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SOX7 and SOX17 Regulate the Parietal Endoderm-specific Enhancer Activity of Mouse Laminin α1 Gene*

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Laminin-1 is the major component of embryonic basement membrane and consists of α1, β1, and γ1 chains. The expression of laminin-1 is induced in mouse F9 embryonal carcinoma cells upon differentiation into parietal endoderm cells. We recently identified a parietal endoderm-specific enhancer in the mouse laminin α1 (*Lama1*) gene and showed that Sp1/Sp3 and YY1 transcription factors were involved in the enhancer activity. Although here we identified that NF-Y binds to the enhancer sequence between Sp1/Sp3- and YY1-binding sites, all these transcription factors are ubiquitously expressed and thus are not sufficient to explain parietal endoderm-specific enhancer activity. In the present study, we further showed that SOX7 and SOX17 are involved in the regulation of parietal endoderm-specific enhancer activity of the mouse *Lama1* gene. Northern blot analysis revealed that the steady-state levels of mouse *Sox7* and *Sox17* mRNAs increased in parallel with that of *Lama1* mRNA during the differentiation of F9 cells. Both SOX7 and SOX17 markedly *trans*-activated the transcription of the *Lama1* enhancer-reporter construct in undifferentiated F9 cells in a manner dependent on high mobility group box-mediated DNA binding. Electrophoretic mobility shift assays and mutational analyses revealed that SOX7 and SOX17 bound specifically to two SOX-binding sites within the *Lama1* enhancer, and that these SOX-binding sites functioned synergistically to confer the *trans*-activation by SOX7 and SOX17. Furthermore, this *trans*-activation was dependent on the integrity of the binding sites for Sp1/Sp3 and NF-Y located at upstream of the two SOX-binding sites. These results indicate that the transcription of the mouse *Lama1* gene during the differentiation of F9 cells is controlled by a combination of the actions of the ubiquitous factors, Sp1/Sp3 and NF-Y, and the parietal endoderm-specific factors, SOX7 and SOX17.

Mouse F9 embryonal carcinoma cells are widely used as an *in vitro* model to study early embryonic development and cell differentiation (1, 2). When treated with retinoic acid (RA),[1] F9 cells differentiate into primitive endoderm-like cells, and then further differentiate into parietal endoderm-like cells by the addition of reagents elevating intracellular cAMP concentration. Parietal endoderm cells are major fetal components of the yolk sac, synthesizing large amounts of basement membrane components including collagen IV and laminin-1. They are organized into a monolayer by a thick sheet of basement membrane, Reichert's membrane, in the early stage embryo (3, 4). Differentiated F9 cells also synthesize and secrete large amounts of basement membrane components, resembling parietal endoderm cells in many of their biochemical properties (5). In recent years, retinoid signaling during this RA-induced differentiation has been well characterized; the action of RA is mediated through nuclear receptors that in turn modulate gene expression. Some of the direct transcriptional targets of RA are known, such as GATA transcription factors (6) and laminin β1 chain (7); however, the mechanisms regulating parietal endoderm-specific gene expression and the overproduction of basement membrane components are, as yet, not fully understood.

To identify transcription factors that control parietal endoderm differentiation, we have analyzed transcriptional regulation of the gene for mouse laminin α1 chain (*Lama1*), a constituent of laminin-1 (8, 9). Laminins are large glycoproteins that are an integral part of the structural architecture of basement membranes (10–12). Each laminin consists of three subunit chains, α, β, and γ, that are assembled and disulfide-bonded in a cross-shaped structure. Through interactions with integrins, dystroglycan, and other receptors, laminins contribute to cell proliferation, migration, and differentiation in various types of cells. To date, five α chains (α1–5), three β chains (β1–3), and three γ chains (γ1–3) have been identified, and these assemble into at least 15 distinct laminin isoforms (13–15). The laminin α1 chain, assembled with β1 and γ1 chains to form laminin-1, has a restricted tissue distribution and is predominantly expressed in epithelial basement membrane during embryonic development (16–19), suggesting that the *Lama1* gene is regulated by parietal endoderm-dependent mechanisms in the early stage embryo.

In an earlier study, we identified a minimal sequence in the 5′-flanking region of the *Lama1* gene that is sufficient to direct parietal endoderm-specific expression using the F9 cell model system (9). The minimal sequence is 435 bp in length, located from −3516 to −3082 relative to the initiation codon, and acts as an enhancer because it increases heterologous promoter activity when placed upstream of the reporter gene in either orientation. Within this 435-bp region, three transcription factor binding sites were predicted, and Sp1/Sp3 and YY1 were found to be involved in enhancer activity. Because Sp1/Sp3 and YY1 are ubiquitous transcription factors, other factor(s) that bind to the enhancer are required to explain the parietal endoderm-specific enhancer activity.

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[1] The abbreviations used are: RA, all-*trans*-retinoic acid; HMG, high mobility group; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; PBS, protein-binding site.

Recent reports (20) have suggested that *Sox7* and *Sox17* might play roles in specification of parietal endoderm cells. SOX proteins constitute a family of more than 20 transcription factors with a conserved high mobility group (HMG) box DNA binding domain similar to that found in the mammalian testis-determining factor SRY (21–23). SOX proteins have critical functions in a number of developmental processes, including neurogenesis (24, 25), chondrogenesis (26, 27), and lens development (28, 29). *Sox7* and *Sox17* are closely related and are grouped in the same subfamily of SOX proteins. *Sox17*-null mutant embryos are deficient of the definitive endoderm of the embryonic gut (30). *Sox7* are co-expressed with *Sox17* in the extra-embryonic endoderm, suggesting that there may be some functional compensation between them (30–32). A genome-wide expression profile using cDNA microarrays revealed that *Sox7* and *Sox17* were up-regulated during the differentiation of F9 cells into parietal endoderm-like cells (20).

