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Identification of an Upstream Enhancer in the Mouse Laminin *α1* Gene Defining Its High Level of Expression in Parietal Endoderm Cells*

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Laminin-1 is the major component of the 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 through transcriptional up-regulation of the genes encoding these subunits. Here, we identified a 435-bp enhancer in the 5′-flanking region of the mouse laminin α1 (*LAMA1*) gene that activated its transcription in a differentiation-dependent manner. This enhancer was also active in PYS-2 parietal yolk sac-derived cells but not in NIH/3T3 fibroblasts, indicating that it was a parietal endoderm-specific enhancer. This enhancer was also active in Engelbreth-Holm-Swarm (EHS) tumor-derived cells characterized by excessive production of laminin-1 and other basement membrane components, suggesting that EHS tumors have a transcriptional control mechanism similar to that of parietal endoderm cells. Electrophoretic mobility shift analyses revealed four protein binding sites (PBS1-PBS4) in the 435-bp region. However, these DNA-binding proteins were detected not only in parietal endoderm cells (*i.e.* differentiated F9 cells, PYS-2 cells, and EHS tumor-derived cells) but also in undifferentiated F9 cells and NIH/3T3 cells. Mutational analyses revealed that three of these binding sites (PBS2, PBS3, and PBS4) function synergistically to confer the parietal endoderm-specific enhancer activity. The proteins binding to PBS2 and PBS4 were identified as the Sp1/Sp3 family of transcription factors and YY1, respectively.

Laminins are the major basement membrane glycoproteins regulating tissue morphogenesis through their effects on the proliferation, migration, and differentiation of various types of cells (1–3). Laminins consist of three subunit chains, α, β and γ, which are assembled and disulfide-bonded in a cross-shaped structure with three short arms and one long rodlike arm. 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 12 distinct laminin isoforms (4–7). Among these isoforms, laminin-1 is the major component of the early embryonic basement membrane and has been shown to be required for normal development (8, 9).

Mouse F9 embryonal carcinoma cells, a cell culture model of early mammalian embryogenesis, can be induced to differentiate into primitive endoderm- and parietal endoderm-like cells upon treatment with retinoic acid and dibutyryl cAMP with concomitant transcriptional activation of the genes encoding the laminin α1, β1, and γ1 chains (10, 11). The coordinate expression of these subunit genes during F9 cell differentiation suggests that a common mechanism is operating in their transcriptional regulation. Several studies on the transcriptional regulation of laminin subunit expression during the differentiation of F9 cells have been reported previously (12). In the laminin β1 (*LAMB1*) gene promoter, a retinoic acid-responsive element has been identified previously (13–16), whereas differentiation-dependent elements in the first intron have been identified in the laminin γ1 (*LAMC1*) gene (17). However, the molecular mechanisms mediating the coordinate activation of these genes are poorly understood, and the function of the laminin α1 (*LAMA1*) gene promoter has not been studied in any species.

The β1 and γ1 chains are common components of several laminin isoforms (laminin-1, -2, -6, -8, and -10) and have a wide distribution in basement membranes. In contrast, the laminin α1 chain has a restricted tissue distribution and is predominantly expressed in the epithelial basement membrane during embryonic development (5, 18–21). Moreover, the laminin α1 chain expression is thought to be the limiting factor in the secretion of laminin-1, because the β1 and γ1 chains, which are preassembled into a disulfide-linked β1-γ1 dimer, cannot be secreted without the trimeric assembly with the α1 chain (22). These findings prompted us to investigate the mechanism restricting the laminin α1 chain expression in a differentiation-dependent and cell type-specific manner.

In this study, we isolated the 5′-flanking region of the mouse *LAMA1* gene. Using reporter gene assays and deletion analyses, we identified an enhancer in the promoter sequence responsible for laminin α1 expression during F9 cell differentiation. This enhancer was also active in the PYS-2 mouse teratocarcinoma cell line that exhibits parietal endoderm phenotypes (23) but not in NIH/3T3 fibroblasts, suggesting that this enhancer functions in a parietal endoderm-specific manner. Interestingly, this enhancer was also active in Engelbreth-Holm-Swarm (EHS)[1] tumor-derived cells, which are characterized by an excessive secretion of laminin-1 and other basement membrane components (24, 25). We further demonstrated that the synergy of three *cis*-elements was required for the enhancer

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[1] The abbreviations used are: EHS tumor, Engelbreth-Holm-Swarm tumor; BAC, bacterial artificial chromosome; EMSA, electrophoretic mobility shift assay; PBS, protein binding site; kb, kilobase(s).

activity. DNA-binding proteins interacting with two of these *cis*-elements were identified as Sp1/Sp3 and YY1, zinc finger transcription factors widely expressed in many tissues, suggesting that posttranslational modifications of these factors and/or cooperative interactions with other proteins are important for parietal endoderm-specific enhancer activity.

