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Role of intron 1 in smooth muscle α-actin transcriptional regulation in activated mesangial cells *in vivo*

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Role of intron 1 in smooth muscle α-actin transcriptional regulation in activated mesangial cells *in vivo*.

Background. The activation of glomerular mesangial cells is one of the early, important features of progressive glomerular disease. Smooth muscle α-actin (SMαA) is an excellent marker of activated mesangial cells. However, the mechanisms of SMαA regulation are only available from *in vitro* investigation.

Methods. We examined *in vivo* promoter analysis of the SMαA gene-utilizing transgenic mice harboring different promoter regions of the SMαA gene fused to chloramphenicol acetyl transferase (CAT). CAT activities were tested in primary cultured mesangial cells and in glomerular legions of Habu venom glomerulonephritis.

Results. The DNA sequence −891 to +3828, which contains exon 1, intron 1, and the first 14 bp of exon 2 in addition to the 5′-flanking sequence of the SMαA gene, induced high levels of transcription in activated mesangial cells in *in vivo* habu venom glomerulonephritis and in cultured mesangial cells derived from transgenic mice. The DNA region −891 to −124 was a positive element in mesangial cells derived from transgenic mice. Deletions (3316 or 137 bp) in intron 1 reduced transcription to undetectable levels. The 137 bp sequence is highly conserved among several species, containing one CArG box element, which is one of the key motifs for transcriptional activation of contractile-related proteins. *In vitro* transfection analysis failed to demonstrate these positive effects of intron 1 and region −891 to −124.

Conclusions. In vivo promoter analysis of the SMαA gene provided new information about the transcriptional regulation of SMαA in activated mesangial cells. The DNA region −891 to −124 has a positive effect on SMαA transcription in cultured mesangial cells. The intron 1 region (+1088 to +1224) plays a pivotal role in SMαA transcription in activated mesangial cells *in vivo*. Further analysis of this conserved region in intron 1, including the CArG motif, will be of great value in understanding the molecular mechanisms of mesangial activation.

Key words: myofibroblast, Habu venom glomerulonephritis, phenotypic modulation, CarG element, progressive renal disease.

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Mesangial hypercellularity and matrix expansion are hallmarks of glomerular disease and play important roles in the progression of renal disease at late stages. There is increasing evidence that mesangial cells in glomerular disease are activated and change to a secretory phenotype [1–3]. Activated mesangial cells release inflammatory mediators, cytokines, and growth factors and are potent at producing extracellular matrix components. Understanding the nature and mechanism of mesangial cell activation may provide insight and novel therapeutic approaches toward progressive glomerular disease. Several molecular markers in activated mesangial cells are reported. Enhanced expression of several contractile-related proteins, such as caldesmon and SMemb, is one feature of activated mesangial cells *in vivo* in both humans and animals [4–7]; among these, smooth muscle α-actin (SMαA) is the most widely recognized marker of activated mesangial cells [8, 9].

Smooth muscle α-actin is one of six actin isoforms in mammals [10]. Under normal conditions, SMαA is expressed exclusively and is the dominant protein in vascular and visceral smooth muscle cells [11, 12]. Normal glomeruli express only minimal SMαA; however, with progression to various forms of glomerular disease, glomerular mesangial cells begin to express SMαA [8, 13–15]. Activated mesangial cells are also called myofibroblast-like cells because of their extracellular matrix production and expression of contractile-related proteins [9]. Understanding the transcriptional regulation of SMαA may provide an understanding of the phenotypic modulations of mesangial cells to myofibroblast-like cells.

Smooth muscle α-actin expression in smooth muscle cells and in activated mesangial cells has been studied by *in vitro* transient transfection analysis. These studies revealed the importance of the upstream region −125 to +1 for expression of SMαA in both smooth muscle cells and activated mesangial cells [16–21]. This region is called the "core promoter" and contains two CArG

box elements, which are designated as CArG #1 (−62) and CArG #2 (−112). Interaction of serum response factor (SRF) with these two CArG boxes is characterized as one of the most important mechanisms for transcriptional activation of SMαA. Recently, Hautmann, Madsen, and Owens described a novel transforming growth factor-β (TGF-β) control element, −42 in this region [22]. They also demonstrated that MHox, the murine homologue of the homeodomain containing protein Phox 1, can potentiate binding of SRF to CArG #2 [23, 24]. This new finding with regards to MHox, using *in vitro* transfection, enhances our understanding of the mechanism of cell-specific transcription of the SMαA gene. However, the problem with *in vitro* transfection analysis of the "core promoter" region is that the "core promoter" *per se* may not fully confer smooth muscle-specific transcriptional activation of SMαA. Transcriptional activities of a core promoter were reported in rat cultured skeletal myoblasts and in aortic endothelial cells that do not express intrinsic SMαA [20]. To overcome this problem, positive and negative *cis* elements in loci other than the core promoter region must be considered as regulating cell type-specific transcriptional activation of the SMαA gene [17, 20, 25–27]. We previously reported enhancer activity in intron 1 of the SMαA gene [18], leading us to test the importance of intron 1, in addition to the core promoter and its upstream region, in the transcriptional regulation of SMαA in the mesangial cell activation.

The goals of this study were to identify *cis*-acting DNA regions within the human SMαA promoter that regulate transcriptional activation of SMαA during *in vivo* activation of mesangial cells with glomerular disease. We used *in vivo* promoter analysis of SMαA in transgenic mice harboring different promoter regions of SMαA. We found that the regulation of SMαA transcription is different in cultured mesangial cells derived from transgenic mice compared with SMαA transcription in transiently transfected wild-type mesangial cells. This study provides new information about transcriptional regulation of SMαA in activated mesangial cells.