In this study, we sought to ascertain whether *Sox7* and *Sox17* might contribute to the control of *Lama1* gene expression during the differentiation of F9 cells. We demonstrated that SOX7 and SOX17 specifically bind to two SOX-binding sites on the *Lama1* enhancer, and that exogenous expression of SOX7 or SOX17 is sufficient to activate the enhancer in undifferentiated F9 cells. We also showed that Sp1/Sp3, NF-Y, and SOX7/SOX17 synergistically control parietal endoderm-specific expression of the mouse *Lama1* gene.

EXPERIMENTAL PROCEDURES

Cell Cultures—F9 cells were cultured on gelatin-coated dishes in a high glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum. Differentiation of F9 cells was induced by adding 0.1 μM all-*trans*-retinoic acid (Sigma) and 1 mM dibutyryl cAMP (Sigma) into the medium. A parietal yolk sac-derived cell line, PYS-2, was kindly provided by Dr. Atsuhiko Oohira, Institute for Developmental Research, Aichi Human Service Center, Aichi, Japan. PYS-2 cells were cultured in a high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Plasmids—The *Lama1* promoter-reporter plasmids (−2888/−30 and −3516/−30), *Lama1* enhancer-SV40 promoter-reporter plasmids (−3516/−3082), and plasmids with disrupted Sp1/Sp3, NF-Y, and YY1-binding sites have been described previously (9). Potential SOX-binding sites in the −3516/−3082 plasmid were disrupted by using the GeneTailor™ Site-directed Mutagenesis System (Invitrogen) to generate the reporter plasmids SOXA mut, SOXB mut, and SOXA&B mut. The sequences of the mutagenic primers are available upon request. All the mutants were verified by sequencing.

To construct expression vectors for SOX7 and SOX17, full-length mouse SOX7 and SOX17 coding sequences were amplified by reverse transcriptase-PCR and cloned into the pCMV-Script mammalian expression vector (Stratagene). Mutations in the HMG box, which does not affect protein stability but diminishes DNA binding (33), was introduced into SOX7 or SOX17 to produce SOX7-F52A or SOX17-F75A, respectively, using the GeneTailor™ Site-directed Mutagenesis System. For expression of the glutathione *S*-transferase (GST) fusion proteins of SOX7 and SOX17, cDNA fragments of SOX7 and SOX17 were inserted into the pET42a(+) and pET42b(+) expression vectors (Novagen), respectively.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extract preparation from F9 cells and EMSA analysis for the NF-Y-binding site were performed as described previously (9). Briefly, 5 μg of nuclear extracts and, when indicated, unlabeled oligonucleotide competitors were preincubated in 23 μl of the gel mobility shift assay buffer (10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 0.6 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 5 mM dithiothreitol, 0.7 mM phenylmethylsulfonyl fluoride, 2 μg/μl pepstatin A, 2 μg/μl leupeptin, and 87 ng/μl poly(dI-dC) (Amersham Biosciences)) for 10 min on ice. An oligonucleotide probe (1×10^5 cpm) was added to the mixture and incubated for an additional 30 min at room temperature. For antibody supershift analysis, 1 μl of antibody was added, and the incubation was continued for an additional hour. The antibody used for the supershift analysis was raised against the NF-YB subunit (C-20; Santa Cruz Biotechnology). DNA-protein complexes were separated from the free probe by 5% nondenaturing PAGE, after which the gel was blotted onto Whatman No. 3MM paper, dried, and analyzed using a BAS2000 Image Analyzer (Fuji Film, Tokyo, Japan).

EMSA analyses for SOX-binding sites were performed in a similar manner by using recombinant, bacterially expressed GST fusion proteins (10 ng) of full-length SOX7 and SOX17. Each recombinant protein was produced in BL21 (DE3) host bacteria by isopropylthio-β-D-galactoside induction and purified using nickel-nitrilotriacetic acid resin (Qiagen).

Northern Blotting—Total RNAs (10 μg) from undifferentiated or differentiated F9 cells and PYS-2 cells were electrophoresed on a 1% agarose gel containing 0.22 M formaldehyde and blotted onto GeneScreen Plus nylon membranes (PerkinElmer Life Sciences). Filters were serially hybridized with mouse *Sox7*, *Sox17*, *Lama1*, and β-actin as a probe. Hybridization was performed in PerfectHyb™ hybridization solution (Toyobo) at 68 °C for 3 h. The membrane was washed twice with 2× SSC (0.15 M NaCl and 15 mM sodium citrate (pH 7.0)) containing 0.1% SDS at room temperature for 10 min and twice with 0.1× SSC containing 0.1% SDS at 55 °C for 20 min and then analyzed using a BAS2000 Image Analyzer (Fuji Film).

Transfection and Reporter Gene Assays—Cells in 24-well plates at 50–70% confluency were transfected using the Effectene transfection reagent (Qiagen) with 200 ng of reporter plasmid and 10 ng of the *Renilla* luciferase expression vector phRL-null (Promega) as an internal control. In *trans*-activation assays, 100 ng of reporter plasmid, 100 ng of expression plasmid, and 10 ng of the *Renilla* luciferase expression vector phRL-null were co-transfected. Forty eight hours later, the cells were harvested in Passive Lysis Buffer (Promega), and the lysates were assayed for luciferase activity by using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activities of various mouse *Lama1* enhancer constructs were normalized to that of the *Renilla* luciferase and were expressed based on the activity of the pGL3 promoter plasmid as 1. Data are expressed as the mean values ± S.D. of at least three experiments (duplicate samples).