EXPERIMENTAL PROCEDURES

Cell Culture—F9 and NIH/3T3 cells were obtained from Health Science Research Resources Bank (Osaka, Japan). PYS-2 cells were kindly provided by Dr. Atsuhiko Oohira (Institute for Developmental Research, Aichi Human Service Center, Aichi, Japan). These cells were cultured in high glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum in an atmosphere of 95% air, 5% CO_2, and 100% humidity. The 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. EHS tumor-derived cells were prepared cultured as described previously (26) with minor modifications.[2]

Isolation of the Mouse LAMA1 Genomic Clones—A mouse RPCI-23 bacterial artificial chromosome (BAC) library was screened using a BAC/PAC library screening kit (GenoTechs, Tsukuba, Japan). The oligonucleotide primers used for screening were: 5′-GAGTGTGCTCTTC-CCAGCTC-3′ and 5′-CCCCTGGAGGACAGACCT-3′. Genomic DNA fragments containing exon 1 of the mouse *LAMA1* gene were digested with restriction enzymes, subcloned into pBluescript II (Stratagene, La Jolla, CA), and sequenced. All DNA sequencing was carried out using an ABI Prism dye terminator cycle sequencing kit and a model 3100 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Luciferase Reporter Plasmid Construction and Site-directed Mutagenesis—A 9.4-kb *Xba*I fragment containing 2.5 kb of the 5′-flanking sequences of the mouse *LAMA1* genomic DNA was subcloned into the *Xba*I site of pBluescript II. An *Xba*I and blunted *Eco*NI fragment then was inserted into the pGL3-Basic vector (Promega, Madison WI) to generate a −2527/−30 (relative to the initiation codon) plasmid. To generate the longest promoter construct, −6198/−30, a blunted *Nsi*I and *Spe*I fragment was inserted into the −2527/−30 plasmid (see Fig. 1). All of the 5′-deletion constructs were generated in these plasmids by using the endogenous restriction sites and the appropriate restriction sites in the polylinker.

To identify the enhancer element, the *Afl*II fragment (−3684 to −2892) from the mouse *LAMA1* genomic DNA was inserted into the *Afl*II site of the pcDNA3.1(+) vector (Invitrogen). The fragments with appropriate restriction sites at both ends were inserted into the pGL3-Promoter vector (Promega) to generate −3684/−2892, −3684/−3516, −3082/−2892, −3516/−3082, −3516/−3214, and −3214/−3082 plasmids. Using a similar approach, plasmids carrying a double copy and the reverse direction of the −3684/−2892 enhancer, −3516/−3082(+)x2, and 3516/−3082(−), respectively, were constructed.

Site-directed mutagenesis of four potential protein binding sites was carried out in the −3516/−3082 plasmid using the GeneEditor™ *in vitro* site-directed mutagenesis system (Promega) and/or the GeneTailor™ site-directed mutagenesis system (Invitrogen). The sequences of the mutagenic primers are available upon request. All of the mutants were verified by sequencing.

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 20 ng of the *Renilla* luciferase expression vector phRL-null (Promega) as an internal control. 48 h later, the cells were harvested in Passive lysis buffer (Promega), and the lysates were assayed for luciferase activity using the dual-luciferase reporter assay system (Promega). Firefly luciferase activities of various mouse *LAMA1* promoter constructs were normalized to that of the *Renilla* luciferase and expressed based on the activity of the pGL3-Basic or pGL3-Promoter plasmid as 1. The data are expressed as the mean values ± S.E. of at least three experiments (duplicate samples). The *p* values were obtained using Student's *t* test.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts of various cells were prepared as described previously (27). Single-stranded oligonucleotides were annealed at a concentration of 10 μM in annealing buffer (1 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 5 mM NaCl) at 95 °C for 5 min and then cooled to room temperature. Double-stranded DNA was end-labeled with [α-^{32}P]dCTP and the DNA polymerase Klenow fragment (Invitrogen). Labeled DNA was separated from free dCTP by filtration through a ProbeQuant™ G-50 Micro Column (Amersham Biosciences).

Nuclear extracts (5 μg) 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 analyses, 1 μl of antibody was added and the incubation was continued for an additional hour. The antibodies used for the supershift analyses were raised against Sp1 (PEP 2, Santa Cruz Biotechnology, Santa Cruz, CA), Sp2 (K-20, Santa Cruz Biotechnology), Sp3 (D-20, Santa Cruz Biotechnology), and YY1 (H-414, Santa Cruz Biotechnology). DNA-protein complexes were separated from the free probe by 5% non-denaturing polyacrylamide gel electrophoresis. After electrophoresis, the gel was blotted onto Whatman 3MM paper, dried, and analyzed using a BAS2000 Image Analyzer (Fuji film, Tokyo, Japan).

The competitors used in EMSA were obtained by annealing of the following oligonucleotides: wild-type Sp1, 5′-ATTGGATCGGGGCGGG GCGAGC-3′ (forward) and 5′-GCTCGCCCCGCCCCGATCCAAT-3′ (reverse); mutated Sp1, 5′-ATTGGATCGGTTCGGGGCGAGC-3′ (forward) and 5′-GCTCGCCCCGAACCGATCCAAT-3′ (reverse); wild-type YY1, 5′-CGCTCCGCGGCCATCTTGGCGGCTGGT-3′ (forward) and 5′-ACC-AGCCGCCAAGATGGCCGCGGAGCG-3′ (reverse); and mutated YY1, 5′-CGCTCCGCGATTATCTTGGCGGCTGGT-3′ (forward) and 5′-ACC-AGCCGCCAAGATAATCGCGGAGCG-3′ (reverse).

RESULTS

Promoter Activity of the 5′-Flanking Sequence of the Mouse LAMA1 Gene—A *LAMA1* genomic clone was isolated from a mouse BAC genomic library, and a 6.2-kb DNA fragment containing the 5′-flanking region of the mouse *LAMA1* gene was subcloned and fully sequenced. This sequence is available through the GenBank™ data base (GenBank™ accession number AB097426). Previously, Sasaki *et al.* (28) estimated the 5′-untranslated region of the mouse laminin α1 transcript to be 128-bp long. According to this finding, neither a TATA box nor a CCAAT box was found proximal to the putative transcription start site. Mouse and human (GenBank™ accession number AC021879) *LAMA1* genes display a high degree of sequence conservation in the proximal promoter regions (−200 ~+1), suggesting that the *LAMA1* proximal promoter regions contain binding sites for the transcription factors necessary for basal expression in rodents and humans.