METHODS

Promoter chloramphenicol acetyl transferase expression plasmids

To identify regions that control the expression of the SMαA gene *in vivo*, transgenic vectors were constructed that encode the chloramphenicol acetyl transferase (CAT) reporter gene under transcriptional control of SMαA promoter fragments. The p891int-CAT reporter plasmid has a 4.7 kb fragment of the human SMαA gene, which contains the 5′ upstream region, exon 1, intron 1, and the first 14 bp of exon 2; this fragment contains −891 to +3828, followed by the CAT gene. The precise sequence of the SMαA gene has been reported [18]. The p123int-CAT, p891intΔBH-CAT, and p891intΔ0-CAT plasmids contain −123 to +3828 of SMαA, −891 to +3828 of SMαA with a 3.8 kb deletion in intron 1, and −891 to +3828 of SMαA with a 137 bp deletion in intron 1, respectively (Fig. 1). These were subcloned 5′ to the CAT reporter gene in a modified pBluescript II KS (Stratagene, La Jolla, CA, USA). p891-CAT and p123-CAT plasmids, which were used only for transient transfection analysis, contain −891 to +49 of SMαA and −123 to +49 of SMαA, respectively [18].

Transgenic mice

Transgenic mice harboring the varying lengths of the SMαA promoter regions, 891int-CAT, 123int-CAT, 891int ΔBH-CAT, and 891intΔ0-CAT, were generated on a mixed C57BL/6 × DBA/2 background according to standard techniques [28]. To identify transgenic founder mice, Southern blot analysis was performed with a radiolabeled CAT probe (250 bp EcoRI fragment) and high molecular weight DNA prepared from tail biopsies of each potential founder. The number of copies per cell was quantitated by comparing the hybridization signal intensity to standards corresponding to 1, 5, 25, and 125 copies per cell with an image analyzer (BAS 1500 MAC; Fuji-Rebio, Tokyo, Japan). Two independent founder lines were identified in 891int-CAT, 123int-CAT, and 891 intΔ0-CAT transgenic mice, and three lines were identified in 891intΔBH-CAT transgenic mice. The copy numbers of transgenes of SMαA promoter transgenic mice were 85, 120 (891int-CAT); 35, 90 (123int-CAT); 4, 8, and 12 (891intΔBH-CAT); and 35, 80 (891intΔ0-CAT).

Tissue preparation

For immunohistochemistry, mice were anesthetized with pentobarbital and were perfused *in situ* with cold phosphate-buffered saline to remove circulating blood cells. Tissue sections from adult mice were immersed in a cold 4% paraformaldehyde solution or 70% ethanol for 16 to 24 hours. After dehydration, tissues were embedded in paraffin or Tissue-Tec O.T.C. compound (Miles, Elkhart, IN, USA).

Cell culture

Mesangial cells were obtained by culturing glomeruli isolated from kidneys of both transgenic and wild-type BDF1 mice and were characterized as previously described [29]. Cells were maintained in RPMI 1640 supplemented with 17% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2, with medium changes every three to four days. The first medium change was performed seven days after explantation of glomeruli. Fourteen days after explantation, CAT assay, CAT mRNA reverse transcription-polymerase chain re-

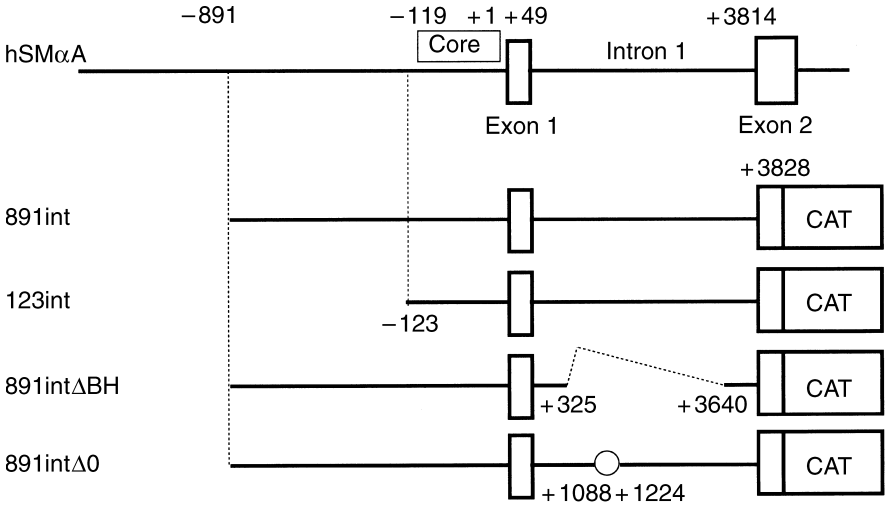

Fig. 1. Schematic representation of transgenes used in this study. Abbreviations are: hSMαA, human smooth muscle α-actin; CAT, chloramphenicol acetyl transferase.

action (RT-PCR) analysis, and CAT immunohistochemistry were performed to test for transcriptional activation of SMαA promoter fragments in cultured mesangial cells derived from transgenic mice.

Transient transfection analysis

For *in vitro* transient transfection analysis, mesangial cells at passages 5 to 7 from wild-type BDF1 mice were employed. In preliminary experiments, we used cultured mesangial cells of earlier passages (2 to 3) for transient transfection, but these cells did not survive as well as cells of passages 5 to 7. Cells were seeded for transfection assays into 6 cm dishes at a density of 4×10^4 cells/cm^2. These densities were chosen so that cells would be at 80% confluence at the time of transfection (48 hr after plating). Transient transfection experiments consisted of transfecting each promoter-CAT plasmid in triplicate using Lipofectamine Reagent (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer's recommendation. Briefly, for each well to be transfected, 3 μg of a reporter gene construct and 1 μg of a luciferase reporter plasmid [pGV-P2; in 20 μl of 10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA)] were incubated for 30 minutes in a tissue culture-sterile microcentrifuge tube with 20 μl of Lipofectamine Reagent and 600 μl of serum-free RPMI 1640. During the incubation, each well was washed twice with 3 ml of transfection medium to remove residual serum, and after the second wash, 2.4 ml of fresh transfection medium and 600 μl of DNA/Lipofectamine/media solutions were added to each well. At four-hours post-transfection, 3 ml of RPMI 1640 supplemented with 17% FCS were added to each well. At 24-hours post-transfection, media were changed. After 48 hours in culture medium, cells were harvested by scraping in buffer.

