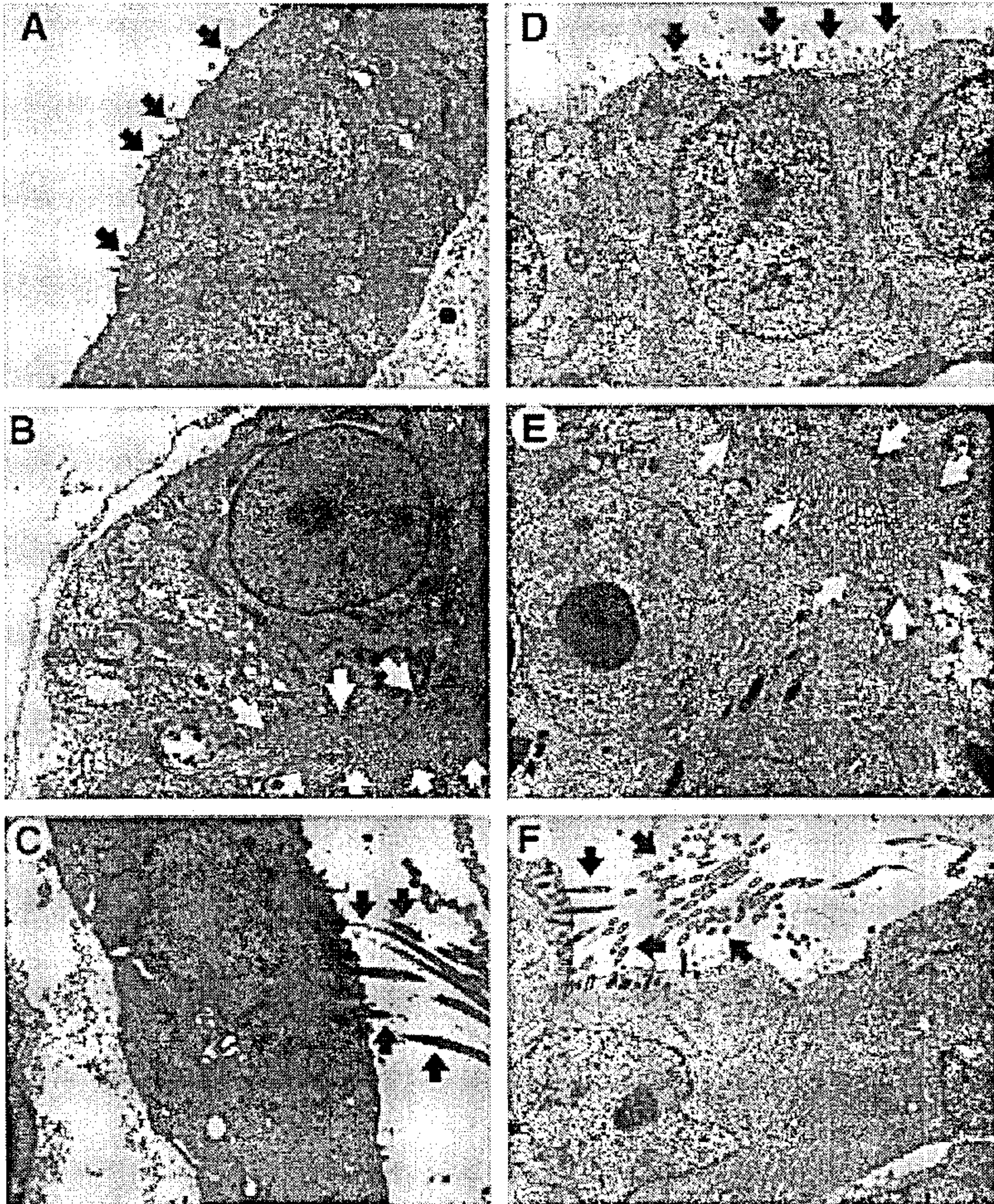

Fig. 2. Electron micrographs of the pronephros of a stage 42 tadpole and *Xenopus* explants treated with activin A and retinoic acid. Three types of tubular structure are observed in the explants (A–C). (A) Cuboidal cells with scant microvilli on their luminal surface (arrow) resemble the pronephric duct cells of normal larvae (D). (B) Tubular cells with abundant microvilli on their luminal surface (arrow) resemble the pronephric tubule cells of normal larvae (E). (C) Cells with cilia consisting of microfilaments (arrow) correspond to the cells of normal larvae located in the intermediate segment connecting the proximal and distal tubules or in the neck segment between the pronephric glomus and proximal tubule (F).

suggesting that the 4A6-positive cells in the explants were different from pronephric tubule cells. Treatment of animal pole explants with activin A and RA may therefore induce pronephric duct differentiation in addition to differentiation of pronephric tubules and glomera.

To examine the relationship between the rate of induction of 4A6-positive cells and the size and location of the ectoderm, the ectoderm was cut into 0.4–0.5 or 0.6–0.7 mm squares. Ectoderm was also removed in 0.4–0.5 mm squares from the ventral and dorsal halves of the animal pole. As shown in Table 1, the larger ectoderm (0.6–0.7 mm) and ventral ectoderm squares had 4A6-positive cells more often than the smaller ectoderm (0.4–0.5 mm) and dorsal ectoderm squares. Immunoreactivity was observed most frequently (96%) in the ventral ectoderm explants. Ectoderm treated with activin A and RA for various periods of culture was examined immunohistochemically. As shown in Table 2, explants cultured to stage 27 and 32 equivalence did not show 4A6 immunoreactivity. The 4A6-positive tubules were first observed at stage 37/38 equivalence, and the induction rate increased to more than 90% at later stages. In normal larvae, 4A6 immunoreactivity is observed at stage 38 or later, indicating that 4A6 antigen is expressed in the explants in the same temporal pattern as in normal larvae. The expression of 3G8 antigen in the explants was detected earlier and was observed at stage 32 equivalence or later (Table 2), also temporally similar to that of normal embryos (stage 31 or later).

Ultrastructure of pronephric ducts induced in vitro

Animal ectoderm explants cultured to stage 42 equivalence were subjected to electron microscopic examination (Fig. 2), and three types of tubular structures were observed. The ultrastructural characteristics of these structures are as follows: (i) cuboidal cells with scant microvilli on the luminal surface and few mitochondria and small vesicles in their cytoplasm (Fig. 2A); (ii) cuboidal cells with abundant microvilli, rich in mitochondria and small vesicles (Fig. 2B); and (iii) cuboidal cells with luminal cilia consisting of microfilaments (Fig. 2C). The first form of tubular cells is similar to those of the pronephric duct in stage 42 larvae (Fig. 2D), whereas the second form resembles both proximal and distal portions of the pronephric tubule (Fig. 2E). The third resembles the intermediate segment connecting proximal and distal tubules or the neck segment between the pronephric glomus and proximal tubule (Fig. 2F; Christensen 1964). The explants therefore contained the pronephric duct-like structure identified by electron microscopy.

Gene expression in pronephric ducts induced in vitro

To investigate whether the *in vitro* induced pronephros expresses the same genes as the pronephric duct of normal embryos, we performed whole-mount *in situ* hybridization using *Gremlin* and *c-ret* as probes. Expression of *Gremlin*, the *Xenopus* dorsalizing factor, largely correlates with neural crest lineages, and at stage 27 or later also appears in the pronephric duct, but not in the pronephric tubules (Hsu *et al.* 1998). *C-ret*, a tyrosine kinase receptor for glial cell line-derived neurotrophic factor (GDNF), is also expressed in the growing pronephric duct (Xenopus Molecular Marker Resource (XMMR) homepage; McLaughlin *et al.* 2000).

In our study, stage 32 equivalent explants treated with activin A and RA expressed transcripts of both *Gremlin* and *c-ret* in the tubular structures of the induced pronephros (Fig. 3A,C,E). However, not all of the tubular structures expressed *Gremlin* or *c-ret*, and these negative structures may correspond to the pronephric tubules, which would be consistent with the double staining of the explants (Fig. 1). Staining of *Gremlin* became weaker in explants cultured to stage 40–42 equivalence, and no *c-ret* expression was observed at all, the same as in normal larvae (data not shown). These results suggest that pronephric ducts induced *in vitro* have the same gene expression profile as the pronephric ducts of normal larvae. Taking together all of these findings, we conclude that animal cap ectoderm treated with activin A and RA contains pronephric ducts, the third component of the pronephros.

Discussion

In vitro induction of pronephric tissue from *Xenopus* ectoderm was first reported by Moriya *et al.* (1993), who found that combined treatment with activin A and RA induced pronephric tubules. Brennan *et al.* (1999) used the reverse transcription (RT)–PCR to show the presence of the Wilms' Tumor-1 gene, *xWT-1*, and demonstrated that the pronephric glomus also formed in animal cap ectoderm treated with activin A and RA. They also discovered that treatment with basic fibroblast growth factor (bFGF) and RA led to the formation of the pronephric glomus. In this study, we demonstrated that *Xenopus* blastula-stage ectoderm treated with activin A and RA frequently contains pronephric ducts, and therefore contains all three components of the pronephros.