To identify the *cis*-regulatory elements controlling the mouse *LAMA1* gene transcription, a series of reporter plasmids driven by the 5′-flanking region of the *LAMA1* gene of different lengths were constructed and transfected into mouse F9 cells before and after induction of differentiation by retinoic acid and dibutyryl cAMP (Figs. 1*A*–*C*). When compared with the −103/−30 plasmid, the −178/−30 plasmid showed significantly higher activities in both undifferentiated and differentiated F9 cells (designated F9-stem and F9-PE cells, respectively), indicating that the basal promoter activity is localized within the −103 to −178 region (*Fsp*I to *Sfo*I). Six other reporter plasmids with longer 5′ sequences (*i.e.* −237/−30, −676/−30, −1036/−30, −2046/−30, −2527/−30, and −2888/−30) showed transcriptional activity similar to −178/−30 in both F9-stem and F9-PE cells. Intriguingly, the transcriptional activity in F9-PE cells was dramatically elevated when the 5′-flanking region was extended to −3516, although such potentiation in transcriptional activity was not observed in F9-stem cells. These results indicate that the 630-bp region encompassing −3516 to −2888 contains an enhancer that is only effective in F9 cells in the differentiated state. Because differentiated F9 cells exhibit a parietal endoderm-like phenotype, we examined the transcriptional activity of these deletion constructs in the PYS-2 parietal yolk sac-derived cells as well as EHS tumor-derived cells that secrete a large amount of laminin-1 (Fig. 1, *D* and *E*).

[2] Y. Hayashi, unpublished observations.