Immunohistochemical study

Smooth muscle α-actin and CAT proteins were identified with anti-SMαA monoclonal antibody (1A4; Sigma Co., St. Louis, MO, USA) and digoxigenated sheep anti-CAT polyclonal antibody (Boehringer Mannheim GmbH, Mannheim, Germany). The anti-SMαA antibody was biotinylated with a Biotin Protein Labeling Kit (Boehringer Mannheim). Cultured cells or cryosections (10 μm) obtained from tissues were fixed in 4% paraformaldehyde for 30 minutes and in ice-cold acetone for 30 minutes. All sections were incubated in a 99:1 methanol/H_2O_2 solution for 30 minutes at room temperature to inactivate endogenous peroxidase activity. Primary antibodies were then applied to the sections and incubated for 16 hours at 4°C. Sections for SMαA were then incubated with an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 40 minutes at room temperature. Peroxidase activity was visualized with a solution containing 0.6 mg/ml of p-dimethyl aminobenzaldehyde and 10 mg/ml of H_2O_2 in 50 mM Tris-HCl (pH 6.85) using DAB buffer tablets (Merck, Darmstadt, Germany). After counterstaining in methylgreen, sections were mounted.

Sections for CAT immunostaining were incubated with the second antibody (peroxidase-conjugated sheep antidigoxigenin polyclonal antibody; Boehringer Mannheim) for 16 hours at 4°C. After washing, TSA™-Indirect for Immunohistochemistry Renaissance™ (Dupont/New England Nuclear, Boston, MA, USA) was used to intensify CAT staining. Peroxidase activity was visualized as described earlier here, and counterstaining was performed. Negative controls used omission of the first antibody or substitution of normal mouse IgG for the anti-SMαA monoclonal antibody or normal sheep serum for anti-CAT polyclonal antibody.

Chloramphenicol acetyl transferase assay

Monolayers of mesangial cells were rinsed three times with ice-cold phosphate-buffered saline and were incubated for 15 minutes at room temperature with 400 μl PicaGene™ Cell Culture Lysis Reagent LCβ (Toyo Inki Co., Tokyo, Japan). Cells were then scraped off and collected by centrifugation for two minutes at 12,000 *g*. The supernatant was either assayed directly or stored frozen at −20°C. The assay for CAT activity in cell lysates was performed essentially as described [18] using [^{14}C]-chloramphenicol (57 mCi/mmol; Amersham Co., Buckinghamshire, UK) and equivalent amounts of cytoplasmic extract. Acetylated products were separated by thin-layer chromatography using MERCK Silica Gel 60 F254 and exposed to MAC BAS IIIs (Fuji). The percentage of conversion of [^{14}C]-chloramphenicol to its acetylated derivatives was calculated by BAS 1500 MAC (Fuji). Protein in cell lysates was measured by the Coomassie brilliant blue dye binding method with Bio-Rad protein assay reagent using bovine γ-globulin as a standard (BioRad, Hercules, CA, USA) [30]. In transient transfection analysis, to account for possible differences in transfection efficiency within the same experiment, cells were cotransfected with pGV-P2, which directs luciferase activity from the viral long-terminal repeat. Luciferase activity in cell lysates was measured with a PicaGene™ Luminescence Kit (Toyo Inki Co.).

Reverse transcription-polymerase chain reaction of chloramphenicol acetyl transferase mRNA

Total RNA of cultured mesangial cells was extracted by an acid guanidinium thiocyanate-phenol-chloroform extraction procedure using TRIzol reagent (GIBCO BRL) according to the manufacturer's instructions. Extracted RNA was dissolved in sterile water, and contaminating DNA was digested with DNase I (GIBCO BRL). Reverse transcription was performed using the GeneAmp RNA PCR Kit (Perkin-Elmer, Norwalk, CT, USA) using 2.5 μg of total RNA as template, and oligo(dT) as a primer (42°C × 60 min and 95°C × 7 min) [31]. The cDNA was amplified by PCR using "SMA exon 1" (5′-CTGTCAGGAATCCTGTGAAGC-3′) and "CAT 1" (5′-AGTTGTCCATATTGGCCACG-3′), which yielded a 572 bp amplified product. A set of primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense, 5′-TCCCTCAAGATTGTCAGCAA-3′; antisense, 5′-AGATTCACAACGGATACATT-3′; predicted product size, 309 bp) was used as a control (25 cycles, 95°C × one min, 59°C × one min, and 72°C × one min). Samples were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

Habu snake venom-induced glomerulonephritis

Glomerulonephritis (GN) was induced in six-week-old male transgenic mice. The left kidneys were removed after a dorsolateral incision under pentobarbital anesthesia. After a recovery period of 24 hours, lyophilized venom from the Habu pit viper, *Trimeresurus flavoviridis* (Wako Pure Chemical Industries, Osaka, Japan), was dissolved in saline and injected into a tail vein at a dose of 1.5 mg/kg body wt. Eight days after the infusion of habu venom, the right kidney was removed. Kidney sections were examined by immunohistochemistry.