RESULTS

The Transcription Factor NF-Y Binds to the Lama1 Enhancer Element—Previously, we identified a 435-bp region, which confers parietal endoderm-specific enhancer activity, at 3.5 kb upstream of the mouse *Lama1* transcription start site, and we identified three protein-binding sites (PBS2, PBS3, and PBS4) in the 435-bp region (schematically presented as Fig. 1*A*) (9). We have also shown that Sp1/Sp3 and YY1 transcription factors bind to PBS2 and PBS4, respectively. However, a factor that binds to PBS3 remained to be determined. A data base search for potential binding proteins to PBS3 (core sequences are CCATCCTATAG) using a lower stringency indicated that PBS3 resembles the consensus binding motif for the CCAAT box-binding proteins (34). Possible binding of several CCAAT box-binding proteins, C/EBP (CCAAT/enhancer-binding protein), NF-Y/CBF (CCAAT-binding factor), CTF/NF-1 (CCAAT transcription factor), and CDP (CCAAT displacement protein), to PBS3 was examined by EMSA combined with oligonucleotide competition and antibody supershift assays (Fig. 1*B* and data not shown). As shown in Fig. 1*B*, competitor oligonucleotide with an authentic NF-Y-binding motif abolished protein binding to PBS3, whereas those with mutations in the NF-Y motif did not. Antibody to the NF-YB subunit produced supershifted complex formation. Thus, the protein binding to PBS3 was identified as the NF-Y transcription factor. Disruption of these protein-binding sites by site-directed mutagenesis resulted in a marked reduction in enhancer activity (Fig. 1*C*).

Identification of Parietal Endoderm-specific Transcription Factors for Activation of Lama1 Enhancer—The expression of Sp1/Sp3, YY1, and NF-Y is ubiquitous and not unique to parietal endoderm cells, suggesting that other factor(s) may be required to regulate the parietal endoderm-specific gene expression. We have recently shown that SOX7 and SOX17 were highly expressed in differentiated F9 cells in microarray analyses (20). Time course analyses of the gene expression profiles during F9 differentiation showed that up-regulation of these