In contrast to our own findings, Brennan *et al.* (1999) rarely found pronephric ducts in explants treated with activin A and RA. However, they cultured the treated

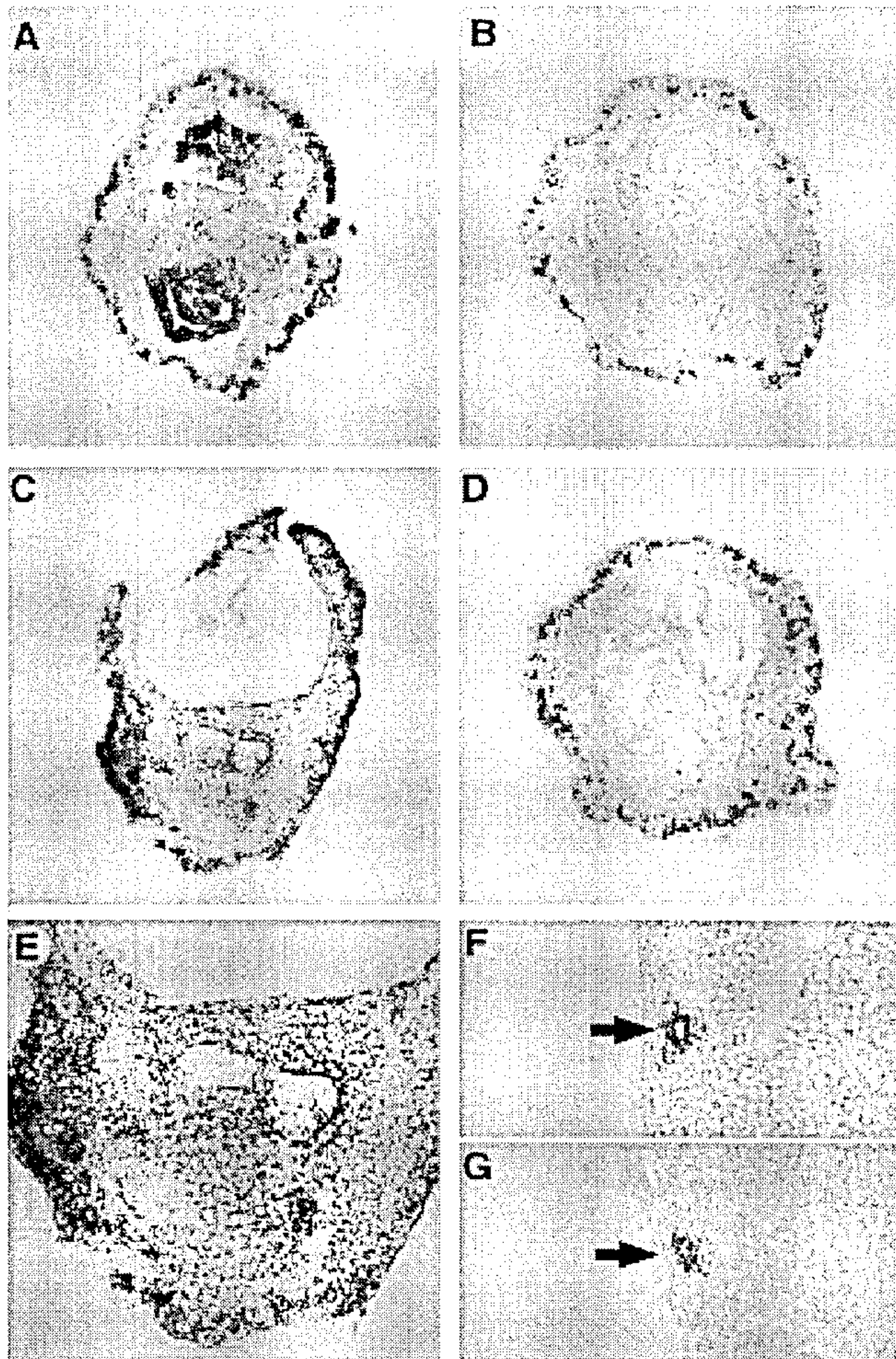

Fig. 3. Whole-mount *in situ* hybridization of *Gremlin* and *c-ret* in treated *Xenopus* explants cultured until stage 32 equivalence. Expression of *Gremlin* and *c-ret* mRNA is seen in explants treated with activin A and retinoic acid (A–E). (A) Antisense *Gremlin* probe; (B) sense *Gremlin* probe; (C) Antisense *c-ret* probe; (D) sense *c-ret* probe; (E) higher magnification of (C). *Gremlin* antisense (F) and *c-ret* antisense (G) patterns in stage 32 larvae (arrow) are also shown.

explants only up to stage 33/34 equivalent, and our results showed that the 4A6 antigen first appeared in explants at stage 37/38 equivalent or later, displaying the same temporal expression as in normal larvae. The pronephros that forms in explants expresses the marker genes *xlim-1* and *xlcaax-1* with the same timing and pattern as in normal embryos (Uochi & Asashima 1996) and we also confirmed that the expression pattern of 3G8 antigen in explants was temporally similar to that of normal larvae. These results indicate that the pronephros in explants may share common molecular mechanisms with those of normal embryos and that this *in vitro* model can serve as a simple and useful system for analyzing the mechanisms of kidney differentiation. Indeed, an essential gene for mammalian kidney development has been identified using this *in vitro* system (Onuma *et al.* 1999; Nishinakamura *et al.* 2001).

Treatment of animal pole ectoderms with activin A and RA in the ventral halves led to frequent formation of pronephric ducts in almost 100% of explants, as compared to only 60% of the dorsal halves. *Xenopus* ectoderm in dorsal halves treated with activin showed the presence of eye or notochord, whereas the ectoderm in ventral halves only rarely included eye or notochord (Sokol & Melton 1991). It was concluded that the dorsal and ventral regions of the ectoderm respond differently to the same concentration of activin, and that mesoderm patterning is determined by the different competence of cells in the region as well as by a localized inducer. Similarly, our results suggest that *Xenopus* ectoderm in the ventral halves might have greater competence to form pronephric ducts in response to activin A and RA.

In conclusion, treatment of *Xenopus* presumptive ectoderm with activin A and RA frequently induces differentiation of the pronephric duct, in addition to the pronephric tubule and glomus. This *in vitro* system can serve as a simple and effective model for analysis of pronephric kidney development.

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Identification of a Renal Proximal Tubular Cell-Specific Enhancer in the Mouse 25-Hydroxyvitamin D 1α-Hydroxylase Gene

TADASHI YOSHIDA, JUN YOSHINO, MATSUHIKO HAYASHI, and TAKAO SARUTA
Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

Abstract. The active form of vitamin D is synthesized by 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase), which is expressed predominantly in renal proximal tubular cells. To clarify the mechanism of cell-specific gene expression of this enzyme, the 5'-flanking region of the mouse 1α-hydroxylase gene was investigated. Investigation began with mRNA expression of 1α-hydroxylase in cultured cells, including LLC-PK1, NIH/3T3, HepG2, MDCK, and OK cells. Expression of 1α-hydroxylase mRNA was restricted in LLC-PK1 cells. Several lengths of the 5'-flanking region of 1α-hydroxylase gene were linked to a pGL3-basic luciferase vector and introduced into these cells. Only LLC-PK1 cells had a substantial luciferase activity. Deletion analyses revealed that luciferase activity was detected in constructs extending from the transcription initiation site to −1652 to −105 bp, whereas further deletion to −80 bp resulted in a marked decrease in activity. The region from −105 to −80 bp contained two ternary complex factor-1 (TCF-1) sites, and mutations in the proximal TCF-1 site decreased the activity. Electrophoretic mobility shift assay demonstrated binding of LLC-PK1 nuclear proteins to this region. Tests of enhancer function in LLC-PK1 cells indicated that the 26-bp fragment behaved as a classical enhancer, *i.e.*, independently of position and orientation. Moreover, a decoy oligonucleotide corresponding to this region substantially inhibited the promoter activity of 1α-hydroxylase gene. This study suggests that the −105 to −80 bp element of mouse 1α-hydroxylase gene contains an enhancer to be necessary for renal proximal tubular cell-specific expression.

Vitamin D plays important roles in calcium and phosphate homeostasis, bone growth, and cell differentiation. The biologically active form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25-$(OH)_2$D], is synthesized by sequential hydroxylation steps before it can bind to and activate the vitamin D receptor. The first hydroxylation is catalyzed in the liver by vitamin D 25-hydroxylase to 25-hydroxyvitamin D, and the second hydroxylation is catalyzed in the kidney by 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase) to 1α,25-$(OH)_2$D (1). The 1α-hydroxylase is a mitochondrial cytochrome P450 enzyme essential for vitamin D metabolism. Its enzyme activity is tightly regulated by several factors including parathyroid hormone, 1α,25-$(OH)_2$D itself, calcitonin, and serum concentrations of calcium and phosphate (2). Most of the studies to date have demonstrated that renal 1α-hydroxylase activity is localized exclusively in the proximal tubules (3–5).

We and other investigators have recently cloned rat (6,7), mouse (8), human (9,10), and porcine (11) cDNAs of 1α-hydroxylase. The complete sequences of mouse and human 1α-hydroxylase genes have also been reported (9,12–14). It has been demonstrated that mutations in the 1α-hydroxylase gene cause vitamin D dependency rickets type I, which is characterized by early onset of hypocalcemia, secondary hyperparathyroidism, and severe rachitic lesions (10,15,16). Northern blot analyses have revealed 1α-hydroxylase mRNA expression to be localized mainly in the kidney (9,15). This result is consistent with the previous studies that the kidney is the principal site of 1α,25-$(OH)_2$D synthesis, although several extrarenal cells, including keratinocytes (17), placental decidual cells (18), and pulmonary macrophages (19) also have 1α-hydroxylase activity. Extrarenal 1α-hydroxylase does not contribute to circulating 1α,25-$(OH)_2$D concentrations and acts in an intracrine manner.

The mechanism that the 1α-hydroxylase gene is predominantly expressed in renal proximal tubular cells clearly warrants study. Little is known about proximal tubular cell-specific gene expression, although several investigations have focused on the mechanism responsible for the kidney-specificity or other nephron segments-specificity (20–24). We hypothesized that some cell-specific transcriptional regulators bound to DNA elements that are located in the 5'-flanking region of 1α-hydroxylase gene because cell type-specific gene expressions in a variety of cells are controlled at the transcriptional level. Thus, this study was undertaken to identify the *cis*-element that is necessary for renal proximal tubular cell-specific expression in the 5'-flanking region of mouse 1α-hydrox-

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Correspondence to: Dr. Matsuhiko Hayashi, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. Phone: 81-3-5363-3796; Fax: 81-3-3359-2745; E-mail: matuhiko@sc.itc.keio.ac.jp
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ylase gene. As will be shown, the −105 to −80 bp element in mouse 1α-hydroxylase gene contains an enhancer that contributes to this cell-specific expression.

Materials and Methods

Cell Culture

LLC-PK1 (porcine renal proximal tubular cells), NIH/3T3 (mouse fibroblasts), HepG2 (human hepatoblastoma), MDCK (canine renal distal tubular cells), and OK (opossum renal proximal tubular cells) cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS) (Life Technologies, Grand Island, NY).

Poly (A)$^+$ RNA Extraction and Northern Blot Analyses

Poly (A)$^+$ RNA was prepared using a MicroPoly (A) Pure mRNA Purification Kit (Ambion, Austin, TX). Three micrograms of poly (A)$^+$ RNA were denatured with glyoxal and DMSO, fractionated on a 1% agarose gel, and transferred onto a Hybond N$^+$ nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out in PerfectHyb solution (TOYOBO Co., Ltd., Osaka, Japan) at 66°C for 8 h with a ^{32}P-labeled human 1α-hydroxylase cDNA probe (9). A final wash was performed at 66°C in 0.1 × standard saline citrate and 0.1% sodium dodecyl sulfate, and the membrane was exposed to a BAS Imaging Plate (Fuji Photo Film Co., Ltd., Tokyo, Japan). The membrane was rehybridized with a β-actin probe (Clontech Laboratories, Inc., Palo Alto, CA).