RESULTS

Analysis of CAT transcription in transgenic mice *in vivo* under normal conditions

To determine the significant promoter regions for transcriptional regulation of SMαA *in vivo* in activated mesangial cells, varying lengths of the promoter region were ligated to a CAT reporter gene; these transgenes were used for construction of transgenic mice (Fig. 1). Figure 2 shows the representative appearance of CAT immunostaining in a normal glomerulus (Fig. 2 C, E, G, I) and abdominal aorta (Fig. 2 D, F, H, J) from transgenic mice. Endogenous SMαA expression was shown in kidney arterioles and the aortic media layer (Fig. 2 A, B). Expression of CAT in 891int-CAT (Fig. 2 C, D) and 123int-CAT (Fig. 2 E, F) transgenic mice showed localization of CAT protein identical to that of endogenous SMαA. By contrast, partial removal of either large (3316 bp; Fig. 2 G, H) or small (137 bp; Fig. 2 I, J) portions of intron 1 of the SMαA gene abolished tissue-specific expression of CAT. These results demonstrate that the region from −123 to +3828 (containing exon 1, intron 1, and the first 14 bp of exon 2) confers smooth muscle-specific transcriptional activation of SMαA *in vivo*. The 137 bp region in intron 1, especially from +1088 to +1224, was also demonstrated to play an important role in smooth muscle-specific expression of SMαA. No detectable CAT expression was observed in the glomeruli of normal kidneys from transgenic mice.

Analysis of loci that control SMαA transcription in cultured mesangial cells derived from transgenic mice

Although mesangial cells *in situ* do not express detectable amounts of SMαA, mesangial cells in culture express substantial amounts of SMαA [32]. Mesangial cells in culture are assumed to share the characteristics of *in vivo* activated mesangial cells, as seen in glomerular disease, including SMαA expression. Thus, cultured mesangial cells are considered a good model of activation [1, 9]. To investigate the effects of different promoter regions for induction of SMαA transcription in activated mesangial cells in culture, CAT immunostaining and enzymatic activities were tested in mesangial cells derived from transgenic mice harboring different SMαA promoter regions. Figure 3 shows immunostaining of CAT in primary cultured mesangial cells 14 days after the