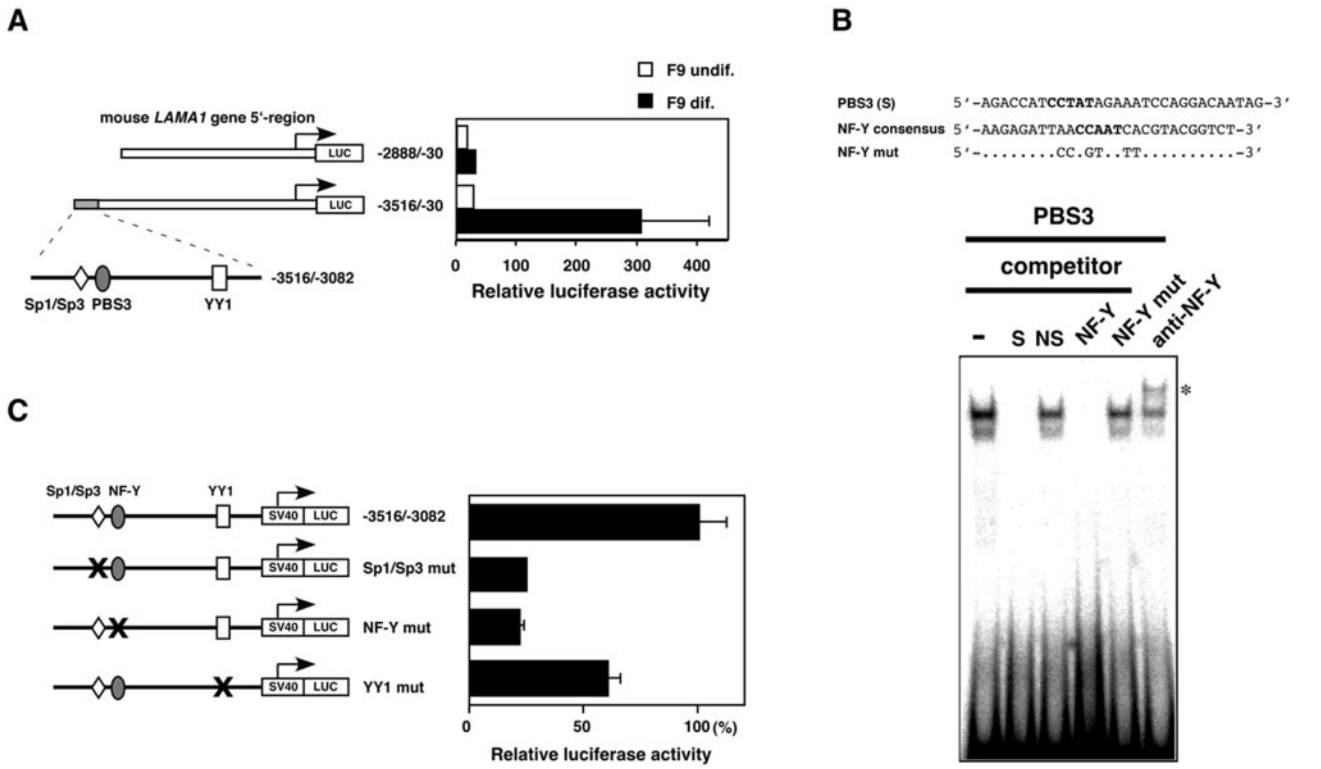


FIG. 1. **Identification of NF-Y as a binding protein to PBS3 in the *Lama1* enhancer.** *A*, the upstream parietal endoderm-specific enhancer element (−3516/−3082) of the *Lama1* gene and transcriptional activity of the *Lama1* promoter. The protein-binding sites identified in our earlier study (9) are schematically presented. Transcriptional activity of *Lama1* promoter-reporter constructs with or without the enhancer element in differentiated (*dif*) and undifferentiated (*undif*) F9 cells is indicated. *B*, EMSA using ^{32}P-labeled PBS3 oligonucleotide and nuclear extracts from differentiated F9 cells. Competition assays were performed with a 100-fold excess of unlabeled specific (*S*), nonspecific (*NS*), wild-type consensus (*NF-Y*), or mutated (*NF-Ymut*) oligonucleotides. For the antibody supershift analysis, NF-YB subunit-specific polyclonal antibody was added to the reaction mixture. The *asterisk points* to the supershifted band. *C*, functional analysis of the protein-binding sites on the *Lama1* enhancer activity. Schematic representation of the mutated constructs is shown on the *left*. The regions altered by site-specific mutagenesis are indicated by ×. pGL3 promoter plasmids containing the wild-type *Lama1* enhancer (−3516/−3082) and the mutated enhancers were transfected into differentiated F9 cells and tested for luciferase activity. The activities are shown as the means ± S.D. of three separate experiments (duplicate samples).

transcription factors was associated with those of basement membrane components, suggesting that they play important roles in the specification of parietal endoderm cells. Therefore, we hypothesized that SOX7 and SOX17 are responsible for parietal endoderm-specific expression of the *Lama1* gene. To test this possibility, we initially examined mRNA expression of *Sox7* and *Sox17* genes in undifferentiated and differentiated F9 cells. Northern blot analysis of total RNA from undifferentiated F9 cells detected little or no transcripts of *Sox7* and *Sox17* as well as of *Lama1* (Fig. 2). However, the intensity of these transcripts increased markedly after the differentiation of F9 cells and parietal yolk sac-derived PYS-2 cells. As other investigators have reported, multiple transcripts were observed for SOX17, and the most abundant transcript exhibiting a size of 2.8 kb was likely an authentic form (35). Thus, it appears that expression of *Lama1* correlated with high levels of SOX7 and SOX17 RNAs in parietal endoderm cells.

Next, we examined whether the exogenous expression of SOX7 and SOX17 drives the *Lama1* enhancer activity in undifferentiated F9 cells. When expression plasmids for these transcription factors were co-transfected with the *Lama1* −3516/−3082 reporter plasmid, both SOX7 and SOX17 increased the transcriptional activity of the *Lama1* enhancer (Fig. 3*A*). This activation was dose-dependent, as increasing amounts of SOX7 and SOX17 augmented the *trans*-activity of the *Lama1* enhancer. This *trans*-activation was not observed in NIH/3T3 or HeLa cells (data not shown), suggesting that SOX7 and SOX17 up-regulate the *Lama1* enhancer activity in a cell type-dependent manner. We further analyzed the involvement of HMG box-mediated DNA binding on *trans*-activation by

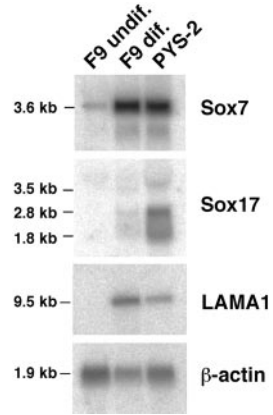


FIG. 2. **Northern blot analysis of *Sox7*, *Sox17*, and *Lama1* transcripts.** Total RNAs (10 μg/lane) from undifferentiated F9 (*F9 undif.*), differentiated F9 (*F9 dif.*), and PYS-2 cells were sequentially hybridized with ^{32}P-labeled probes for mouse *Sox7*, *Sox17*, *Lama1*, and β-actin under stringent conditions. The sizes of the transcripts are shown on the *left*.

introducing an amino acid substitution into SOX7 or SOX17, because SOX proteins bind DNA through their HMG boxes. Because substitution of phenylalanine at amino acid 12 in the HMG box in Sry has been reported to diminish DNA binding without affecting protein folding or stability (33), homologous substitution was introduced into SOX7 or SOX17 to produce SOX7-F52A or SOX17-F75A. In contrast to SOX7 or SOX17, SOX7-F52A or SOX17-F75A marginally, if at all, activated the *Lama1* −3516/−3082 reporter (Fig. 3*B*). These results demon-