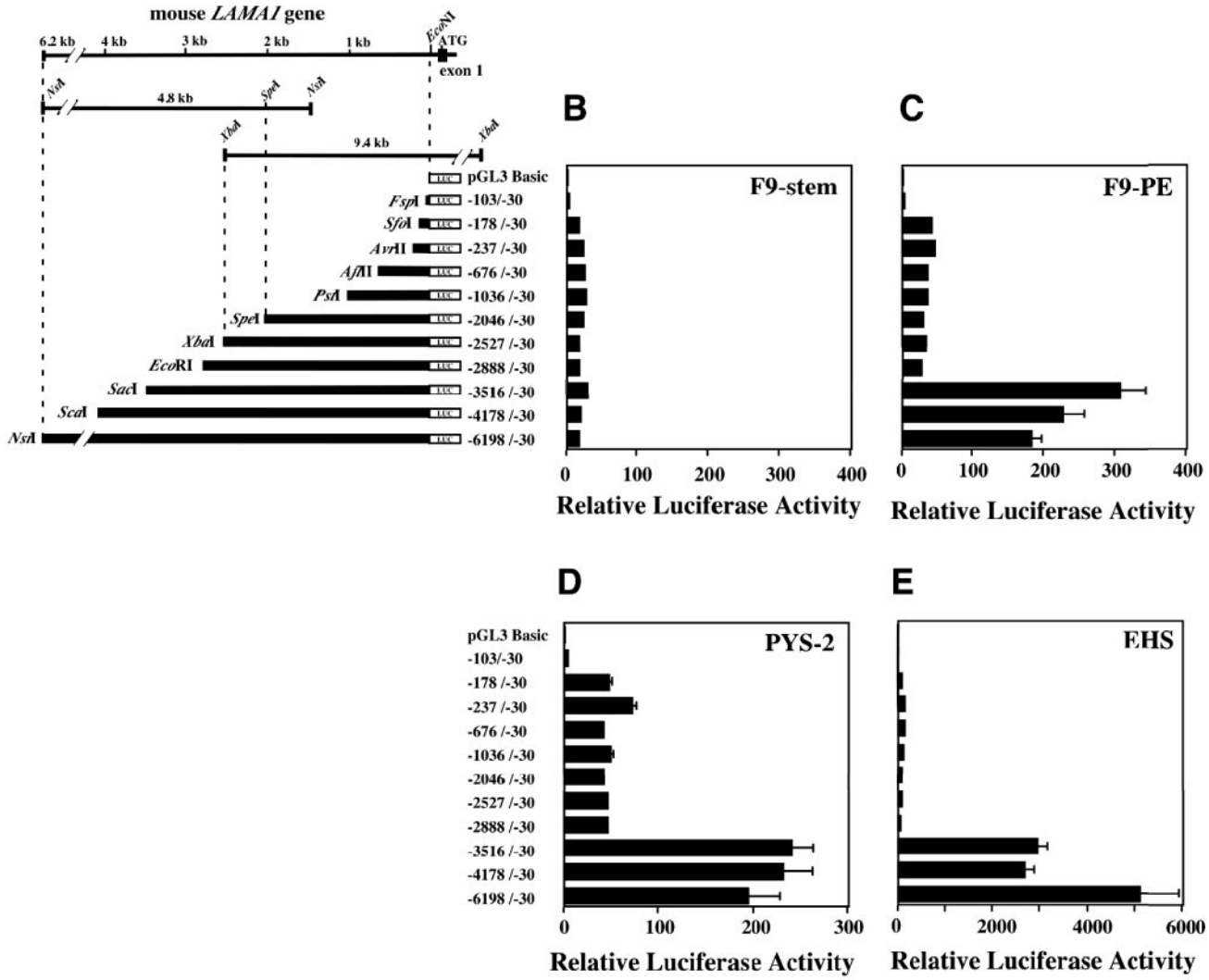

FIG. 1. ***LAMA1* promoter reporter gene constructs and cell type-specific promoter activity in transient transfection assays.** *A*, a schematic representation of the region around exon 1 of the *LAMA1* gene. A series of *LAMA1* promoter reporter genes were constructed as described under "Experimental Procedures." These constructs were transfected into F9-stem (*panel B*), F9-PE (*panel C*), PYS-2 (*panel D*), and EHS tumor-derived cells (*panel E*) together with phRL-null as an internal control. 48 h after transfection, the cells were harvested for the luciferase assay. The relative luciferase activities are shown as the means ± S.E. of at least three experiments (duplicate samples).

A dramatic increase in the transcriptional activity was also detected with the −3516/−30 construct, but not with the −2888/−30 construct, in both PYS-2 and EHS tumor-derived cells, whereas a basal promoter activity was also detectable within the −103 to −178 region. These results suggest that the 630-bp region from −3516 to −2888 harbors an enhancer activity closely associated with parietal endoderm cells. Although the exact origin of the EHS tumor has not been determined, overproduction of extracellular matrix proteins similar to those of Reichert's membrane (29) as well as gene expression profiles determined by microarray analysis[3] indicates that EHS tumor cells are also parietal endoderm-like cells, lending support for the parietal endoderm-specific enhancer activity within the −2888/−3516 630-bp region.

Characterization of a Cell Type-specific Enhancer—To further localize the region critical for the enhancer activity, an 800-bp *Afl*II fragment from −3684 to −2892 and its 5′- and 3′-deletion constructs were tested directly for their enhancer activity using the heterologous SV40 promoter. The 800-bp *Afl*II fragment showed high enhancer activity (*i.e.* a 100-fold increase relative to the basal promoter activity) in EHS tumor-derived cells (Fig. 2) as well as in F9-PE and PYS-2 cells (data not shown). Deletion from the 3′-end to −3516 (*Afl*II-*Sac*I fragment) and from the 5′-end to −3082 (*Bgl*II-*Afl*II fragment) abolished the enhancer activity. In contrast, a 435-bp *Sac*I-*Bgl*II fragment covering nucleotides −3516 to −3082 retained the full enhancer activity, although further deletion constructs (−3516/−3214 and −3214/−3082) did not. These results indicate that both regions (*Sac*I-*Xmn*I and *Xmn*I-*Bgl*II) contain the regulatory element required for the high expression of *LAMA1* in EHS tumor-derived cells, making it likely that the 435-bp region from nucleotides −3516 to −3082 is sufficient for the enhancer activity in EHS tumor-derived cells. Similar results were also obtained with F9-PE and PYS-2 cells (data not shown).

To verify the activity of the 435-bp region as a cell type-specific enhancer, this fragment was cloned 5′ to the SV40 promoter in both the forward and reverse orientation or as two copies in tandem and their enhancer activities were examined in F9-stem cells, F9-PE cells, PYS-2 cells, EHS tumor-derived cells, and NIH/3T3 fibroblasts (Fig. 3). The 435-bp fragment conferred high luciferase activity independent of its orientation in F9-PE, PYS-2, and EHS tumor-derived cells but not in F9-stem and NIH/3T3 fibroblasts. The tandem repeat of the

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

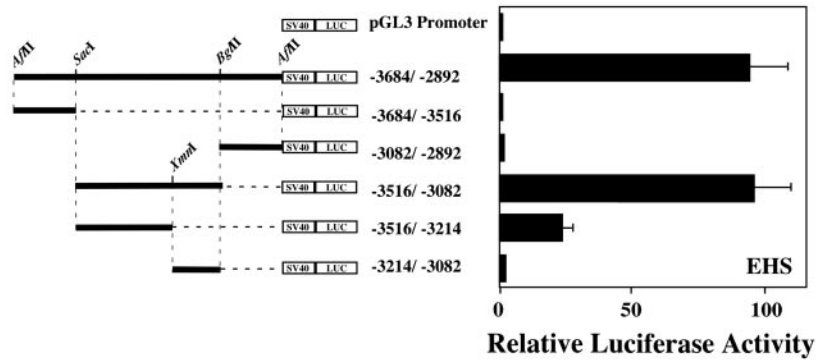

FIG. 2. **Identification of a minimal enhancer region (−3516/−3082) by deletion analysis.** The *Afl*II fragment (−3684 to −2892) subcloned into the pGL3-Promoter vector showed full enhancer activity in EHS tumor-derived cells. As shown schematically on the *left*, several deletion mutants were constructed from this *Afl*II fragment and tested for their luciferase activity. The data are the means ± S.E. of at least three experiments (duplicate samples).