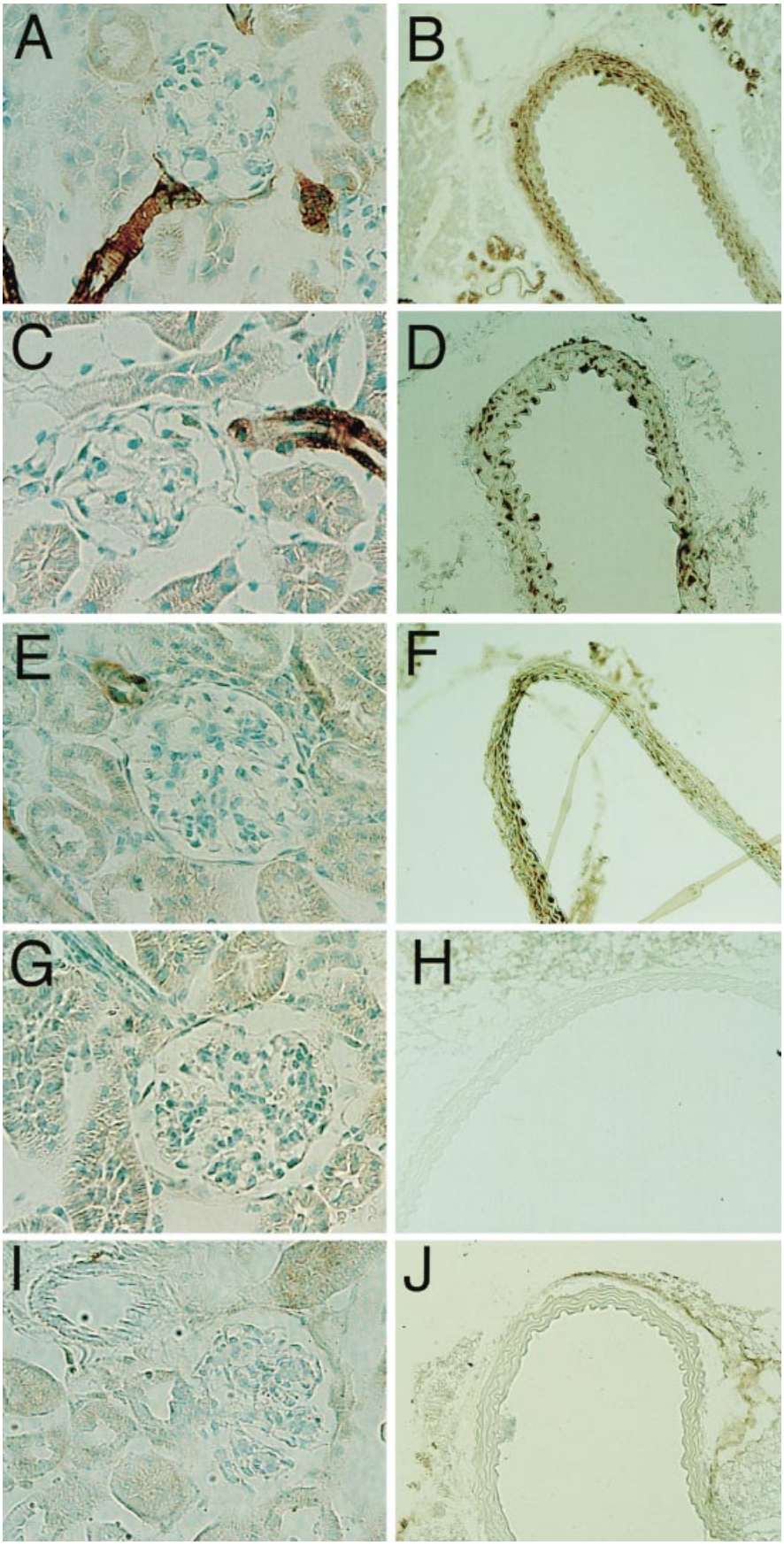

Fig. 2. Immunostaining for smooth muscle α-actin (SMαA) and CAT in normal kidneys (*A, C, E, G,* and *I*) and abdominal aorta (*B, D, F, H,* and *J*) from transgenic mice. Sections from 891int-CAT transgenic mice were used for SMαA and CAT staining in glomerulus (A and C) and in aorta (B and D). (A) SMαA staining in normal kidney of 891int-CAT transgenic mice. (C, E, G, I) CAT staining in normal kidney of 891int-CAT (C), 123int-CAT (E), 891intΔBH-CAT (G), and 891int Δ0-CAT (I) transgenic mice (original magnification ×400). (B) SMαA staining in aorta of 891int-CAT transgenic mice. (D, F, H, J) CAT staining in aorta of 891int-CAT (D), 123int-CAT (F), 891intΔBH-CAT (H), and 891int Δ0-CAT (J) transgenic mice (original magnification ×100).

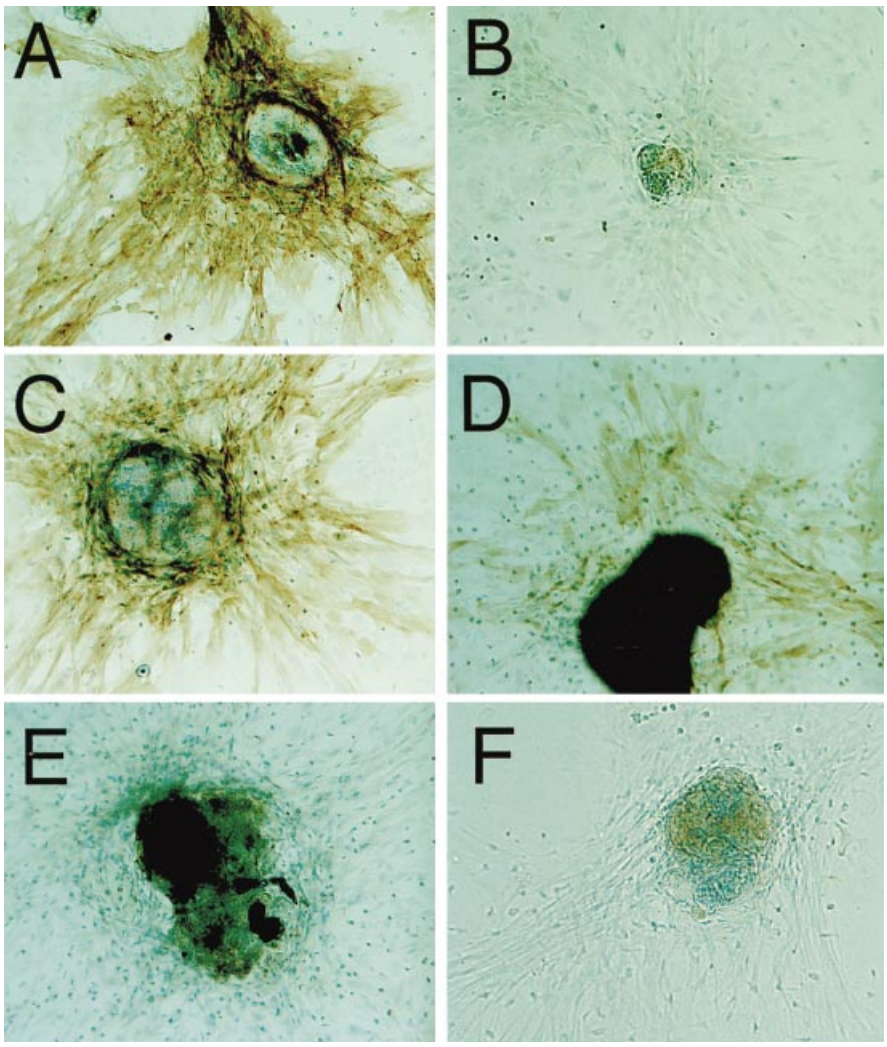

Fig. 3. Immunostaining for SMαA and CAT in cultured mesangial cells derived from transgenic mice. (*A*) SMαA staining in cultured mesangial cells from 891int-CAT transgenic mice. (*B–E*) CAT staining in cultured mesangial cells from wild-type BDF1 mice (B), 891int-CAT (C), 123int-CAT (D), 891intΔBH-CAT (E), and 891intΔ0-CAT (F) transgenic mice (original magnification ×100).