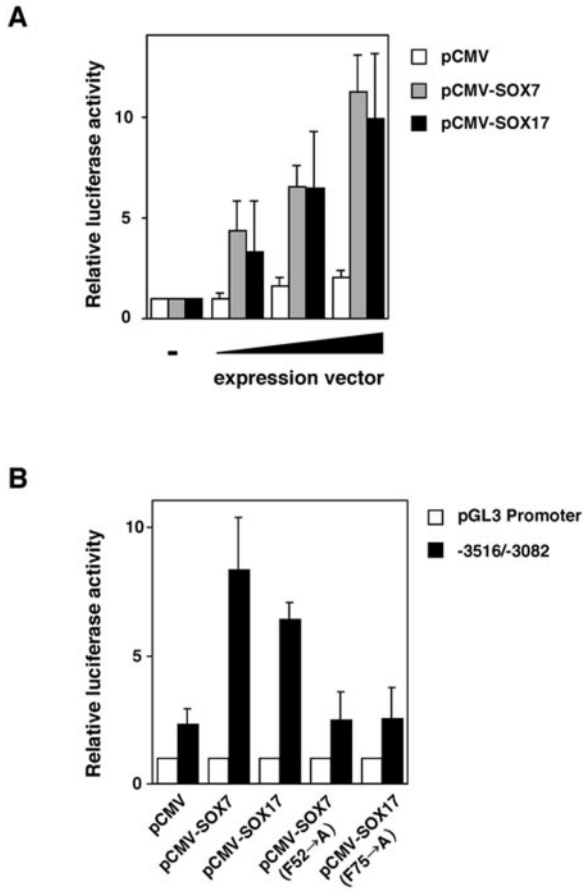


FIG. 3. **Potentiation of *Lama1* enhancer activity by SOX7 and SOX17.** *A,* dose-dependent activation of the *Lama1* enhancer-reporter by SOX7 and SOX17. pGL3 promoter plasmids containing the *Lama1* enhancer (−3516/−3082) and increasing amounts (50, 100, and 200 ng) of SOX7, SOX17, or empty expression vectors were co-transfected into undifferentiated F9 cells and assayed for luciferase activity. *B,* HMG boxes in SOX7 and SOX17 are essential for *trans*-activation of the *Lama1* enhancer. The *Lama1* enhancer (−3516/−3082)-reporter and SOX7, SOX17, or their HMG box mutant expression vectors were co-transfected into undifferentiated F9 cells and assayed for luciferase activity. The activities are shown as the means ± S.D. of three separate experiments (duplicate samples).

strated that HMG boxes in SOX7 and SOX17 are essential for *trans*-activation of the *Lama1* enhancer.

SOX7 and SOX17 Bind to the Lama1 Enhancer Sequence—SOX proteins contain HMG domains that bind DNA in a sequence-specific manner. These proteins have been reported to bind *in vivo* and *in vitro* to sites with a core motif resembling (A/T)(A/T)CAA(A/T)G (36), and it has been reported that SOX7 and SOX17 bind to AACAAT or GACAAT sequences (31, 35, 36). Because the *Lama1* enhancer contains three GACAAT sequences at positions −3349, −3295, and −3246, we performed an EMSA with oligonucleotides spanning putative SOX-binding sites to investigate whether SOX7 and SOX17 proteins bind to these sequences (Fig. 4). Both SOX7 and SOX17 bound to two of the putative SOX-binding sites (at positions −3295 and −3246, designated as SOXA and SOXB, respectively) but not to the site at position −3349 (Fig. 4*C*). The protein-DNA complexes in SOXA and SOXB sites were displaced by the addition of 100- and 500-fold excesses, respectively, of unlabeled wild-type oligonucleotides but not by mutated oligonucleotides. These results indicated that both SOX7 and SOX17 bind in a sequence-specific manner to SOXA and SOXB sites.

Contribution of Individual Elements to Enhancer Activity—To confirm the functional relationship between the two SOX-binding sites and *Lama1* enhancer activity, mutations were introduced into the SOX-binding sites of the −3516/−3082 reporter construct (Fig. 5*A*). Disruption of the SOXA or SOXB sites by site-directed mutagenesis resulted in a 4- or 2-fold, respectively, reduction of transcriptional activity in undifferentiated F9 cells under the expression of SOX7 or SOX17 (Fig. 5*B*, *left*). When both binding sites were mutated, *trans*-activation by SOX7 or SOX17 was completely abolished. Similar results were obtained with differentiated F9 and PYS-2 cells that express endogenous SOX7 and SOX17 (Fig. 5*C, left*). These results demonstrated that the SOXA and SOXB sites in the *Lama1* enhancer are required for the transcriptional activation by SOX7 or SOX17.

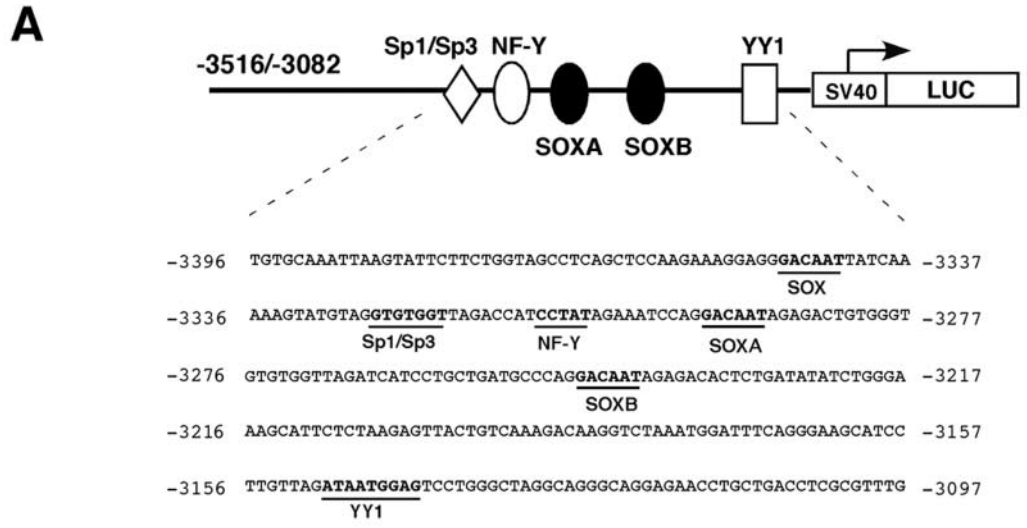
We next examined whether the binding sites of ubiquitous transcription factors (Sp1/Sp3, NF-Y, and YY1) contribute to the *trans*-activation of the *Lama1* enhancer by SOX7 or SOX17. Mutations in the Sp1/Sp3 and NF-Y-binding sites significantly reduced *trans*-activation (Fig. 5*B, right*). In contrast, mutations in the YY1-binding site had little effect on *trans*-activation. The functional importance of these transcription factor-binding sites was also confirmed by transfection into differentiated F9 and PYS-2 cells (Fig. 5*C, right*). Taken together, it was concluded that Sp1/Sp3, NF-Y, and SOX7/SOX17 act synergistically on the *Lama1* enhancer to confer parietal endoderm-specific expression of the mouse *Lama1* gene.