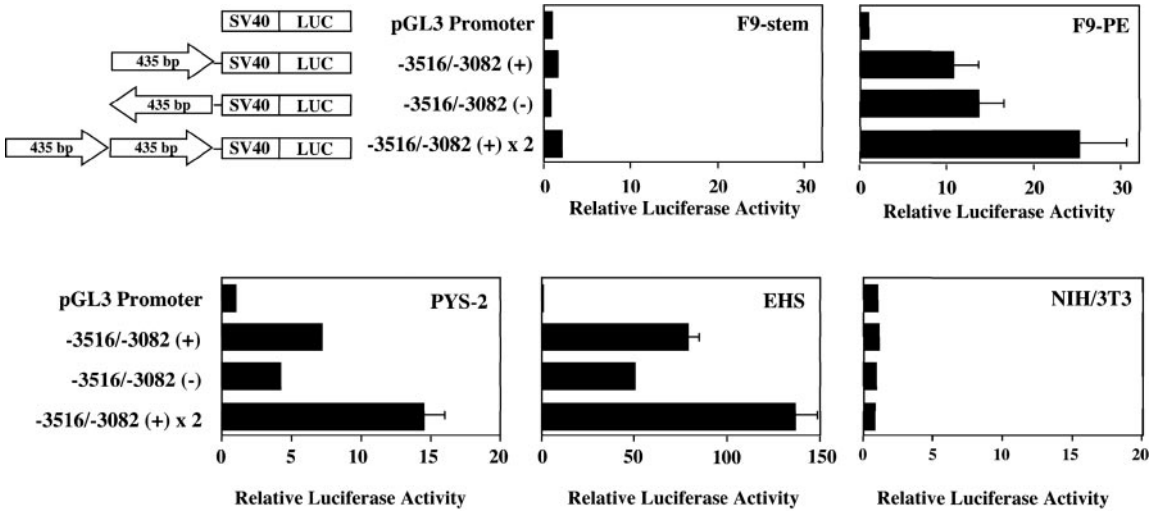

FIG. 3. **Parietal endoderm-specific enhancer activity of the 435-bp fragment (−3516/−3082).** The 435-bp fragment (−3516 to −3082) was inserted to pGL3-Promoter in the forward (+) or reverse (−) orientation as well as in a tandem repeat ((+) × 2). The constructs were introduced into non-parietal endoderm cells (F9-stem and NIH/3T3) or parietal endoderm cells (F9-PE, PYS-2, and EHS tumor-derived cells). The activities are shown as the means ± S.E. of at least three experiments (duplicate samples).

435-bp fragment was more potent than a single copy in the enhancer activity. Given that the enhancer activity was only detected in cells with parietal endoderm phenotypes, we concluded that the 435-bp *Sac*I-*Bgl*II (−3516 to −3082) fragment acts as a parietal endoderm-specific enhancer.

Characterization of Nuclear Protein Binding by EMSA—To determine the regions in the 435-bp enhancer that interact with DNA-binding proteins, we prepared a series of overlapping double-stranded oligonucleotides (data not shown) altogether covering the whole segment and used them as probes for EMSA analyses. Among 24 sets of oligonucleotides, four oligonucleotides designated protein binding sites (PBS) 1–4 formed DNA-protein complexes with nuclear extracts from EHS tumor-derived cells (Fig. 4). All of the four DNA-protein complexes were detected not only with nuclear extracts from F9-PE and PYS-2 cells but also with those from F9-stem and NIH/3T3 cells, implying that the binding proteins are not unique to parietal endoderm cells.

To narrow down the enhancer activity within these four DNA segments, a series of mutant double-stranded oligonucleotides with 6-bp substitutions were used as competitors for the complex formation of a ^{32}P-labeled probe and nuclear proteins (Fig. 5). For PBS1, an excess amount of unlabeled oligonucleotides mut1-2 and mut1-3 competed with the protein binding, whereas mut1-1 failed to compete. These results indicate that a substituted sequence in mut1-1 (ATTAAG) is critical for the DNA-protein complex formation. Similarly, the nucleotide sequences substituted in mut2-3 (TAGGTG), mut3-1 (CCATCC), and mut4−2 (ATAATG) were identified to be critical for protein binding in PBS2, PBS3, and PBS4, respectively.

Contribution of Individual Elements to Enhancer Activity—We next examined the contribution of these putative enhancer elements to the overall enhancer activity of the 435-bp fragment by introducing mutations in the 6-bp core sequences of the PBS1, PBS2, PBS3, and PBS4 segments (Fig. 6). Mutation at PBS1 had no significant effects on the 435-bp enhancer activity, although mutation in PBS2, PBS3, and PBS4 reduced the enhancer activity by 72%, 93%, and 48%, respectively. Double mutations in these three elements resulted in further reduction of the enhancer activity to 2–5% of the control, and mutations of all three sites completely abolished the enhancer activity. Similar results were observed in PYS-2 cells, but not in NIH/3T3 cells (data not shown). Mutation in the PBS3 element alone eliminated more than 90% of the enhancer activity, suggesting that PBS3 is the most critical for the enhancer activity. In contrast, mutation in the PBS4 element had only a modest effect. These data are consistent with the results that the *Xmn*I-*Bgl*II fragment (−3214/−3082) lacking PBS1 through PBS3 showed little, if any, enhancer activity, whereas the *Sac*I-*Xmn*I fragment (−3516/−3214) lacking only PBS4 had significant enhancer activity (Fig. 2). Together, these results indicate that synergy of three protein binding sites (PBS2, PBS3, and PBS4) accounts for the bulk of the activity of the 435-bp enhancer.