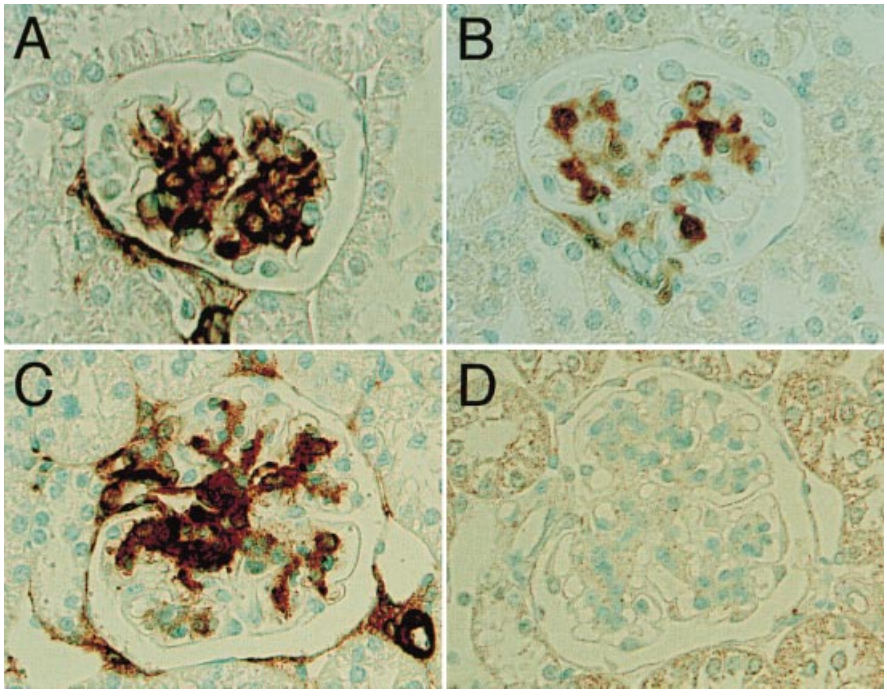

Fig. 7. Immunostaining for SMαA and CAT in the glomerular lesions of Habu venom–induced glomerulonephritis mice. SMαA (*A* and *C*) and CAT (*B* and *D*) were examined in the consecutive sections from the kidneys of 891int-CAT (A and B) and 891intΔ0-CAT (C and D) transgenic mice eight days after the intravenous injection of Habu venom (original magnification ×400).

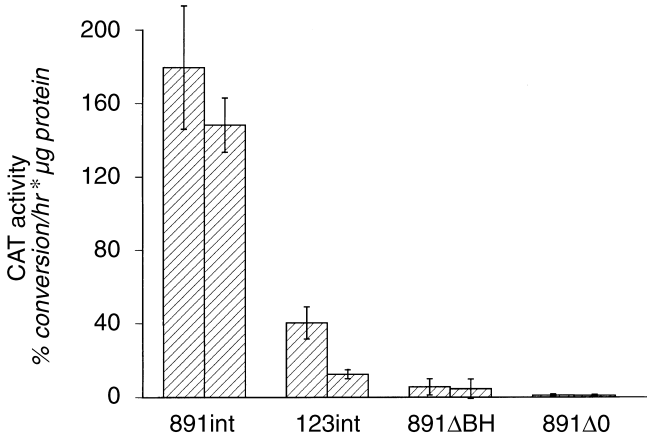

Fig. 4. CAT activities in mesangial cells derived from SMαA-promoter transgenic mice. Two independent lines of each strain were tested. Enzyme activities are shown in arbitrary units/mg protein. Values are expressed as mean ± SD from three different cultures.

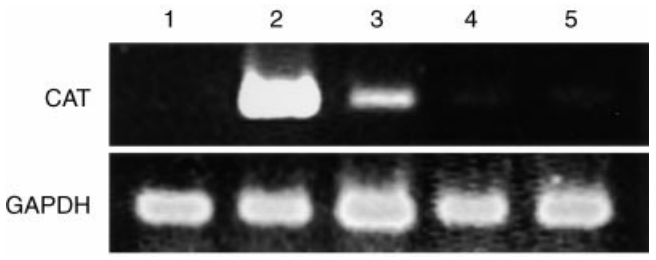

Fig. 5. RT-PCR detection of CAT (*top*) and GAPDH (*bottom*) in cultured mesangial cells derived from transgenic mice. RNA samples from wild-type BDF1 mice (lane 1), 891int-CAT (lane 2), 123int-CAT (lane 3), 891intΔBH-CAT (lane 4), and 891intΔ0-CAT (lane 5).

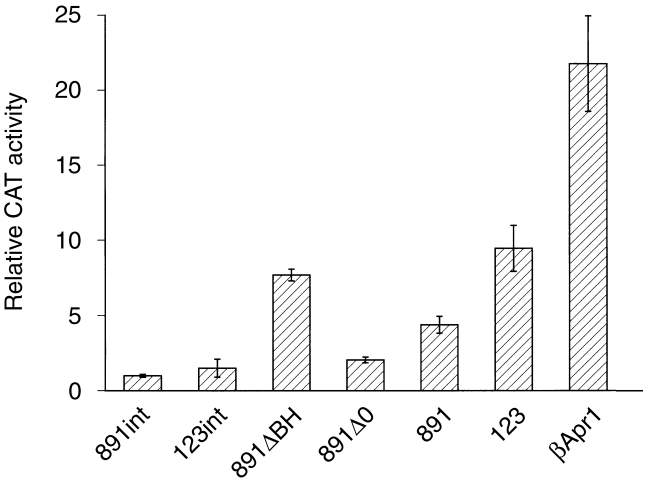

Fig. 6. *In vitro* transient transfection analysis of SMαA-promoter activities in mesangial cells. Assays were performed 48 hours after transfection. The variance in transfection efficiency was monitored and normalized by the luciferase activity from the cotransfected pGV-P2 expression vector. CAT activity was expressed relative to values obtained with the 891int-CAT.

explantation of isolated glomeruli. The glomerulus is placed in the middle of each photograph, and the outgrowth of SMαA-positive mesangial cells from a glomerulus is shown in Figure 3A. Mesangial cells obtained from wild-type BDF1 mice did not show positive staining for CAT, demonstrating the specificity of the antibody (Fig. 3B). Immunostaining demonstrated that mesangial cells derived from 891int-CAT and 123int-CAT strains were positive for CAT (Fig. 3 C, D), whereas mesangial cells derived from 891intΔBH-CAT or 891intΔ0-CAT strains did not show detectable levels of CAT protein (Fig. 3 E, F).

To analyze CAT expression quantitatively, enzymatic activities of CAT were measured (Fig. 4). Two lines of each strain were tested. Strong CAT activity was detected in 891int-CAT–derived mesangial cells, followed by 123int-CAT. There was a 4- to 10-fold difference in CAT activities between 891int-CAT– and 123int-CAT–derived mesangial cells. Marginal CAT activities were observed in mesangial cells derived from 891intΔBH-CAT or 891intΔ0-CAT. CAT mRNA in mesangial cells was confirmed by RT-PCR analysis (Fig. 5). The intensity of the PCR product was the strongest in 891int-CAT followed by 123int-CAT. Only trace levels were seen in mesangial cells from transgenic mice with a deletion in intron 1, which is consistent with the results of CAT activity. These results demonstrate that the region from −123 to +3828 is necessary for transcriptional activation of SMαA during mesangial cell activation *in vitro*. In addition, the 5′ upstream region from −891 to −124 was shown to promote SMαA transcription in cultured mesangial cells derived from transgenic mice. The region in intron 1 from +1088 to +1224 is required for efficient activation of the SMαA promoter in cultured mesangial cells derived from transgenic mice.