Construction of Reporter Plasmids

A 1.7-kb *SpeI-XhoI* DNA fragment was extracted from a pBluescript KS (−) vector in which the 5'-flanking region of mouse 1α-hydroxylase gene had been subcloned (12). This fragment contains −1652 to +15 bp of the gene (+1 bp: transcriptional initiation site shown in reference 12) and was subcloned into a pGL3-basic vector (Promega Corp., Madison, WI), which is designated pGL1α (−1652). The pGL1α (−1652) vector was digested with *KpnI* and *PstI*, Van 91I, *BstXI*, or Cel II to generate pGL1α (−1392), pGL1α (−656), pGL1α (−291), or pGL1α (−105) vectors, respectively. The inserts were eliminated, and vectors including the 1α-hydroxylase gene promoter were blunted at both ends before religation.

Several 5'-deletion mutants of the 1α-hydroxylase gene constructs, pGL1α (−80), pGL1α (−74), pGL1α (−51), and pGL1α (−30), were generated by PCR with the pGL1α (−105) vector as a template using the respective upstream pGL1α primers and a downstream pGL1α rev. primer (Table 1). The PCR products were digested with *KpnI* and *XhoI* and ligated into the pGL3-basic vector.

Site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Primers used for construction are shown in Table 1.

To test for enhancer function, synthetic double-strand oligonucleotides with appropriate restriction sites were subcloned into the Mlu I-BglII site of pGL3-promoter vector.

All constructs were confirmed by DNA sequencing. The DNA sequence was analyzed for putative *cis*-acting regulatory elements by the GENETYX-MAC version 9 (Software Development Co., Ltd., Tokyo, Japan).

Transient Transfection

Approximately 16 h before transfection, cells were seeded at 50% confluence onto 12-well plates. Cells were transiently transfected with plasmid DNA vectors using Tfx-20 (LLC-PK1 and HepG2) or Trans-

Table 1. Oligonucleotides used for generation of pGL1α reporter constructs[a]

Name	Sequence
Plasmid constructs	
pGL1α (−80) primer	5'-TATGGTACCGATTGGCTGAAGAGCTTG-3'
pGL1α (−74) primer	5'-TATGGTACCTGAAGAGCTTGGAGAGGG-3'
pGL1α (−51) primer	5'-TATGGTACCTTCACCTCCAGGACCAAG-3'
pGL1α (−30) primer	5'-TATGGTACCTATATATGGGTCCAGGCA-3'
pGL1α rev. primer	5'-CATAAAGAATTGAAGAGAGTT-3'
Site-directed mutagenesis	
pGL1α (−105)M1 primer	5'-CTCTATCGATAGGAGAAACACAGACCACTTGCAAAGGG-3'
pGL1α (−105)M2 primer	5'-CTCTATCGATAGGAGCTATTCAGACCACTTGCAAAGGG-3'
pGL1α (−105)M3 primer	5'-CGATAGGAGCTACATTGACCACTTGCAAAGGGATTGGC-3'
pGL1α (−105)M4 primer	5'-CGATAGGAGCTACACATTCCACTTGCAAAGGGATTGG-3'
pGL1α (−105)M5 primer	5'-CGATAGGAGCTACACAGACTTCTTGCAAAGGGATTGGC-3'
pGL1α (−105)M6 primer	5'-CGATAGGAGCTACACAGACCAAATGCAAAGGGATTGG-3'
pGL1α (−105)M7 primer	5'-CGATAGGAGCTACACAGACCACTAACAAAGGGATTGG-3'
pGL1α (−105)M8 primer	5'-CGATAGGAGCTACACAGACCACTTGTTAAGGGATTGGC-3'
pGL1α (−105)M9 primer	5'-CGATAGGAGCTACACAGACCACTTGGGAAGGGATTGGC-3'
pGL1α (−105)M10 primer	5'-GGAGCTACACAGACCACTTGCAGGGGGATTGGCTG-3'
pGL1α (−105)M11 primer	5'-GGAGCTACACAGACCACTTGCAAATTGATTGGCTGAAG-3'
pGL1α (−105)M12 primer	5'-CGATAGGAGCTATTTTGACCACTTGCAAAGGGATTGGC-3'
pGL1α (−105)M13 primer	5'-GGAGCTACACAGACCACTTGTTTTGGGATTGGCTG-3'
pGL1α (−105)M14 primer	5'-CGATAGGAGCTACATTGACCACTTGTTAAGGGATTGGC-3'

[a] Underlined sequences indicate Kpn I restriction site. For primers used in site-directed mutagenesis, only the sense strands are illustrated.

fast (NIH/3T3, MDCK, and OK) reagents (Promega Corp.). The optimal transfection reagent and conditions for particular cell types were systematically tested among Tfx-10, Tfx-20, Tfx-50, and Transfast. After transfection, cells were incubated in DMEM supplemented with 5% FCS for 48 h. The Luciferase Assay System (Promega Corp.) was used to harvest cellular lysates and to perform luciferase assays. Luciferase activity was measured with a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Cellular protein concentrations were measured to normalize the experiments. Each sample was examined in triplicate in a single experiment and this was repeated in four different experiments.

Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides used in this study, as listed in Figure 6A, were annealed to their respective complementary sequence in the annealing buffer containing 200 mM Tris HCl (pH 7.5), 100 mM $MgCl_2$, and 500 mM NaCl. Double-strand oligonucleotides were labeled with ^{32}P-ATP by T4 polynucleotide kinase. Nuclear extracts from LLC-PK1 cells were prepared by the method of Dignam *et al.* (25). Ten micrograms of nuclear protein were incubated with ^{32}P-labeled double-strand oligonucleotides. Reactions were carried out in binding buffer containing 10 mM Tris HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, and 0.1 μg/μl poly(dI-dC)poly(dI-dC). For competition assays, 20- to 50-fold excess amounts of unlabeled oligonucleotides were mixed with the nuclear extracts and the labeled probes were then added. The reaction mixtures were separated on a 4% nondenaturing polyacrylamide gel. The gel was dried and autoradiographed. The antibody for TEL (Santa Cruz Biotechnology, Santa Cruz, CA) was used for supershift.

Decoy Experiments

Decoy (GAGCTACACAGACCACTTGCAAAGGG) or scrambled (CGATTGCCGTACCTGACTTAACTCATTG) phosphorothioate oligonucleotides, which are relatively resistant to nucleases due to modified phosphodiester bonds (26), were annealed to their respective complementary sequences. LLC-PK1 cells were transfected with 1.0 μg of pGL1α vector and either decoy (0.1 to 1.0 μM) or scrambled oligonucleotides using Tfx-20. After a 24-h incubation, luciferase activity was measured and normalized to protein content.

Statistical Analyses

Data are presented as the mean ± SE. Statistical analyses were performed by use of one-way ANOVA followed by Scheffe's F *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Only LLC-PK1 Cells Express the 1α-Hydroxylase Gene

The activity of 1α-hydroxylase is most abundant in the renal proximal tubular cells (1,3–5), and our previous studies showed gene expression of 1α-hydroxylase to be restricted to the kidney (9,15). To investigate the mechanism responsible for this cell-specific expression, we first examined the expression of 1α-hydroxylase gene in cultured cell lines by Northern blot analyses. Three types of mammalian kidney cells, including LLC-PK1 (porcine renal proximal tubular cells), OK (opossum renal proximal tubular cells), and MDCK (canine renal distal tubular cells), and other cells, including NIH/3T3 (mouse fibroblasts) and HepG2 (human hepatoblastoma) were tested. Because they are derived from different species, the relaxed stringency were adapted for hybridization. Using human 1α-hydroxyalase cDNA as a probe, expression of 1α-hydroxylase mRNA was detected only in porcine LLC-PK1 cells (Figure 1). NIH/3T3, HepG2, MDCK, and OK cells did not express 1α-hydroxylase gene.

Promoter Activity of 1α-Hydroxylase Gene is Detected Selectively in LLC-PK1 Cells

To examine the involvement of promoter elements in renal proximal tubular cell-specific gene expression, we investigated the 1.7-kb upstream region of mouse 1α-hydroxylase gene. A series of deletion mutants of the upstream region were constructed with a luciferase reporter gene and used for transfection into five lines of cultured cells. The cells used in the transfection study were the same as those in Northern blot analyses. Although the luciferase activity in each cell line was extremely elevated by transfection with a pGL3-control vector (a positive control vector), the activity was selectively detected in LLC-PK1 cells when they were transfected with pGL1α (−1652), pGL1α (−1392), pGL1α (−656), pGL1α (−291), or pGL1α (−105) vectors (Figure 2). In LLC-PK1 cells, luciferase activity was significantly higher in cells transfected with the pGL1α (−105) vector than in cells transfected with other pGL1α vectors. These results suggested that this shorter construct contained *cis*-acting enhancer elements sufficient for renal proximal tubular cell-specific gene expression.

To identify the *cis*-elements, we made much shorter constructs such as pGL1α (−80), pGL1α (−74), pGL1α (−51), and pGL1α (−30) vectors. Luciferase activities of these shorter constructs were assayed by transfection into LLC-PK1 and other cells (Figure 3). The luciferase activities of pGL1α (−80) and pGL1α (−74) vectors were undetectable in LLC-PK1 cells, whereas that of the pGL1α (−105) vector was significantly elevated. Moreover, the luciferase activity of the pGL1α (−51) vector, which contains only a TATA box, were elevated again. The pGL1α (−30) vector, which lacks a TATA box, had no luciferase activity. In contrast, no luciferase activity of these vectors was detected in NIH/3T3, HepG2, MDCK, and OK cells. We considered the nucleotides between −105 and −80 bp of mouse 1α-hydroxylase gene contained

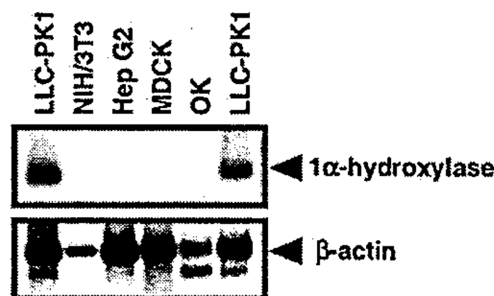


Figure 1. Expression of 1α-hydroxylase mRNA in cultured cells. 1α-Hydroxylase mRNA expressions in LLC-PK1, NIH/3T3, HepG2, MDCK, and OK cells were assessed by Northern blot analyses of poly (A)$^+$ RNA (3 μg/lane) using ^{32}P-labeled human 1α-hydroxylase and β-actin probes.