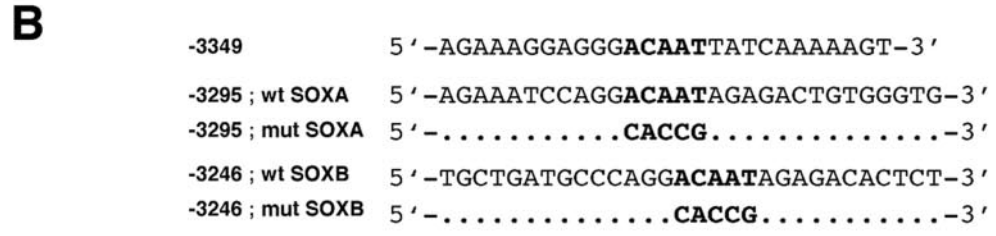
DISCUSSION

In the present study, we demonstrated that SOX7 or SOX17 specifically bind and activate parietal endoderm-specific enhancers of the *Lama1* gene, the products of which are then incorporated into laminin-1 heterotrimer constituting basement membranes in embryos as well as into Reichert's membrane. Members of the *Sox* gene family share similar DNA binding specificity, especially within the same group. *Sox7* and *Sox17* belong to the *Sox* subgroup F with *Sox18* (21). Among these, the RNA levels of *Sox7* and *Sox17* increase in parallel with the *Lama1* RNA level during differentiation of F9 cells in Northern analysis (see Fig. 2). In contrast, *Sox18* expression is not up-regulated during this period.[2] *Sox7* and *Sox17* have similar spatiotemporal expression patterns in the extra-embryonic endoderm (30). In *Sox17*-null mutant embryos, no early developmental defects are found in the extra-embryonic endoderm, suggesting that there may be functional compensation by *Sox7* (30). In parietal yolk sac-derived PYS-2 cells, both *Sox7* and *Sox17* RNAs were highly expressed in association with the high expression levels of *Lama1* RNA. These observations support the possibility that *Sox7* and *Sox17* may play important roles in the specification of parietal endoderm cells.

An earlier study of ours demonstrated that Sp1/Sp3 and YY1 transcription factors were involved in *Lama1* enhancer activity (9). Here we identified that another transcription factor, NF-Y, binds to the protein-binding site (PBS) 3 within the *Lama1* enhancer. NF-Y is a ubiquitous heteromeric transcription factor, composed of three subunits, NF-YA, NF-YB, and NF-YC, which are all necessary for DNA binding (37–39). Although NF-Y is unable to activate transcription on its own, it increases the activity of neighboring enhancer motifs as well as participating in the correct positioning of other transcription factors. For example, the cooperative interactions of NF-Y and Sp1 have been shown to be responsible for the up-regulation of the expression of the major histocompatibility complex class II-associated invariant chain gene (40). Additionally, interactions between NF-Y and Sp1 have been reported in promoter regions of various genes (41–44). Our mutational analysis revealed that both Sp1/Sp3 and NF-Y-binding sites synergistically confer *Lama1* enhancer activity, suggesting that Sp1/Sp3 and

[2] S. Futaki and Y. Hayashi, unpublished observations.