Analysis of transcriptional activity of different promoter regions in a transient transfection assay in cultured mesangial cells derived from wild-type BDF1 mice

The pivotal role of intron 1 for SMαA transcription in activated mesangial cells was demonstrated in transgenic mice. There have been no reports of transcriptional activity of promoter regions containing intron 1 in mesangial cells by *in vitro* transient transfection analysis. We examined promoter activities of various SMαA promoter-CAT fusion genes in transgenic mice by a transient transfection assay in mesangial cells obtained from wild-type BDF1 mice (Fig. 6). The highest transcriptional activity was observed in −123 to +1, the core promoter region. There was an increase in CAT activity when the 5′ upstream region adjacent to the core promoter (−891 to −124) was deleted with or without intron 1 in the promoter region. Thus, the region from −891 to −124 is

likely to have a negative effect on SMαA transcription by *in vitro* transfection analysis. These results agree with previous reports by us and others [16–21]. CAT activities driven by the promoter region of 891intΔBH or 891intΔ0, both of which had little transcriptional activity in transgenic mice, were the same or higher than for 891int or 123int, a sharp contrast to the results in transgenic mice. These results suggest a negative role for intron 1 in transient transfection.

Analysis of *in vivo* transcriptional activation of the SMαA promoter in activated mesangial cells in experimental proliferative glomerulonephritis

To test transcriptional activation of the SMαA promoter in activated mesangial cells *in vivo*, habu snake venom-induced GN was induced in transgenic mice. Two strains of transgenic mice, 891int-CAT and 891intΔ0-CAT, were chosen for *in vivo* analysis, based on the previously mentioned results of promoter activation. Eight days after infusion of habu venom, kidneys were analyzed by morphologic examination and immunohistochemistry for SMαA and CAT. Glomerular lesions were assessed by mesangial hypercellularity and matrix expansion on hematoxylin eosin-stained sections (data not shown). Proliferative GN was observed in both strains of transgenic mice (891int-CAT and 891intΔ0-CAT), and intense immunostaining for SMαA was demonstrated (Fig. 7 A, C). Intraglomerular CAT immunostaining in 891int-CAT showed a similar distribution to that of SMαA (Fig. 7B), indicating that the promoter region from −891 to +3814 confers transcriptional activation of SMαA in *in vivo* mesangial cell activation. Deletion of the 137 bp region in intron 1 from the 891int-CAT resulted in a reduction of CAT expression to undetectable levels (Fig. 7D).

DISCUSSION

Smooth muscle α-actin is an excellent marker of activated mesangial cells. This study provides new information about transcriptional regulation of SMαA. Our results highlight the difference in transcriptional regulation of SMαA that occurs between transiently transfected cultured mesangial cells and mesangial cells derived from transgenic mice. Transcriptional activation of the reporter gene CAT was measured in cultured mesangial cells from transgenic mice and in experimental mesangial proliferative GN. These results suggest a complex regulatory mechanism for transcription of SMαA in activated mesangial cells. Novel findings are as follows: (*a*) The region from −891 to +3828 (containing exon 1, intron 1, and the first 14 bp of exon 2) causes transcriptional activation of SMαA in both cultured mesangial cells from transgenic mice and *in vivo* activated mesangial cells from experimental GN. (*b*) Intron 1, especially the locus at +1088 to +1224, is essential for high transcriptional activation of SMαA in both cultured mesangial cells derived from transgenic mice and *in vivo*-activated mesangial cells, and (*c*) the region from −891 to −124 fosters transcriptional activation of the SMαA promoter in cultured mesangial cells from transgenic mice.

The most striking observation of this study is that the SMαA gene intron 1 plays a significant role in transcriptional activation of SMαA in activated mesangial cells. We found a dramatic decrease in CAT activities in 891intΔBH-CAT transgenic mice in both smooth muscle cells and activated mesangial cells compared with the high CAT activities in 891int-CAT transgenic mice. However, the copy numbers of ΔBH strains were lower than 891int strains, and it was possible that the difference of copy numbers affected the results. In intron 1 of the SMαA gene, there is one region where the sequence is highly conserved among species [18]. To investigate the importance of this locus, we deleted a small portion (+1088 to +1224) spanning this well-conserved region (Fig. 8) and produced transgenic mice (891intΔ0-CAT). As expected, activities of CAT were undetectable in both *in vivo* smooth muscle cells and activated mesangial cells of this strain. The results obtained from 891Δ0 strain with regard to the importance of intron 1 (+1088 to +1224) are thought to overcome the weakness of lower copy numbers of 891ΔBH, as mentioned earlier here. The region (+1088 to +1224) contains the CArG motif (+1098), named as CArG #0. The CArG motifs, characterized by the consensus sequence $CC(A/T\text{-rich})_6GG$, were first identified in the upstream regions of immediate-early genes and work as a binding site for SRF [33]. The CArG motif is also present in the upstream regions of skeletal, cardiac, and smooth muscle-specific genes [34]. In cooperation with other muscle-specific *cis* elements, the CArG motif contributes to muscle-specific transcriptional activation of these genes. Enhanced expression of contractile-related proteins is a characteristic of activated mesangial cells. These facts suggest an important role for CArG in the phenotypic modulation of mesangial cells. Indeed, in the transcriptional regulation of SMαA in cultured mesangial cells, the importance of two CArG motifs, CArG #1 (−62) and CArG #2 (−112), has been described by *in vitro* promoter analysis [21]. Some CArG motifs are binding loci for SRF; however, SRF is a ubiquitously expressed transcription factor, and it has been suggested that SRF alone is not sufficient to explain the functional diversity of CArG motifs. Accumulated evidence suggests that the multiplicity of CArG-dependent gene regulation is produced by post-translational modification of SRF, by interaction of SRF with SRF-binding proteins, and by a combination with other *trans*-factors that bind *cis* elements around the CArG motif. Understanding CArG-dependent transcriptional activation of SMαA in activated mesangial cells may

