. 3. A: Testis-specific expression of *Solt* mRNA. RT-PCR was carried out as described in Section 2. As an internal control, a pair of primers for mouse β-actin was added to the same PCR reactions. Positions of size markers in bp are shown on the left. B: Nuclear localization of Sox6 and Solt expressed in CHO cells. The cells were transiently transfected with expression vectors encoding FLAG-tagged SoxLZ/Sox6 (A and C) or FLAG-tagged Solt (B and D). Immunofluorescence was carried out with the anti-FLAG antibody and an FITC-labeled secondary antibody (A and B). Nuclei in the cells were visualized with Hoechst 33258 in the same samples (C and D).

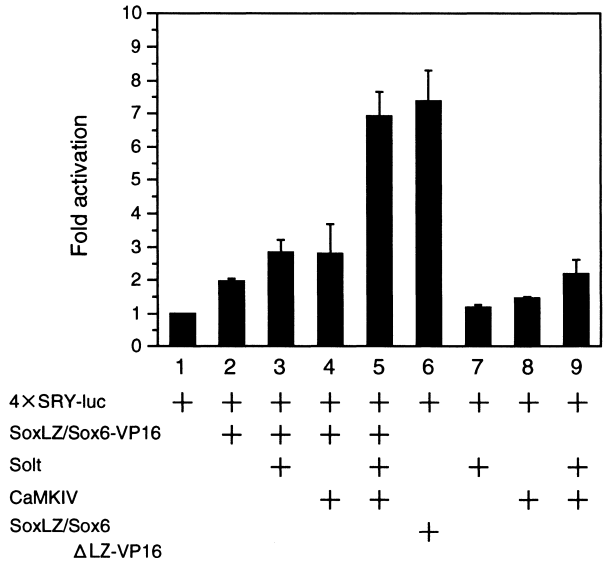

Fig. 4. Effect of Solt and CaMKIV on the reporter gene expression driven by SoxLZ/Sox6. CHO cells were transiently cotransfected with 200 ng of the 4×SRY-luc reporter carrying four copies of the AACAAT sequence, 2.5 ng of the *Renilla* luciferase control vector, and effector plasmids (10 ng of SoxLZ/Sox6-VP16, 10 ng of SoxLZ/Sox6ΔLZ-VP16, 200 ng of Solt, and/or 100 ng of a constitutively active form of CaMKIV) in the indicated combinations. Total DNA was kept at 1.5 μg per transfection with empty pRc/CMV vector. 4×SRY-driven luciferase activity was normalized with *Renilla* luciferase activity. Each error bar represents the standard deviation of three independent experiments.

Solt protein has no phosphorylation consensus sequence. Moreover, it has been reported that transiently expressed CaMKIV localizes to nuclei [23], suggesting that CaMKIV is expressed in the right place at the right time to interact with SoxLZ/Sox6. Therefore, we hypothesized that phosphorylation of SoxLZ/Sox6 by CaMKIV is involved in the function of SoxLZ/Sox6 as a transcription factor. To test this possibility, we constructed an expression vector encoding CaMKIV with constitutive kinase activity. Co-expression of CaMKIV and SoxLZ/Sox6-VP16 alone had little influence on the reporter expression (Fig. 4, lane 4). However, when both Solt and CaMKIV were co-expressed with it, SoxLZ/Sox6-VP16 was able to transactivate the reporter expression (Fig. 4, lane 5) at levels similar to SoxLZ/Sox6ΔLZ-VP16 (Fig. 4, lane 6).

These results suggest that CaMKIV might increase the DNA binding activity of the HMG box of SoxLZ/Sox6 by facilitating its interaction with Solt, although it is still unknown how SoxLZ/Sox6 could have activity of transactivation or participate in any transcriptional activating complex. It is possible that phosphorylation of SoxLZ/Sox6 by CaMKIV leads to a conformational change in the heterodimer complex of Solt and SoxLZ/Sox6 that permits SoxLZ/Sox6 to bind DNA. Further study is required to determine the roles of these proteins during spermatogenesis.

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