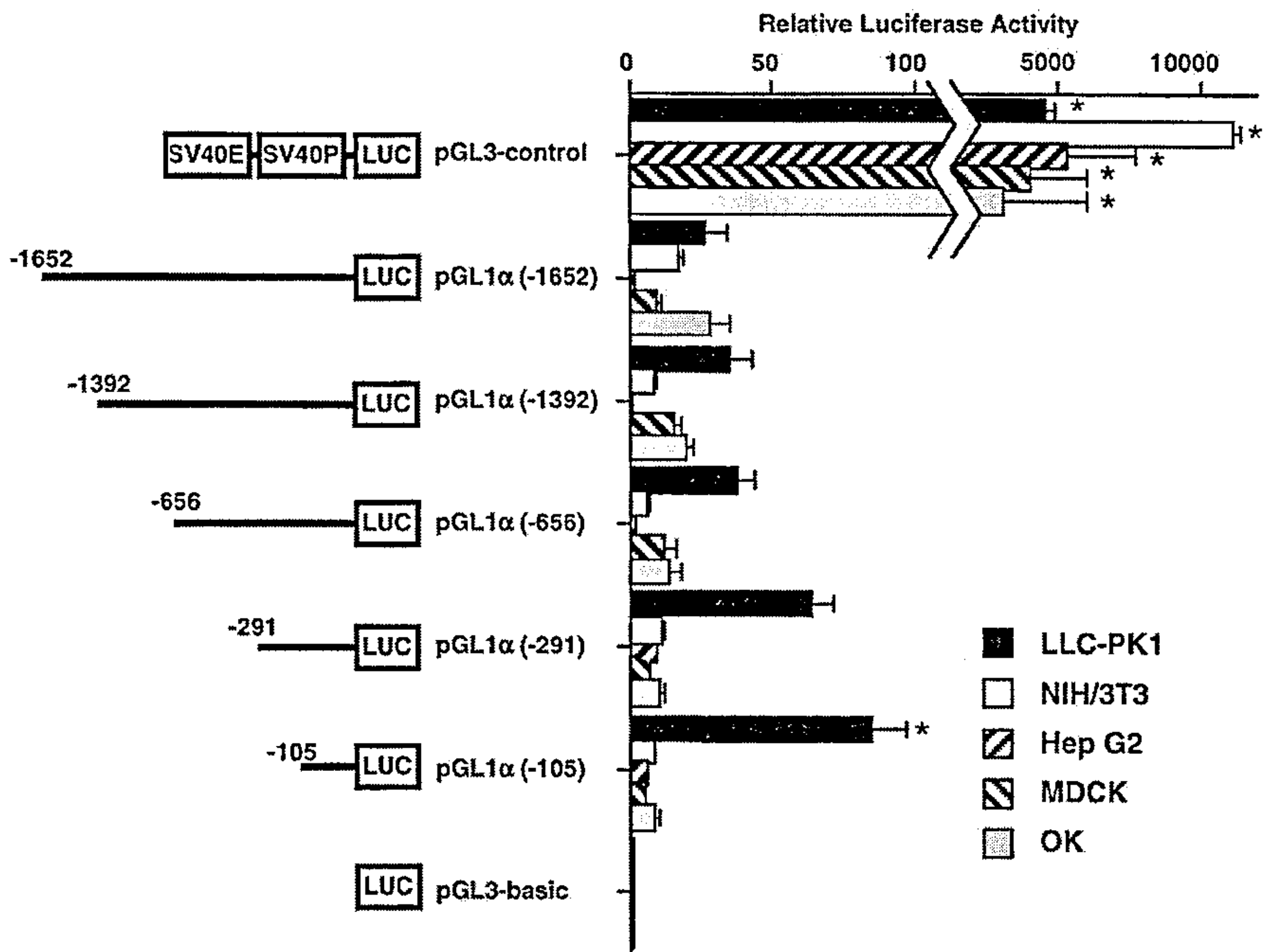


Figure 2. Transcriptional activities of 1α-hydroxylase gene promoter in various cells. LLC-PK1, NIH/3T3, HepG2, MDCK, and OK cells were transiently transfected with pGL3-control, pGL1α (−1652), pGL1α (−1392), pGL1α (−656), pGL1α (−291), pGL1α (−105), or pGL3-basic vector and assayed for luciferase activity as described in Materials and Methods. The activity was normalized for protein content. An arbitrary value of 1 was assigned to the activity of the pGL3-basic vector. Values represent the mean ± SE. *$P < 0.05$ compared with pGL3-basic vector.

enhancers to be necessary for cell-specific expression, and the nucleotides between −74 and −51 bp contained repressors. In this study, we focused our attention on the −105 and −80 bp region of the 1α-hydroxylase gene, and we designated this region the proximal tubular cell-specific element (PSE).

Mutational Analyses of Proximal Tubular Cell-Specific Element of the 1α-Hydroxylase Gene Promoter

DNA sequence analyses revealed the presence of two ternary complex factor-1 (TCF-1) consensus sites in PSE: the −105 to −80 bp region of the 1α-hydroxylase gene promoter (Figure 4A). We introduced mutations in the pGL1α (−105) vector by site-directed mutagenesis, and LLC-PK1 cells were transfected with various mutated vectors. As shown in Figure 4B, mutations in the distal TCF-1 site had a slight decrease in luciferase activity. On the other hand, mutations in the proximal TCF-1 site dramatically decreased luciferase activity.

Nuclear Proteins from LLC-PK1 Cells Bind to Proximal Tubular Cell-Specific Element of 1α-Hydroxylase Gene Promoter

EMSA were performed to examine whether the proximal TCF-1 site was critical for proximal tubular cell-specific gene expression. We used nuclear extracts from LLC-PK1 cells to assess protein-DNA complex formation. When we used a wild-type oligonucleotide containing the −105 to −80 bp region of 1α-hydroxylase gene (PSE −105/−80) as a probe, a specific protein-DNA complex was observed (Figure 5). This complex was competed away by the wild-type oligonucleotide, but not by a nonspecific oligonucleotide. The AP-2 consensus sequence, that is 5'-GATCGAACTGACCGCCCGCGGCCCGT-3', was used as a nonspecific oligonucleotide.

We then used the PSE (−105/−80) oligonucleotide as a probe in EMSA and unlabeled PSE (−114/−87), PSE (−120/−93), or PSE (−101/−77) oligonucleotides as competitors. The nucleotide sequences of unlabeled competitors overlap

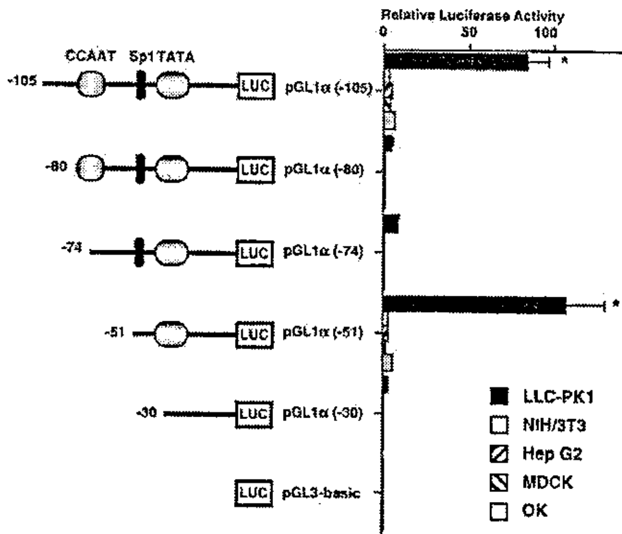
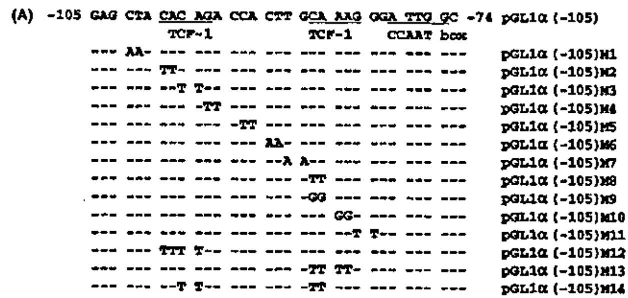


Figure 3. The −105 to −80 bp region of 1α-hydroxylase gene mediates proximal tubular cell-specific transcriptional activity. LLC-PK1, NIH/3T3, HepG2, MDCK, and OK cells were transiently transfected with pGL1α (−105), pGL1α (−80), pGL1α (−74), pGL1α (−51), pGL1α (−30), or pGL3-basic vector, and assayed for luciferase activity as described in Materials and Methods. The activity was normalized for protein content. An arbitrary value of 1 was assigned to the activity of the pGL3-basic vector. Values represent the mean ± SE. * $P < 0.05$ compared with pGL3-basic vector.



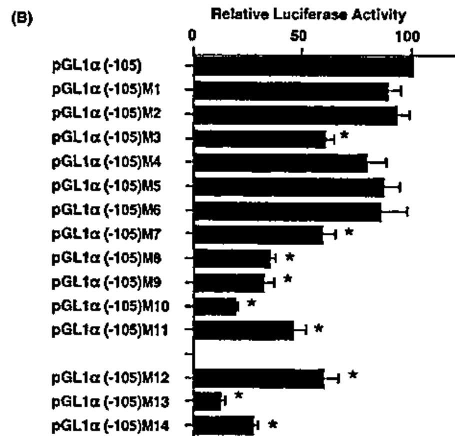


Figure 4. Mutational analyses of the −105 to −80 bp region of 1α-hydroxylase gene promoter. (A) Schematic representation of mutations covering the −105 to −80 bp region of 1α-hydroxylase gene promoter. (B) The promoter activity of the mutant constructs was evaluated by transfection into LLC-PK1 cells. The luciferase activity was measured and normalized for protein content. An arbitrary value of 100 was assigned to the activity of cells transfected with the pGL1α (−105) vector. Values represent the mean ± SE. *$P < 0.05$ compared with pGL1α (−105) vector.

with the PSE (−105/−80) oligonucleotide (Figure 6A). The complex between the ^{32}P-labeled wild-type oligonucleotide and LLC-PK1 nuclear extracts was abolished by PSE (−114/−87) and PSE (−101/−77) competitors, whereas the PSE (−120/−93) competitor could not abolish complex formation (Figure 6B). These results suggest that the adjacent region, including the proximal TCF-1 sequence, is a binding site for nuclear proteins derived from LLC-PK1 cells.