Computer analyses using the TFSEARCH program (30) suggested that PBS2 and PBS4 contained putative binding motifs for Sp1-like (GTGTGG) and YY1 (TAATGG) transcription factors, respectively. To test whether these factors were responsible for the observed protein binding to PBS2 and PBS4, we performed competition and supershift assays. Competitor oli-

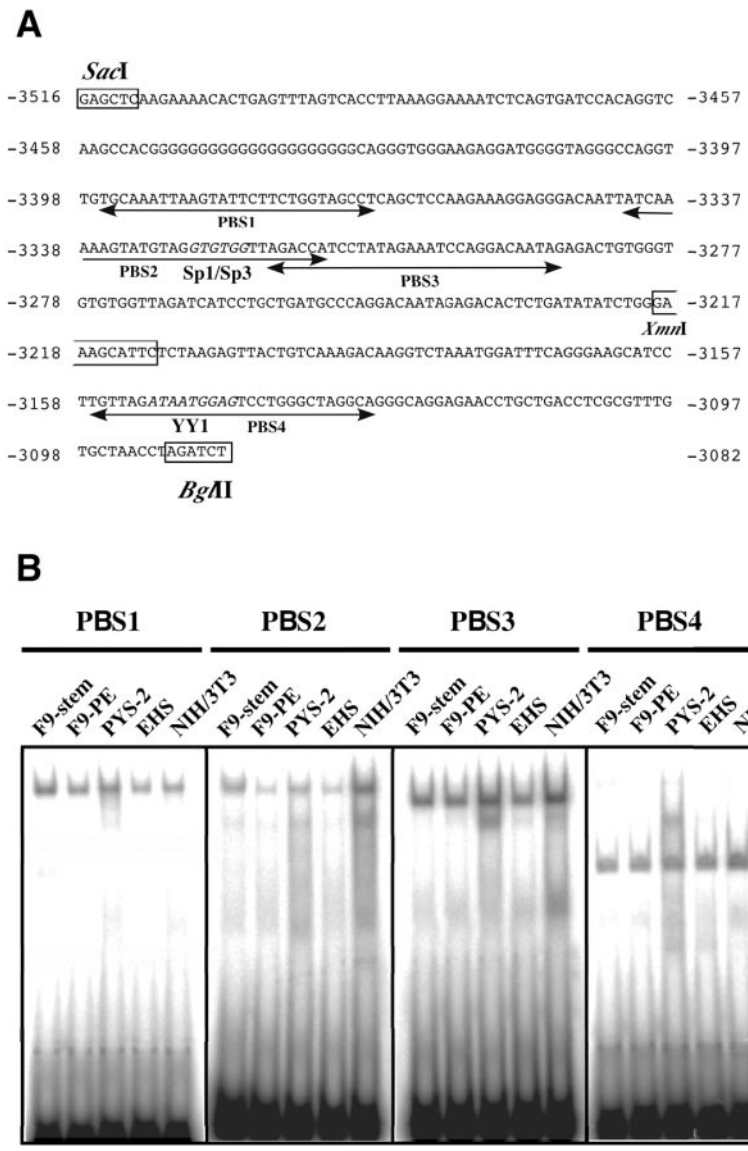

FIG. 4. **Localization of protein binding sites within the 435-bp enhancer.** *A*, sequence of the mouse *LAMA1* 435-bp enhancer (−3516 to −3082). The regions forming DNA-protein complexes in EMSA (from PBS1 to PBS4) are indicated with *lines* with *arrowheads* at both ends. The putative Sp1/Sp3 and YY1 binding sites are shown in *bold* and *italics*. The restriction sites depicted in Fig. 2 are *boxed*. *B*, DNA-protein complex formation on PBS1–PBS4. Nuclear extracts prepared from F9-stem, F9-PE, PYS-2, EHS, and NIH/3T3 cells were used with the indicated oligonucleotide as probes.

gonucleotides with an authentic Sp1 (GGGGCGGGGC) or YY1 (GCGGCCATCT) binding motif abolished protein binding to PBS2 and PBS4, respectively, although those with mutations in the Sp1 or YY1 motif failed to compete (Fig. 7, *A* and *B*). Furthermore, antibodies to Sp1 and Sp3 produced supershifted complexes, whereas antibodies to YY1 inhibited PBS4-protein complex formation. Therefore, it seems probable that Sp1/Sp3 and YY1 bind to the PBS2 or PBS4 sequences, respectively.

DISCUSSION

Parietal endoderm derives from the primitive endoderm, which in turn derives from the inner cell mass of the blastocyst at 4.0–4.5 days post coitum in the mouse (31). Parietal endoderm cells are the major fetal components of the yolk sac, synthesizing large amounts of laminin and collagen IV, which are incorporated into Reichert's membrane (32). Reichert's membrane plays a critical role in the maternofetal exchange of nutrients (33) and is important for the postgastrulation development of the murine embryo. Because parietal endoderm cells continually secrete large amounts of Reichert's membrane components during development, they may be regarded as an active *in vivo* protein biosynthetic system. However, the regulatory mechanisms of genes encoding Reichert's membrane components remain poorly understood. Elucidation of such mechanisms could have a significant impact on developing a system for the large scale biosynthesis of basement membrane components *in vitro*.

In this study, we have cloned the promoter region of the mouse *LAMA1* gene and identified the distal enhancer (−3516 to −3082) responsible for the expression of the laminin α1 chain during the parietal endoderm differentiation of F9 cells. The enhancer was also active in PYS-2 and EHS tumor-derived cells but not in NIH/3T3 cells, suggesting that the enhancer activity is parietal endoderm-specific. Consistent with the definition of an enhancer, the 435-bp sequence enhanced luciferase gene expression in either the forward or reverse orientation from the heterologous SV40 promoter. By EMSA analysis, four protein binding sites (PBS1-PBS4) were identified in the 435-bp enhancer. Although the proteins binding to these elements were detected not only in parietal endoderm cells but also in undifferentiated F9 and NIH/3T3 cells, three of these elements (PBS2, PBS3, and PBS4) appear to be essential for the parietal endoderm-specific enhancer activity. The proteins binding to PBS2 were identified as Sp1/Sp3, and the proteins binding to PBS4 were identified as YY1.