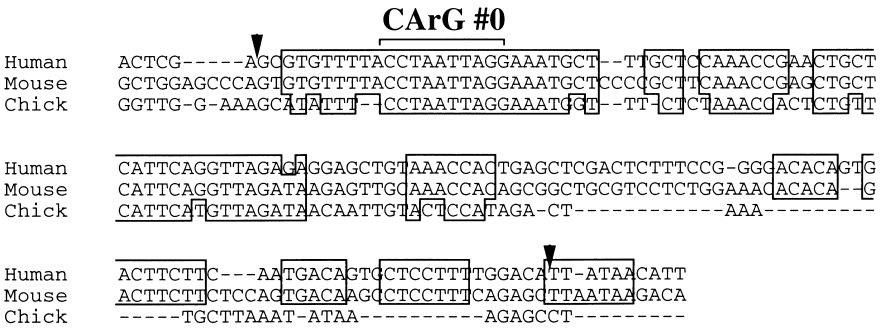

Fig. 8. Sequence comparison of SMαA gene intron 1 of human, mouse, and chicken. Sequences conserved between at least two of the depicted species are boxed. CArG #0 is 100% conserved in human, mouse, and chicken. Arrows indicate the region of the 137 bp deletion in 891intΔ0-CAT transgene.

uncover the molecular mechanisms for phenotypic modulation of mesangial cells. The functions of the "core promoter" region are reported, but little is known about the functional role of the CArG motif in intron 1. Future investigation of intron 1, especially the region around CArG #0, may provide a better understanding of the molecular mechanisms of transcriptional activation of SMαA in activated mesangial cells and their role in glomerular disease.

Another important observation is that the SMαA gene from −891 to −124 contains positive elements for transcriptional activation of SMαA in activated mesangial cells derived from transgenic mice. Because of the negative effects of this region on SMαA transcription, as demonstrated by *in vitro* transient transfection studies [20, 21], little attention has been paid to the regulatory role of this region in the transcriptional activation of SMαA in activated mesangial cells. However, this region contains several interesting elements. One M-CAT–like element is located at −178 [26]. Mar and Ordahl revealed that TGF-β–mediated activation of the skeletal α-actin gene was dependent on an M-CAT site, which binds members of the transcriptional enhancer factor-1 family [35]. TGF-β promotes phenotypic modulation of cells to myofibroblasts in a variety of tissues [36], and TGF-β is induced in glomeruli in *in vivo* GN [37]. Inhibition of the TGF-β action has beneficial effects on glomerulosclerosis and phenotypic modulation of mesangial cells [38, 39]. In addition, Hautmann, Madsen, and Owens recently found a TGF-β control element in the SMαA core promoter region at −42 [22]. These data support a significant role for TGF-β in the phenotypic modulation of mesangial cells in the pathogenesis of GN.

Another interesting element in this region is a p53-responsive element located at −312 to −291 [25]. p53 is an important tumor suppressor; alterations in p53 are common in human tumors [40]. The functions of p53 are to arrest cell cycle progression and induction of cell death. Enhanced glomerular expression of p53, especially in proliferative lesions, has been reported in human glomerular diseases such as IgA nephropathy, focal glomerular sclerosis, and lupus nephritis [41, 42]. Further investigation of the role of p53 in activated mesangial cells may contribute to our understanding of GN. In regions −257 to −189 and −151 to −123, smooth muscle-specific positive elements may exist [20, 27].

The mechanism of differential regulation of the SMαA promoter between transient transfection using wild-type mesangial cells and mesangial cells derived from transgenic mice is not clear. We attempted to determine whether differences in cell passage influenced the transcriptional activities of the SMαA promoter in mesangial cells derived from transgenic mice. However, there were no substantial changes in CAT activities through seven passages (data not shown). Another possible reason for differential regulation is the status of transfection, stable versus transient. Of note, in the previous study, we showed that the sequence (+972/+1152) in intron 1 has an enhancer activity under the artificial condition when this sequence was fused to SV40 promoter [18]. In this study, we studied the effect of the authentic intron 1 sequence for the first time and found that the region (+1088/+1224) plays a significant role for the *in vivo* study. The positive effect of intron 1 was not detected in *in vitro* study. The result of previous *in vitro* studies seems opposite to present *in vitro* study. However, as discussed in a previous study, we are considering that in natural chromatin structure, the intron 1 might be located in close proximity to the promoter region and might work as an enhancer. In *in vitro* study, transgene cannot integrate into genome or form chromatin structure, and, thus, the status of transgene, in chromatin or not, may influence the differential result of *in vivo* and *in vitro.* Although more precise investigations are necessary, this study suggests the limitations of *in vitro* transient transfection analysis.

In conclusion, *in vivo* promoter analysis of the SMαA gene provides new information about the transcriptional regulation of SMαA in activated mesangial cells. The DNA region −891 to −124 has a positive effect on SMαA transcription in cultured mesangial cells. The intron 1 region, including the CArG motif, plays a pivotal role in SMαA transcription in activated mesangial cells *in vivo.* Further analysis of this conserved region in intron

1 will be of great value in understanding the molecular mechanisms of mesangial activation.

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