Moreover, the effects of mutations in the proximal TCF-1 site on protein-DNA complex formation were assessed by EMSA. Three mutant oligonucleotides were generated in the distal TCF-1 site, in the proximal TCF-1 site, and in the middle positions of these regions (Figure 6A). As shown in Figure 6C, the mutant oligonucleotide at the proximal TCF-1 site, PSE (−105/−80)M3, could not compete for the labeled PSE (−105/−80) oligonucleotide, whereas PSE (−105/−80)M1 could. These experiments support the finding that nuclear factors from LLC-PK1 cells bind to the DNA sequence including the proximal TCF-1 site.

The TCF-1 site is a target for Ets transcriptional factor family members, which can be classified according to their homology with Ets-1 (27). Class I members, including Ets-1, have extensive overall sequence similarity. Class II members, including Fli-1, show homology to Ets-1 in both their N-terminal transactivation and C-terminal DNA binding domains, whereas class III members, such as Elk-1, PU.1, and TEL, show homology only in the DNA binding domain. We investigated the ability of TEL antibody to interfere with the protein-DNA complex, because the kidney expresses TEL gene (28). Supershift assay employing TEL antibody showed that it had no effect (data not shown).

Proximal Tubular Cell-Specific Element of 1α-Hydroxylase Gene Behaves as a Classical Enhancer

To determine whether PSE, the −105 to −80 bp region of mouse 1α-hydroxylase gene, could enhance the activity of a heterologous promoter, the DNA was placed upstream and in both orientation of the luciferase gene driven by the SV40 promoter in the enhancerless vector pGL3-promoter. The luciferase activities were elevated in LLC-PK1 cells transfected with these vectors, which are designated pGL3p (−105/−80) and pGL3p (−105/−80)inv vectors (Figure 7). To assess the role of proximal TCF-1 site in the PSE, the same 4-bp mutations as PSE (−105/−80)M3 (Figure 6A) were introduced in the pGL3p (−105/−80) vector and the mutated vector was named as pGL3p (−105/−80)mut vector. The luciferase activity of pGL3p (−105/−80)mut vector was not different from the pGL3-promoter vector in LLC-PK1 cells. Moreover, we made a pGL3p (−105/−80)x3 vector, which has three tandem

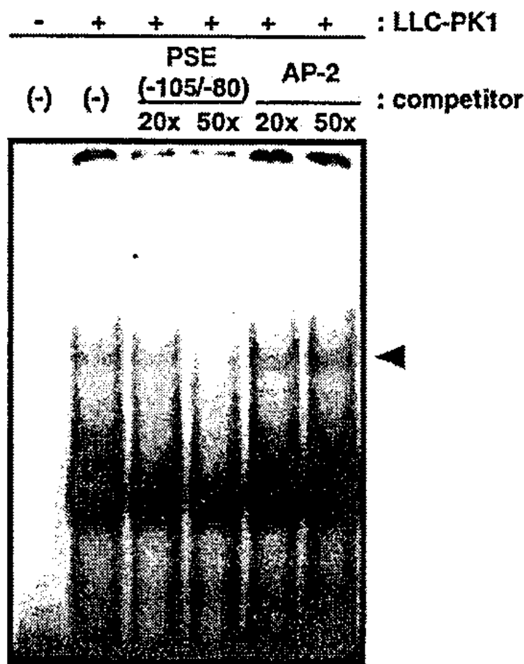


Figure 5. Proximal tubular cell-specific element in 1α-hydroxylase gene promoter binds to nuclear proteins from LLC-PK1 cells. LLC-PK1 nuclear extracts were incubated with a ^{32}P-labeled probe corresponding to the −105 to −80 bp region of 1α-hydroxylase gene promoter in the absence or presence of a 20- or 50-fold molar excess of unlabeled competitor oligonucleotides. The competitors were either the wild-type PSE (−105/−80) oligonucleotide as a specific competitor (CTGAGCTACACAGACCACTTGCAAAGGG) or the AP-2 consensus sequence as a nonspecific competitor (GATCGAACTGACCGCCCGCGGCCCGT). Arrowhead indicates the DNA-protein complex.

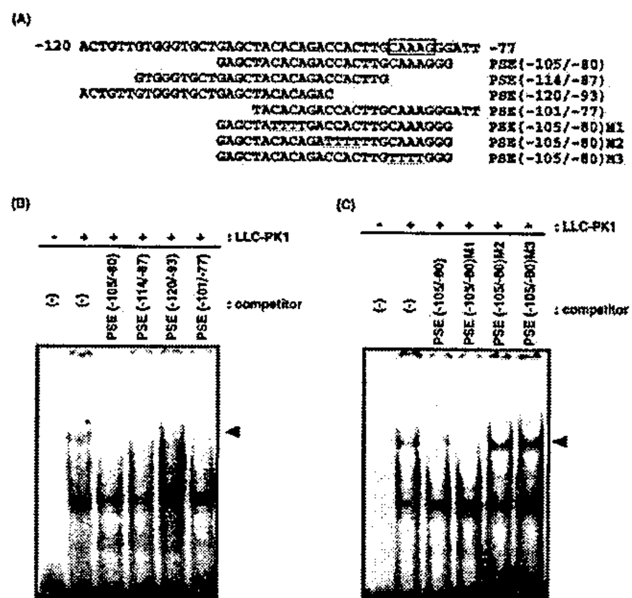


Figure 6. Mutations of proximal ternary complex factor-1 (TCF-1) site abolish ability to bind to nuclear proteins from LLC-PK1 cells. LLC-PK1 nuclear extracts were incubated with a ^{32}P-labeled probe corresponding to the −105 to −80 bp region of 1α-hydroxylase gene promoter in the absence or presence of a 50-fold molar excess of unlabeled competitor oligonucleotides. (A) Only the sense strands of specific and nonspecific competitors are illustrated. Underlined sequences indicate mutations. Proximal TCF-1 site is boxed. (B and C) Representative electrophoretic mobility shift assay (EMSA) are shown. Arrowheads indicate the DNA-protein complex.

repeats of PSE in the pGL3-promoter vector. The luciferase activity of pGL3p (−105/−80)x3 vector was also elevated in LLC-PK1 cells. On the other hand, the luciferase activities of these vectors were not elevated in NIH/3T3, HepG2, MDCK, and OK cells. The PSE could behave in a classical enhancer-like manner, *i.e.*, independently of position and orientation, selectively in LLC-PK1 cells.

PSE (−105/−80) Decoy Oligonucleotide Reduces 1α-Hydroxylase Gene Promoter Activity

To provide further evidence that the −105 to −80 bp region of 1α-hydroxylase gene is required for proximal tubular cell-specific gene expression, we developed a double-stranded decoy oligonucleotide corresponding to PSE. The rationale for this approach was the assumption that the decoy would compete for transcriptional factor binding to the promoter and thus block transcriptional responses. A double-stranded decoy oligonucleotide or a scrambled control was introduced into LLC-PK1 cells together with the pGL1α (−105) vector or the pGL1α (−291) vector, using Tfx-20 reagent. As shown in Figure 8, the PSE (−105/−80) decoy oligonucleotide dose-dependently attenuated the activity of both luciferase plasmids. The scrambled oligonucleotide did not inhibit transcriptional activity. These data suggest that the decoy oligonucleotide is capable of suppressing transcriptional activity and that PSE in mouse 1α-hydroxylase gene is necessary for renal proximal tubular cell-specific gene expression.

Discussion

The serum concentration of 1α, 25-$(OH)_2D$ is very low or undetectable in patients who have severe renal failure or have undergone a bilateral nephrectomy. This suggests that the kidney is the principal and the sole physiologic source of 1α,25-$(OH)_2D$ production. Several studies have demonstrated that renal 1α-hydroxylase activity is localized exclusively in the cells of proximal tubules (3–5). To clarify the mechanism underlying the proximal tubular cell-specific expression of 1α-hydroxylase, we investigated the 5′-flanking region of mouse 1α-hydroxylase gene *in vitro*. Herein, we showed 1α-hydroxylase gene expression and its promoter activity to be selectively detectable in LLC-PK1 cells. Using transfection studies and EMSA, we identified the −105 to −80 bp region of mouse 1α-hydroxylase gene with PSE. PSE bound to nuclear proteins from LLC-PK1 cells, and it behaved as a cell-specific enhancer. Decoy experiments confirmed that PSE was required for renal tubular cell-specific 1α-hydroxylase gene expression.

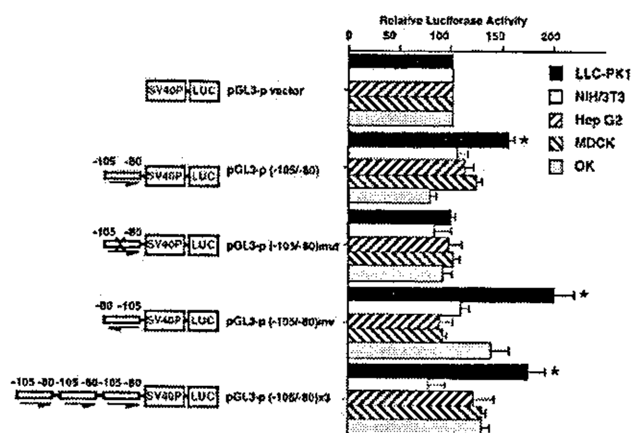


Figure 7. Proximal tubular cell-specific element in 1α-hydroxylase gene acts as an enhancer. LLC-PK1, NIH/3T3, HepG2, MDCK, and OK cells were transiently transfected with pGL3p (−105/−80), pGL3p (−105/−80)mut, pGL3p (−105/−80)inv, pGL3p (−105/−80)x3, or pGL3-promoter vector and assayed for luciferase activity as described in Materials and Methods. The activity was normalized for protein content. An arbitrary value of 100 was assigned to the activity of the pGL3-promoter vector. Values represent the mean ± SE. * $P < 0.05$ compared with pGL3-promoter vector.