Sp1/Sp3 and YY1 have broad tissue distribution and have been implicated in the regulation of several tissue-specific genes as well as housekeeping genes (34–37). Sp1 binding sites have also been identified in heat shock protein 47 (38) and the

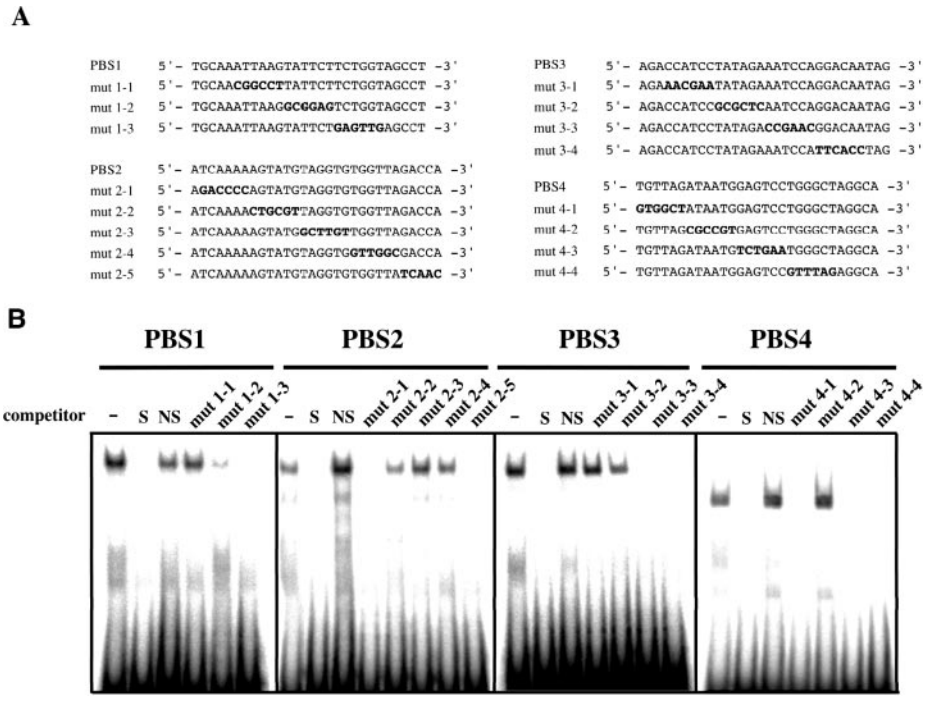

FIG. 5. **Delineation of sequence motifs essential for nuclear protein binding using mutated oligonucleotide competitors**. *A*, sequences of the oligonucleotides used as competitors in EMSA. Wild-type and mutant oligonucleotide sequences containing 6-bp substitutions are shown. *B*, the interaction of ^{32}P-labeled oligonucleotide probes with DNA-binding proteins were analyzed in the presence of a 100-fold excess of unlabeled specific (*S*), nonspecific (*NS*), and mutated competitors to delineate regions critical for DNA-protein interaction. Nuclear extracts were prepared from EHS tumor-derived cells.

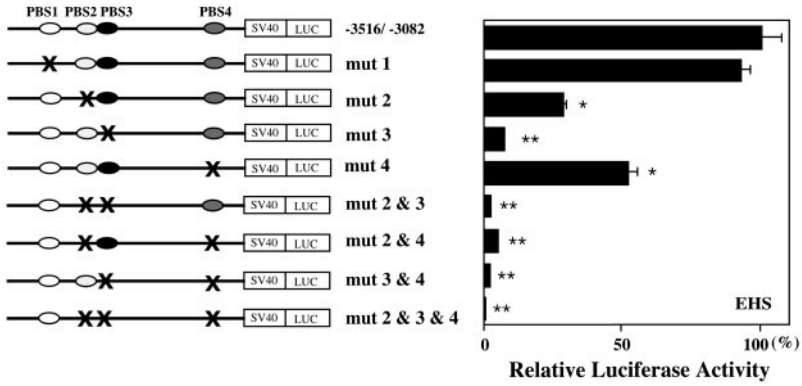

FIG. 6. **Functional analysis of the protein binding sites on the 435-bp enhancer activity.** pGL3-Reporter plasmids containing the wild-type 435-bp enhancer (−3516/−3082) and the mutated enhancers (mut1–4) were transfected into EHS tumor-derived cells and tested for luciferase activity. The regions altered by site-specific mutagenesis are indicated by ***X***. The values represent the percentage of the luciferase activity (mean ± S.E.) of three separate experiments (*versus* the activity of the wild-type construct (−3516/−3082). **, $p < 0.01$; *, $p < 0.05$).

laminin γ1 genes (39), both of which are highly expressed in F9 cells differentiated into parietal endoderm cells. However, it remains to be determined whether these sites are involved in their parietal endoderm-specific expression. Supershift analyses with anti-Sp1, anti-Sp2, and anti-Sp3 antibodies revealed that either Sp1 or Sp3 could bind to PBS2. It has been reported that Sp3 can function as a positive regulatory factor or as a repressor of Sp1-mediated transcription depending on its alternatively spliced isoforms (40, 41). Further studies are required to determine which isoforms are involved in the 435-bp enhancer activity.