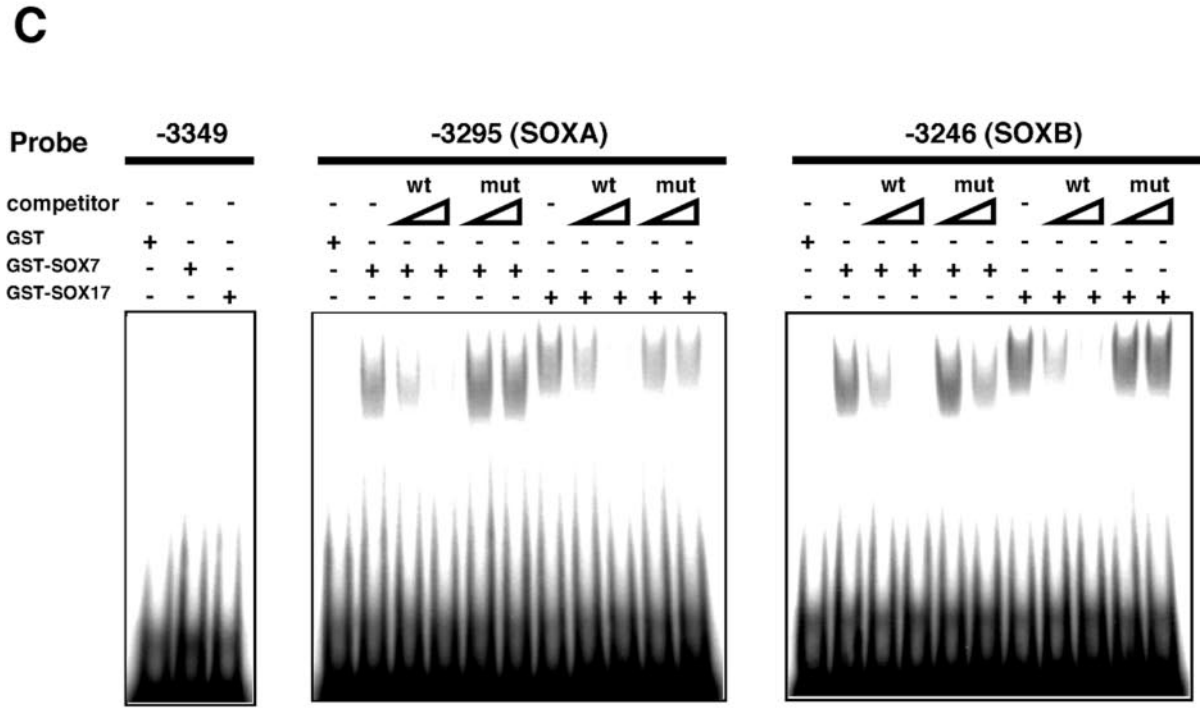


FIG. 4. **Binding of SOX7 and SOX17 to the *Lama1* enhancer.** *A*, schematic representation of the luciferase reporter gene constructs containing the *Lama1* enhancer (−3516/−3082). The nucleotide sequences of the region containing the transcription factor-binding sites are shown. Binding sites are shown in *boldface* and *underlined*. *B*, sequences of oligonucleotides containing either wild-type (*wt*) or mutated (*mut*) SOX-binding sites used as probes and competitors in EMSA. *C*, EMSA using bacterially produced GST fusion proteins and ^{32}P-labeled −3349, −3295, and −3246 probes. Neither GST-SOX7 nor GST-SOX17 protein showed detectable DNA binding to −3349 probe, whereas they clearly bound to −3295 and −3246 probes. These SOX-binding sites were designated as SOXA and SOXB, respectively. Competition assays were performed with 100- and 500-fold molar excesses of unlabeled wild type or mutant oligonucleotides.