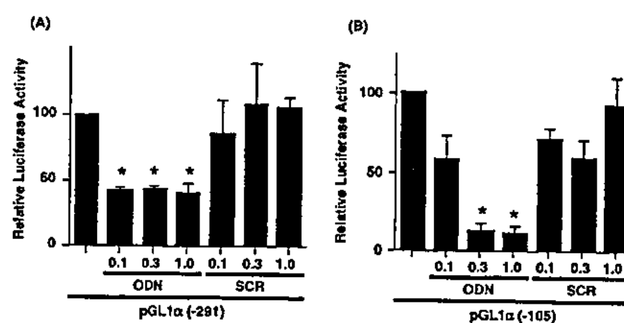


Figure 8. PSE (−105/−80) decoy oligonucleotide inhibits 1α-hydroxylase gene transcription. A decoy corresponding to the −105 to −80 bp region of 1α-hydroxylase gene (ODN) or a scrambled (SCR) double-stranded phosphorothioate oligonucleotide at 0.1 to 1.0 μM were transfected together with 1 μg of (A) pGL1α (−291) or (B) pGL1α (−105) vector into LLC-PK1 cells. Cells were incubated in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum for 24 h, and luciferase activity was then measured. The luciferase activity was normalized for protein content. An arbitrary value of 100 was assigned to the activity of the cells transfected with the pGL1α (−291) or the pGL1α (−105) vector only. Values represent the mean ± SE. * $P < 0.05$ compared with pGL1α (−291) or the pGL1α (−105) vector only.

In this study, we tested three types of mammalian kidney cells and other cells. LLC-PK1, OK, and MDCK cells are the representative renal tubular cell lines used in a wide variety of *in vitro* studies. Of the cells examined, NIH/3T3, HepG2, and MDCK cells are not derived from renal proximal tubular cells and were expected to not express 1α-hydroxylase gene. Actually, we could not detect 1α-hydroxylase mRNA expression by Northern blot analyses and its promoter activity by transient transfection study. In contrast, both porcine LLC-PK1 and opossum OK cells retain many functional characteristics of renal proximal tubular cells. Both were expected to have 1α-hydroxylase gene expression and its promoter activity, but OK cells had neither. These results are consistent with a previous report by Condamine *et al.* (29). They demonstrated that LLC-PK1 cells expressed a detectable ability to synthesize 1,25-$(OH)_2D$, whereas OK cells did not. This lack of expression may be an argument in favor of the pars recta origin of OK cells. OK cells would not have a sufficient capacity to express the 1α-hydroxylase gene.

By means of transient transfection studies using deletion constructs, we provided evidence that PSE, the −105 to −80 bp region of 1α-hydroxylase gene promoter, is necessary for renal proximal tubular cell-specific gene expression. This element contains two TCF-1 consensus sites. We found that mutations in the proximal TCF-1 site caused a substantial decrease in promoter activity by site-directed mutagenesis and transfection assay, whereas those in the distal TCF-1 site did not. EMSA also demonstrated the binding of PSE with nuclear proteins from LLC-PK1 cells. It should be noted that the EMSA competition assay supported the importance of the proximal TCF-1 site for binding. Double-stranded oligonucleotides including the native proximal TCF-1 site only had the ability to compete for wild-type PSE oligonucleotide, whereas oligonucleotides with a mutated or deleted proximal TCF-1 site did not have this ability. Taken together, these observations indicate the proximal TCF-1 site of PSE to be a strong candidate for the core enhancer element responsible for the cell-specificity of 1α-hydroxylase gene expression.

The TCF-1 site is a target sequence for the Ets transcriptional factor family characterized by a conserved DNA-binding domain (27). This family currently has more than 30 known members, from organisms as diverse as sponges and humans. A class III member, TEL has been shown to be expressed in the kidney (28). In the present study, however, the antibody against TEL could not interfere with the protein-DNA complex formation by the supershift assay. An unidentified factor belonging to the Ets-family may be expressed in a proximal tubular cell-specific manner and may regulate some proximal tubular cell-specific genes by binding to *cis*-acting elements.

Although we focused our attention on PSE, the −105 and −80 bp region of mouse 1α-hydroxylase gene in this study, it is possible that the −74 to −51 bp region may play a suppressive role in cell-specific gene expression. This region includes Sp-1 binding sequence. In general, Sp1 binding to its recognition element leads to activation of transcription. However, Sp1 has also been shown to function as a negative regulator. For example, it was demonstrated that Egr-1, immediate-early growth response gene product, displaces Sp-1 from a GC-rich composite element to activate expression of the platelet-derived growth factor A chain (30). It was additionally demonstrated that Sp-1 activates and inhibits transcription from separate elements in the promoter of the human adenine nucleotide translocase 2 gene (31). The possibility also remains that other *cis*-elements in this region contribute to gene suppression.

However, it should be noted that the suppression of 1α-hydroxylase gene transcription occurred in LLC-PK1 cells, not in non-1α-hydroxylase expressing cells such as NIH/3T3, HepG2, MDCK, and OK cells. It is hard to understand how this suppression contributes to proximal tubular cell-specific gene expression. Further studies are required to resolve the problem.

It is interesting that shorter constructs, such as pGL1α (-291) and pGL1α (-105) vectors, had higher luciferase activity than longer constructs in our transfection studies, whereas the entire upstream 5'-flanking region is actually used *in vivo*. Although our findings would reflect that the expression of 1α-hydroxylase gene is very low at basal levels (32), it is somewhat difficult to understand why the shorter promoter had higher selectivity in gene expression than the longer one. We can speculate, as follows. First, it is possible that transcriptional repressors may bind only to longer constructs, not to shorter constructs, thereby suppressing the promoter activity of longer constructs in LLC-PK1 cells. Tissue-specific gene expression requires the combined action of tissue-specific and promoter-specific activators and repressors. For instance, it is known that 1α,25-$(OH)_2D$ inhibits 1α-hydroxylase gene expression transcriptionally (12,13), although the mechanism of repression has not been identified. The *cis*-acting elements responsible for 1α,25-$(OH)_2D$-mediated transcriptional repression may exist in the upstream region over -105 bp of the 1α-hydroxylase gene promoter. Second, it is also possible that some other regions located upstream from the 1.7-kb flanking sequence cooperatively play a role in determining renal proximal tubular cell-specific expression of the 1α-hydroxylase gene. Various binding sites for ubiquitous and cell-specific transcriptional factors would participate in the restricted 1α-hydroxylase gene expression specific for renal proximal tubular cells. Further cloning of the 5'-flanking region of the gene will be needed to fully understand the underlying mechanism.

Because expression of 1α-hydroxylase gene is highly restricted *in vivo* and *in vitro*, we used only LLC-PK1 cells as 1α-hydroxylase expressing cells in this study. In spite of renal proximal tubular origin, OK cells did not express 1α-hydroxylase gene. To verify the role of PSE in proximal tubular cell-specific expression, it would be helpful to examine whether PSE works as an enhancer in other renal proximal tubular cell lines as well as LLC-PK1 cells. Moreover, the roles of PSE in nonrenal 1α-hydroxylase–expressing cells, such as keratinocytes and macrophages, are also interesting.

In conclusion, our study suggests that nuclear proteins derived from LLC-PK1 cells bind to the -105 to -80 bp enhancer region of mouse 1α-hydroxylase gene, and the protein-DNA complex thereby activates gene transcription in a renal proximal tubular cell-specific manner. Further studies identifying these transcriptional factors are required. These factors are anticipated to serve as new therapeutic targets for diseases affecting predominantly renal proximal tubular cells.

Acknowledgments

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Original Article

Role of Genetic Polymorphism in the SA Gene on the Blood Pressure and Prognosis of Renal Function in Patients with Immunoglobulin A Nephropathy

Ichiei NARITA, Noriko SAITO, Shin GOTO, Arimasa SHIRASAKI, Yoshio MORIOKA, Song JIN, Kentaro OMORI, Minoru SAKATSUME, Masaaki ARAKAWA, and Fumitake GEJYO

The SA gene has been shown to be much more highly expressed in the kidneys of spontaneously hypertensive rats than in the corresponding wild-type strain. Genetic polymorphism of this gene has been shown to play a role in human hypertension, although the details of this association remain controversial. We investigated the possible associations between SA gene polymorphism and both hypertension and the prognosis of renal function in patients with immunoglobulin A nephropathy (IgAN). Genomic DNA was isolated from the peripheral blood of 367 individuals, including 274 patients with histologically proven IgAN and 100 controls without any history of renal disease. The SA genotype was determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) with Pst I. The frequencies of genotypes and alleles were not different between the patients with IgAN and those without renal disease. In the group without renal disease, the SA gene polymorphism was not associated with hypertension. However, in the patients with IgAN the A1 allele frequency was significantly higher in the hypertensives than in the normotensives. The renal survival of the patients with the A2 allele tended to be better than that of those without the A2 allele. The findings thus suggest that SA gene polymorphism may be associated with the renal prognosis of IgAN through its effect on blood pressure. Further, they suggest that the sensitivity to this gene polymorphism increases in patients with renal injury. (*Hypertens Res* 2002; 25: 831–836)

***Key Words*: SA gene, gene polymorphism, immunoglobulin A nephropathy**

Introduction

Immunoglobulin A nephropathy (IgAN), which is characterized by immunoglobulin A (IgA) deposits in the glomerular mesangium, is the most prevalent form of primary glomerulonephritis and the major cause of end stage renal disease (ESRD) (*1, 2*). IgAN is a complex disease in which familial clustering is suggestive of an inherited genetic predisposition (*3, 4*). It has a variable clinical course, with one-third of IgAN patients progressing to ESRD within 10–20 years of onset (*5*). The mechanism of the inter-individual differences in the rate of disease progression are still unclear. It has been well documented that high blood pressure is a substantial risk factor for the progression of renal dysfunction (*6*). Hypertension is a polygenic, heterogeneous, and multifactorial disease with a high genetic predisposition.