YY1 is also known to act as a transcriptional activator or repressor depending on its promoter context. The transcriptional activity of YY1 appears to be regulated at the posttranslational level, possibly through interaction with other proteins. In fact, a wide variety of transcription factors including Sp1 and nuclear receptor co-activators have been shown to associate with YY1 (34, 36, 42–44). Considering these findings, the parietal endoderm-specific activation of the *LAMA1* gene may be controlled by complex transcriptional pathways involving interactions among three ubiquitous factors (Sp1/Sp3, YY1, and an unidentified factor), tissue-specific co-factors, and posttranslational modification such as phosphorylation and acetylation. Recently, it was demonstrated that Akt/protein kinase B activates the transcription of all three chains of laminin-1 as well as type IV collagen (45). It has also been shown that the

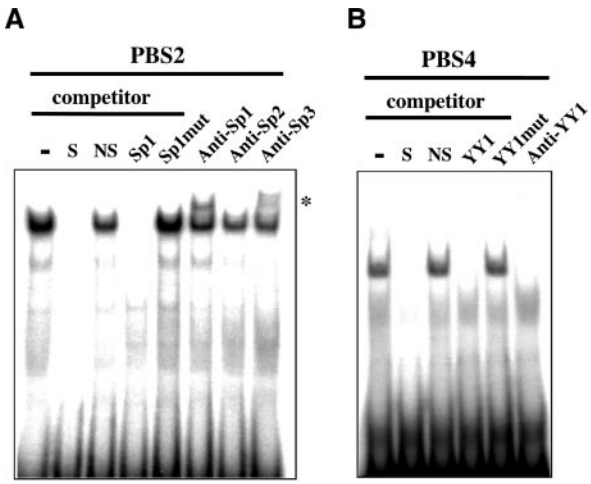

FIG. 7. **Binding of Sp1/Sp3 and YY1 to the 435-bp enhancer.** The ^{32}P-labeled PBS2 (*panel A*) and PBS4 (*panel B*) oligonucleotides were incubated with nuclear extracts from EHS tumor-derived cells. Competition assays were performed with a 100-fold excess of unlabeled specific (*S*), nonspecific (*NS*), wild-type consensus (*Sp1*, *YY1*), or mutated (*Sp1mut*, *YY1mut*) oligonucleotides. For the antibody supershift analysis, Sp1-, Sp2-, Sp3-, or YY1-specific polyclonal antibodies were added to the reaction mixture. The *asterisk* points to the supershifted band. Note that the DNA-protein complex formation was completely abrogated in the presence of the YY1 antibody.

DNA binding and transcriptional activities of YY1 and Sp1/Sp3 are regulated by acetylation and deacetylation (44, 46, 47). It remains to be explored whether Sp1/Sp3, YY1, and an unidentified factor binding to PBS3 are targets of such modifications.

Although parietal endoderm-specific enhancer elements have been identified in the α1(IV) and α2(IV) collagen genes (48, 49), the proteins binding to these elements have not been identified. There is no clear sequence similarity between the enhancer elements in the collagen IV genes and the presently identified 435-bp enhancer. A parietal endoderm-specific enhancer has also been identified in the 5′-flanking region of the platelet-derived growth factor α receptor gene (50), the expression of which is also induced in F9 cells during the differentiation into parietal endoderm cells. GATA-4, a member of the GATA transcription factor family, is considered to be responsible for the platelet-derived growth factor α receptor enhancer activity. This is consistent with a recent report that GATA-4 and GATA-6 are key regulators of differentiation of the extraembryonic endoderm (51). The 435-bp enhancer has several GATA-like motifs, but it seems unlikely that these motifs are involved in the DNA-protein complex formation, because the double-stranded oligonucleotides containing the GATA-like motifs did not produce any significantly shifted band in the EMSA analysis and GATA-4 failed to activate the 435-bp enhancer (data not shown). These observations indicate that the parietal endoderm-specific gene expression can be conferred by either GATA-dependent or GATA-independent mechanisms. In search of the parietal endoderm-specific enhancer of the *LAMB1* and *LAMC1* genes, we cloned ~4-kb and ~7-kb genomic DNA segments covering the 5′-flanking regions of the *LAMB1* and *LAMC1* genes and examined their enhancer activity in PYS-2 cells. However, none of these DNA segments showed as strong transcriptional activity as the 435-bp enhancer.[4] Further sequences upstream of these region or the introns of the mouse *LAMB1* and *LAMC1* genes may contain a regulatory element similar to the 435-bp enhancer.

In conclusion, we have identified a parietal endoderm-specific enhancer of the mouse *LAMA1* gene, which could explain the increased mRNA levels of laminin-1 during early mouse development. Further characterization of this enhancer, *i.e.* identification of the nuclear protein(s) binding to PBS3 and/or other factors interacting with Sp1/Sp3 and YY1, will clarify the novel mechanism(s) operating in the regulation of parietal endoderm-specific gene expression. This 435-bp enhancer system may also provide a clue to understanding the molecular basis of the large amount of production of basement membrane components in EHS tumor and parietal endoderm cells.

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[4] T. Niimi, unpublished observation.

Identification of an Upstream Enhancer in the Mouse Lamininα*1* Gene Defining Its High Level of Expression in Parietal Endoderm Cells

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