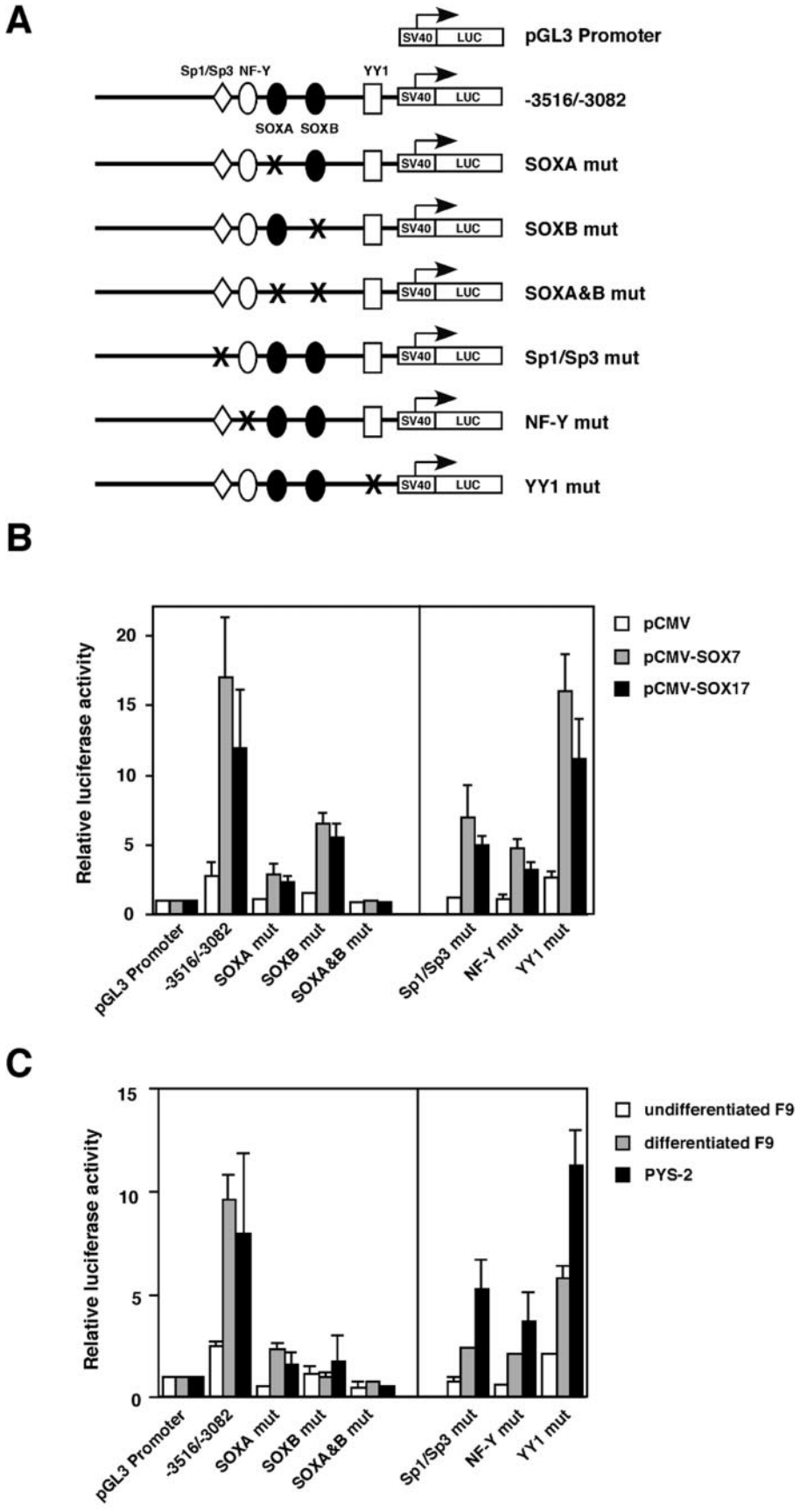


FIG. 5. **Functional analyses of transcription factor-binding sites in the *Lama1* enhancer.** *A*, schematic representation of the reporter constructs. The regions altered by site-specific mutagenesis are indicated by ×. *B*, pGL3 promoter plasmids containing the wild-type *Lama1* enhancer (−3516/−3082) and the mutated (*mut*) enhancers were co-transfected with SOX7, SOX17, or empty expression vectors into undifferentiated F9 cells and assayed for luciferase activity. *C*, pGL3 promoter plasmids containing the wild-type *Lama1* enhancer (−3516/−3082) and the mutated enhancers were transfected into undifferentiated or differentiated F9 cells and PYS-2 cells. The luciferase activities are shown as the means ± S.D. of three separate experiments (duplicate samples).

NF-Y bind cooperatively to the *Lama1* enhancer.

In general, SOX proteins interact with DNA through their HMG domains and recognize short 6-bp core DNA sequences (22). SOX proteins have a relatively low affinity for DNA (a dissociation constant of 10^{-8}–10^{-9} M *in vitro*) and thus are thought to be unable to activate a target promoter *in vivo* unless tightly tethered to DNA through protein-protein interactions (22). Several transcription factors have been identified

to interact with SOX proteins, either to activate or to repress transcription of target genes (22, 23). Our EMSA experiments showed that SOX7 and SOX17 bind as a monomer, although the DNA binding was relatively low affinity in nature, which may have accounted for our failure to identify the SOX-binding sites by EMSA-based screening using nuclear extracts of F9 cells in our earlier study (9). Nevertheless, the binding of SOX7 and SOX17 to these sites is of physiological relevance, because disruption of the SOX-binding sites abrogated the *Lama1* enhancer activity. Moreover, the mutation in HMG boxes diminishing DNA binding in SOX7 and SOX17 also abrogated *trans*-activation of the enhancer. Because our data from a mutagenesis experiment showed that *trans*-activation of the *Lama1* enhancer by SOX7 and SOX17 was Sp1/Sp3 or NF-Y-binding site-dependent, SOX7 or SOX17 may form multiprotein complexes with Sp1/Sp3 and NF-Y on the *Lama1* enhancer *in vivo*.

Recently, Hosking *et al.* (45) characterized *VCAM-1* as a target gene of *Sox18*, the other member of the *Sox* subgroup F. Most interestingly, the expression of the *VCAM-1* gene is not up-regulated during parietal endoderm differentiation of F9 cells, suggesting that the spectrum of the target genes of *Sox7* or *Sox17* is different from that of *Sox18* not only because of differences in spatiotemporal expression patterns but also because of functional differences, including the specificity for binding sites. The finding that the BLAST homology between murine SOX7 and murine SOX17 is higher than the homology to murine SOX18 further supports this interpretation. In the two SOX-binding sites (SOXA and SOXB) identified here, the core GACAAT motifs are preceded and followed by at least two purine residues, which is not the case for the other GACAAT motif in the *Lama1* enhancer (Fig. 4*A, SOX*). On the other hand, the core (G/A)ACAAT motif in the SOX18-binding sites identified by Hosking *et al.* (45) is preceded by pyrimidine residues and followed by a guanine and pyrimidines, suggesting that nucleotide residues surrounding the core motif define the binding specificities for different members of the *Sox* subgroup F.

The observation that the overexpression of SOX7 and SOX17 potentiated the *Lama1* enhancer in undifferentiated F9 cells but not in NIH/3T3 or HeLa cells suggests the involvement of additional mediator proteins or post-translational modifications of the transcription factors specific to undifferentiated F9 cells. Alternatively, an additional mechanism(s) such as remodeling in the chromatin structure or changes in methylation status of the region surrounding the *Lama1* gene may also be involved in SOX7/SOX17-dependent activation of *Lama1* gene expression. In support of the latter possibility, exogenous expression of SOX7 or SOX17 in undifferentiated F9 cells altered the gene expression profile only marginally[2] and failed to induce the endogenous *Lama1* mRNA.

In conclusion, our results provide evidence that the parietal endoderm-specific enhancer of the mouse *Lama1* gene is a direct target for *Sox7* and *Sox17*. To our knowledge, this is the first report clarifying the target gene of *Sox7* and *Sox17*. Further efforts to identify other target genes for *Sox7* and *Sox17* should unveil not only the functions of *Sox* subgroup F members but also the regulatory network of gene expressions involved in parietal endoderm differentiation and the overproduction of basement membranes.

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SOX7 and SOX17 Regulate the Parietal Endoderm-specific Enhancer Activity of Mouse Laminin α1 Gene

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