The SA gene was identified in genetically hypertensive rats using the differential hybridization technique (*7*). It has been shown that the SA gene is predominantly expressed in the rat kidney proximal tubules and that it is located on rat chromosome 1 (*8*). Moreover, the SA gene has been demon-

From the Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.
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Address for Reprints: Ichiei Narita, M.D., Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, 1–757, Asahimachi-dori, Niigata 951–8510, Japan. E-mail: naritai@med.niigata-u.ac.jp
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Table 1. Genotype and Allele Frequencies of SA Polymorphism in Patients with IgAN and Controls without Any Renal Disease

	IgAN	Control
Genotype frequency		
A1A1	197	74
A1A2	70	21
A2A2	7	5
	274	100
Allele frequency		
A1	0.847	0.845
A2	0.153	0.155

IgAN, immunoglobulin A nephropathy.

strated to cosegregate with blood pressure in both the SHR/WKY and Dahl rat models (*9–11*). In humans, the SA gene is located on chromosome 16p13.11, and a difference in allele frequency of the SA polymorphism between hypertensives and controls has been reported (*12*), although the results of other previous reports remain controversial (*13, 14*).

In this study, we attempted to investigate the possible role of the SA gene polymorphism in kidney disease. We also studied any possible associations between the SA gene variation and the clinical manifestations in patients with IgAN as well as those in controls without any history of renal disease.

Methods

The ethics committee of the institution involved approved the protocol for the study, and informed written consent for the genetic studies was obtained from all participants. Genomic DNA of peripheral blood cells was isolated by an automatic DNA isolation system (NA-1000; Kurabo, Osaka, Japan) from 374 Japanese individuals, including 274 patients with histologically confirmed IgAN. Patients with Schonlein-Henoch purpura or secondary IgAN, such as cases of hepatic glomerulosclerosis, were excluded from the analysis. Diagnosis of IgAN was based on a kidney biopsy that revealed the presence of dominant or co-dominant glomerular mesangial deposits of IgA as assessed by an immunofluorescence examination. Investigated clinical characteristics of the patients with IgAN included age, sex, duration of observation (in months), level of urinary protein excretion (g/day), serum creatinine (sCr; mg/dl), and 24-h creatinine clearance (C_{Cr}; ml/min). The time from the first urine abnormality to renal biopsy (in months) was also recorded for the 223 of the 274 patients whose first episode of urine abnormality (proteinuria or hematuria) could be clearly defined. At the time of renal biopsy, the advanced type of glomerulonephritis (GN) was defined by a sCr level greater than 1.2 mg/dl or a 24-h C_{Cr} of less than 70 ml/min. Hypertension was defined by the use of one or more antihypertensive medications and/or a blood pressure greater than or equal to 140 mmHg systolic or 90 mmHg diastolic. The primary endpoint (ESRD; end stage renal disease) was defined as the date at which the sCr levels doubled after the time of diagnosis, or when patients underwent their first hemodialysis.

The administrations of glucocorticoids, antihypertensive agents, and angiotensin converting enzyme inhibitors (ACE-Is) were also recorded for each patient.

The genotypes in 100 patients with no history of renal disease (46 females and 54 males, of which 36 were normotensive and 64 hypertensive; age, 56.3 ± 9.3) were studied, and any associations between the genotype and hypertension were also investigated.

Genotype Determination

We studied a single nucleotide polymorphism in the SA gene by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) according to the method described previously (*14*). PCR of the genomic DNA was performed to amplify a 315-base pair (bp) fragment in the SA gene using the primer pair 5′-GTCACACATTAGGGCA GCTGCACAC-3′and 5′-GCCAGGCATGGTGATGCAAT CCTG-3′. The reaction mixture contained $1 \times$PCR buffer, 1.5 mmol/l $MgCl_2$, 200 mmol/l deoxynucleotide triphosphates (dNTPs), 1 unit Taq DNA polymerase (Takara, Kyoto, Japan), 10 pmol of each primer, and 50–100 ng genomic DNA. The PCR amplification reaction consisted of one cycle at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 62 °C for 15 s, and an extension at 72 °C for 30 s. The final extension was performed at 72 °C for 5 min. The PCR products were digested with restriction endonuclease PstI (Promega, Madison, USA), and electrophoresed on a 3% agarose gel. For A2 alleles, this resulted in a fragment of 230-bp and one of 85-bp fragment, whereas the 315-bp A1 alleles lacked the restrictive site.

Statistical Analysis

Continuous variables were compared using the Mann-Whitney *U*-test. χ^2 analysis was used when comparing allele frequencies and categorical variables between the groups. The Hardy-Weinberg equilibrium was tested by a χ^2 test with 1 *df*. The adjusted odds ratios and 95% confidence interval (CI) for advanced GN at the time of renal biopsy with multivariate factors were calculated using logistic regression analysis. The Kaplan-Meier method and the Cox proportional hazards regression model were use to analyze the time course from the initial renal biopsy to the end point (initiation of dialysis or a doubling in the sCr level after the time of diagnosis). Covariates were selected by a stepwise backward method and the effects of these covariates were expressed by a hazard ratio. Values of $p<0.05$ were considered to indicate statistical significance. Statview 5.0 statistical software (Abacus Concepts, Inc., Berkeley, USA) was used for statistical analyses on a Macintosh G4 computer.

Table 2. Characteristics of Subjects without Renal Disease and Comparison of Characteristics by SA Genotype Group

	Total	SA genotype		
	(N=100)	A1A1 (N=74)	A1A2 and A2A2 (N=26)	
Age (year)	56.3±9.3	57.2±10.2	59.3±9.7	NS
Gender (male %)	54.0	54.1	53.8	NS
BMI	24.4±5.0	23.9±3.6	25.6±7.6	NS
Serum creatinine (mg/dl)	0.6±0.2	0.6±0.2	0.7±0.2	NS
Blood pressure (mmHg)				
Systolic	137.9±21.0	136.5±20.9	138.6±22.0	NS
Diastolic	85.8±14.7	84.2±14.3	86.4±15.6	NS
Incidence of hypertension (%)	64.0	62.2	69.2	NS

BMI, body mass index; NS, not significantly different between groups.

Table 3. Clinical Characteristics of Patients with IgA Nephropathy

	All patients	SA genotype			
	(N=274)	A1A1 (N=197)	A1A2 and A2A2 (N=77)	p value	χ^2
Gender (male %)	46.7	47.7	44.2	NS	
At the time of renal biopsy					
Age (year)	36.9±13.6	37.1±14.0	36.4±12.3	NS	
BMI	22.7±3.4	22.9±3.5	22.1±2.9	NS	
Time from the first urine abnormality to renal biopsy (month)	56.1±66.8	58.7±73.1	54.1±60.7	NS	
Urinay protein excretion (g/day)	1.3±1.3	1.4±1.4	1.3±1.2	NS	
Serum creatinine (mg/dl)	1.0±0.6	1.0±0.5	1.0±0.8	NS	
Creatinine clearance (ml/min)	89.1±33.3	87.0±33.5	94.5±32.3	0.0368	
Blood pressure (mmHg)					
Systolic	128.0±16.9	129.3±18.2	125.0±18.5	NS	
Diastolic	77.2±13.5	78.1±13.5	75.1±13.6	NS	
Incidence of hypertension (%)	37.1	41.4	26.3	0.0216	5.275
Incidence of advanced GN (%)	31.4	35.6	21.1	0.0208	5.598
During the observation					
Observed period (month)	93.4±67.3	92.3±65.6	96.2±71.9	NS	
Incidence of end stage renal disease (%)	31.4	34.9	24.0	NS	
Glucocorticoid (%)	27.5	25.7	32.4	NS	
ACE-inhibitor (%)	41.1	39.0	46.7	NS	
Mean blood pressure (mmHg)					
Systolic	128.8±16.9	130.3±17.2	125.1±15.7	0.0370	
Diastolic	77.5±11.9	78.4±11.9	75.4±11.8	0.0653	
Incidence of hypertension (%)	64.0	67.9	53.2	NS	

BMI, body mass index; NS, not significantly different between each groups; GN, glomerulonephritis; ACE, angiotensin converting enzyme.

Results

The 374 subjects consisting of 274 patients with histologically proven IgAN and 100 without any history of renal disease were genotyped for the SA polymorphism. Table 1 lists the distributions of genotypes and allele frequencies of the SA polymorphism in both groups. No difference in the SA genotype or the allele distribution was observed between them. The frequency of the minor allele, A2, was 0.153 and 0.155 in the IgAN and control groups, respectively. The expected frequency of the genotypes in each group, under the assumption of the Hardy-Weinberg equilibrium, did not differ from the observed genotype frequencies.

Table 2 shows the clinical characteristics of subjects without any renal disease, and compares the clinical characteris-

Table 4. Logistic Regression Analysis for the Advanced Glomerulonephritis at the Time of Renal Biopsy in Patients with IgA Nephropathy

	χ^2	*p* value	Odds ratio	95% CI
Urinary protein excretion				
<1.0 g/day			referent	
>1.0 g/day	7.739	0.0054	2.299	1.279–4.132
Hypertention				
Normotension			referent	
Hypertension	16.757	<0.0001	3.367	1.883–6.022
SA genotype				
A1A2 or A2A2			referent	
A1A1	2.794	0.0946	1.795	0.904–3.571

Table 5. Cox Proportional Hazards Regression Model

Variable	*p* value	HR	95% CI
Urinary protein excretion>1 g/day	0.0002	2.996	1.680–5.342
Advanced GN at the time of diagnosis	<0.0001	2.959	1.811–4.808
Hypertension	0.0050	2.016	1.236–3.288
No ACE-I administration	0.0004	2.644	1.545–4.524

HR, hazard ratio; CI, confidence interval; GN, glomerulonephritis; ACE-I, angiotensin converting enzyme inhibitor. Advanced GN was defined by a serum creatinine level of greater than 1.2 mg/dl or 24 h creatinine clearance less than 70 ml/min.

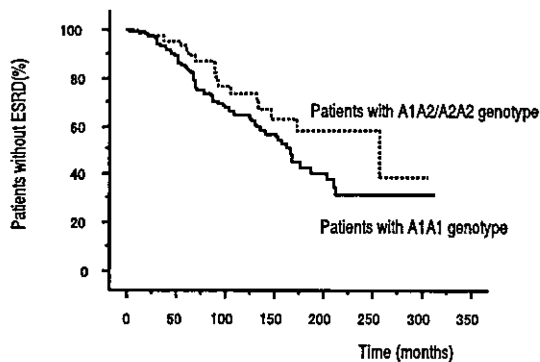

Fig. 1. *Renal survival in IgA nephropathy patients with the A1A2 or A2A2 (dashed line;* N=*77) or A1A1 genotypes (solid line;* N=*197) of SA polymorphism. Kaplan-Meier log rank test,* $\chi^2=3.173$, $p=0.0748$.

tics between the two genotype groups (A1A1 and A1A2/A2A2). The comparisons were made between a group of patients who were homozygous for the major allele, A1A1, and a group of patients with either of the other two genotypes (A1A2 and A2A2), since the number of subjects with the A2A2 genotype was too small to analyze separately. No difference between the two groups was observed for any of the clinical characteristics.

Table 3 shows the clinical manifestations of the patients with IgAN and the comparisons between the two genotype groups. There were no differences in age, gender, body mass index (BMI), or urinary protein excretion between the two groups. The time from the first urinary abnormality to renal biopsy was also not statistically different. However, the C_{cr} of patients with the A1A1 genotype was significantly lower than that observed with the other genotypes (A1A1 *vs.* A1A2/A2A2, 87.0±33.5 *vs.* 94.5±32.3, mean±SD, respectively; $p<0.05$). Moreover, in contrast to the non-renal disease population, the systolic and diastolic blood pressure both at the time of renal biopsy and during observation tended to be higher in patients with the A1A1 genotype. The incidence of hypertension at the time of renal biopsy was also significantly higher in patients with the A1A1 genotype (A1A1 *vs.* A1A2/A2A2, 41.4 *vs.* 26.3%, respectively; $p<0.05$). To investigate whether the SA gene polymorphism affected the renal function at the time of renal biopsy independently of blood pressure, logistic regression analysis was conducted (Table 4). After adjusting for the effect of urinary protein excretion and hypertension, the SA genotype was no longer associated with advanced GN, suggesting that the effect of the gene polymorphism was indirect, occurring *via* the blood pressure.

Among all patients with IgAN ($N=274$), 86 (31.4%) progressed to ESRD during the mean follow-up duration of 93.4±67.3 months. The incidence of ESRD was numerically higher in patients with the A1A1 genotype of SA polymorphism, although the difference was not statistically significant. The effect of the SA gene polymorphism on renal survival was investigated by the Kaplan-Meier method (Fig.

1). The renal survival curve tended to be worse in patient homozygous for A1 than for the other genotypes (log rank test, $\chi^2=3.173$, $p=0.0748$). The mean survival of renal function was 145.3±6.1 and 191.2±13.6 months in the patients with A1A1 and those with A1A2/A2A2, respectively.

Cox proportional hazards analysis was used to further test the significance of the genotype and other clinical covariates at the time of diagnosis as predictors of survival time (Table 5). In this analysis, the SA genotype was no longer a significant risk factor for progression to ESRD after adjustment for other prognostic factors (hazard ratio, 0.977; 95% CI, 0.554 to 1.724; $p=0.937$). The independent predictive risk factors for the development of ESRD in the IgAN patients were a proteinuria level of more than 1 g/day, advanced GN, hypertension, and no administration of ACE-I.

Discussion

The results of the present study indicated that the SA gene polymorphism may have an association with high blood pressure and thus indirectly lead to a deterioration in the renal function of IgAN. Although the effect of the SA polymorphism on renal survival was not statistically significant, a higher proportion of IgAN patients with the A1 homozygote were hypertensive and their C_{cr} was lower than those of patients with the other genotypes at the time of renal biopsy. Both of these clinical manifestations, hypertension and deteriorated renal function, were significant risk factors for the progression to ESRD. Osawa *et al.* have demonstrated that, even among IgAN patients with normal blood pressure, those with a blood pressure level of lower than 120 mmHg in systole and 80 mmHg in diastole show more mild renal histological damage and better renal function (*15*). Thus the SA genotype was likely to have indirectly affected the renal prognosis through its effect on the blood pressure.

Interestingly, this association between the gene polymorphism and blood pressure was not observed within a population without renal disease. To date, we have no data or knowledge available to explain why the association between the SA gene polymorphism and blood pressure was observed only within patients with IgAN. However, the negative result observed in the control group may be inconclusive, because the number of subjects without renal disease in this study was relatively small. Nonetheless, these results may suggest that the impact of SA gene polymorphism on blood pressure is not the same between patients with and without renal involvement and that patients with renal injury have an increased sensitivity to this gene polymorphism.

The SA gene was originally isolated by virtue of its increased expression in the kidney of spontaneously hypertensive rats as compared with the kidney of normotensive WKY rats, and it has been proposed as a candidate gene for essential hypertension (*11*). The SA gene is highly expressed in hypertensive kidneys, and its increased expression correlates with a rise of blood pressure in both SHR and Dahl rat models (*8, 10*). Moreover, congenic substitution mapping studies have suggested that the SA gene is a candidate gene locus for hypertension (*16*). Although the molecular mechanism in which the SA gene expression correlates with blood pressure remains to be elucidated, the subcellular distribution, and the structural and functional characteristics of the SA gene product have recently been demonstrated (*17*). The SA protein is localized in the mitochondrial matrix, where it functions as an enzyme and plays a role in the oxidative degradation of medium-chain fatty acids for the production of energy. Considering the high energy consumption needed for active transport by Na^+,K^+-ATPase in the proximal and distal renal tubules (*18*), an alteration in the expression or the function of the SA gene may have an influence on the renal handling of electrolytes in tubular cells. It may also have an influence on the regeneration process of renal tubular cells after renal injury, and thus have an effect on the renal function of patients with glomerulonephritis. Further study is necessary to clarify the precise mechanism of the association between the SA polymorphism and either renal function or blood pressure. In particular, an investigation into whether the SA gene polymorphism is associated with the expression of the gene products in the renal tubular cells would help us to understand this issue. Recently, Iwai *et al.* demonstrated in a large population study that A/G polymorphism in intron 12 of the SA gene was associated with blood pressure. They also showed that this A/G polymorphism was associated with the expression level of SA mRNA in peripheral mononuclear cells *in vivo* (*19*). The RFLP investigated in this study was located approximately 17 kb upstream from the A/G polymorphism investigated by Iwai *et al.* (*19*). Although we have no data available as to whether or not the RFLP is in linkage disequilibrium with the A/G polymorphism in intron 12, further investigation of this matter might help clarify the mechanism of the association observed in this study.

We chose patients with IgAN as the subjects of this investigation because they were the largest homogeneous population for whom accurate clinical data were available both at the time of renal biopsy and during the clinical course. However, it is likely that the effect of the SA genetic polymorphism on the clinical manifestations observed in this study is not specific to IgAN, but that it affects the clinical phenotypes in other types of glomerular diseases. Investigations of patients with diabetic nephropathy, hypertensive nephropathy, and minimal change nephrotic syndrome are now in progress.

In conclusion, the present study demonstrates for the first time that the SA gene polymorphism may have an impact on the clinical manifestations of patients with renal injury, such as IgAN, which was not observed in the patients without renal involvement. Insight into these issues is not only of theoretical interest, but may lead to new approaches for specific therapies to replace the current use of antihypertensive or immunosuppressive drugs, which have substantial adverse

effects. It is crucial to investigate the molecular mechanism by which the SA gene polymorphism specifically affects the blood pressure in patients with renal disease.

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Identification of genes regulating colorectal carcinogenesis by using the algorithm for diagnosing malignant state method

Group 1: Yasushi Ichikawa,[a,b,1] Takashi Ishikawa,[a,b] Shinji Takahashi,[a,b] Youhei Hamaguchi,[a,b] Tomoyuki Morita,[a,b] Itaru Nishizuka,[a,b] Shigeki Yamaguchi,[a] Itaru Endo,[a] Hideyuki Ike,[a] Shinji Togo,[a] Shigeo Oki,[a] and Hiroshi Shimada[a,*]

Group 2: Koji Kadota,[b,c,1] Shugo Nakamura,[c] Hitoshi Goto,[b,d] Hiroyuki Nitanda,[b,d] Susumu Satomi,[d] Takehito Sakai,[b,e] Ichiei Narita,[e] Fumitake Gejyo,[e] Yasuhiro Tomaru,[b,f] Kentaro Shimizu,[c] Yoshihide Hayashizaki,[b,f] and Yasushi Okazaki[b,*]

[a] *Second Department of Surgery, School of Medicine, Yokohama City University, Yokohama, Japan*
[b] *Laboratory for Genome Exploration Research Group, RIKEN Genomic Sciences Center (GSC), RIKEN Yokohama Institute, 1-7-22 Suechiro-cho, Tsurumi-ku, Yokohama-shi, Kanagawa-ken 230-0045, Japan*
[c] *Department of Biotechnology, The University of Tokyo, Tokyo, Japan*
[d] *Department of Advanced Surgical Science and Technology, Graduate School of Medicine, Tohoku University, Sendai, Japan*
[e] *Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Ibaraki, Japan*
[f] *Institute of Basic Medical Sciences and Biological Sciences, University of Tsukuba, Ibaraki, Japan*

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Abstract

We studied the expression profiles of various stages of colorectal tumors (adenoma (AD), seven samples; carcinoma (CA), 16 samples) by using cDNA microarrays and developed ADMS (algorithm for diagnosing malignant state) method, selecting 335 clones characteristic of CA state. We, then, applied ADMS to 12 additional samples (five from primary lesions with metastasis and seven metastases); all 16 CAs and 12 metastatic tumors were diagnosed correctly as cancerous states. Although three of the seven ADs were diagnosed as "cancerous," the large size of two of these tumors suggested their potential malignancy. Our strategy for selecting clones characteristic of the malignant state is widely applicable to diagnosis and for predicting the stage of progression during multistep carcinogenesis. Of the 335 clones we selected, 135 were known genes. Included in the 135 genes were tumor suppressor and growth factor-related genes and were consistent with the literature. ADMS is a reliable means for identifying genes useful for the diagnosis of cancer. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Gene expression profiling; Molecular classification; Colorectal cancer; Malignancy diagnosis; DNA microarray

Recently, microarray technology has been widely applied to clinical diagnosis, and the expression profiles characteristic of malignancy have been studied extensively. Although several methods have been reported for identifying genes typical of various pathologic states, arguments regarding the utility of the selected genes remain. Here, we have developed a method that is applicable to a variety of clinical samples. We evaluated the utility of our method by using clinically diagnosed colorectal adenomas and cancers.

Colorectal cancer is one of the best-known tumors as developing through multistep carcinogenesis such as adenoma (AD), adenocarcinoma (CA), and metastasis [1]. Although CA almost never reverts to AD, AD can become malignant as it develops. Furthermore, AD occupies a biological position between normal colon

* Correspondence may be addressed to either H.S. or Y.O.
[1] These two authors contributed equally to the work.

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