[CANCER RESEARCH 59, 849–855, February 15, 1999]

Isolation and Characterization of a Rat Homologue of the Human Tuberous Sclerosis 1 Gene (*Tsc1*) and Analysis of Its Mutations in Rat Renal Carcinomas [1]

Nobuo Satake, Toshiyuki Kobayashi, Etsuko Kobayashi, Keisuke Izumi, and Okio Hino[2]

Department of Experimental Pathology, Cancer Institute, Tokyo 170-8455 [N. S., T. K., E. K., O. H.], and Second Department of Pathology, School of Medicine, The University of Tokushima, Tokushima 770-8503 [N. S., K. I.], Japan

ABSTRACT

In the Eker rat, a germ-line mutation in the homologue of the human tuberous sclerosis gene (*Tsc2*) causes renal cell carcinomas (RCs) with a complete penetrance in all heterozygotes. *Tsc2* mutations have also been found in a subset of chemically induced non-Eker rat RCs. Because tuberous sclerosis patients with alteration of either of the two predisposing genes (*TSC1* and *TSC2*) show identical symptoms, the products of these two genes are thought to be involved in a common biological pathway. In this study, to analyze the possible overlap between the functions of Tsc2 and Tsc1 gene products, we isolated and characterized a rat homologue of the *TSC1* gene (*Tsc1*). The rat *Tsc1* gene, which has an identical exon-intron structure to that of human *TSC1* and is localized on rat chromosome 3, has been shown to encode a protein (hamartin) that is highly homologous to the human counterpart with an ~86% amino acid sequence identity. Using PCR-single-strand conformational polymorphism analysis, we identified two splicing donor site mutations in one chemically induced rat RC (1 of 15). This suggests that alterations of the *Tsc1* gene may be involved in the development of a subset of rat RCs.

INTRODUCTION

Renal carcinogenesis in the Eker rat is an excellent animal model of dominantly inherited cancer (1). We and others have identified a germ-line mutation in the rat homologue of the human *TSC2* gene as a tumor-predisposing factor in the Eker rat (2–4). All rats heterozygous for *Tsc2* mutation develop RCs[3] by the age of 1 year (5). LOH and intragenic *Tsc2* gene somatic mutations are characteristic of Eker rat RCs, indicating that a second hit of the *Tsc2* gene is a cause of RC development (6, 7). The introduction of the *Tsc2* gene into *Tsc2*-deficient cell lines suppresses their growth and tumorigenicity (8, 9), clearly supporting a tumor suppressor function. We have constructed transgenic Eker rats with a wild-type *Tsc2* gene and ascertained that germ-line suppression of the Eker phenotype is responsible for both embryonic lethality in homozygotes and tumor predisposition in heterozygotes, and we have finally confirmed that tumors in the Eker rat are caused by the *Tsc2* germ-line mutations (10). We have also identified *Tsc2* mutations in chemically induced rat RCs, suggesting a more general involvement of *Tsc2* alterations in RC development in rats (11).

Tsc2 encodes tuberin, a ~200-kDa protein that contains a Rap1-GAP homology region near its COOH terminus (12). Recently, Rap1-GAP, Rab5-GAP, and transcriptional activator activities, as well as other possible functions of tuberin, were reported (13–16). However, the precise *in vivo* function of tuberin and the molecular mechanisms of tumor development associated with *Tsc2* mutations have not been fully elucidated.

TSC is an autosomal dominantly inherited disease characterized by hamartomatous benign tumors in multiple organs such as the brain, kidney, and heart (17). Approximately half of TSC patients are sporadic cases without any familial history (18). In addition to the *TSC2* gene localized on chromosome 16p13.3, another TSC-predisposing gene (*TSC1*) was recently identified on chromosome 9q34 (19, 20). *TSC1* encodes hamartin, which contains a predicted coiled-coil region and one potential transmembrane region, with a calculated molecular mass of ~130 kDa (20). The symptomatic similarity among human TSC patients associated with either *TSC1* or *TSC2* mutation suggests that hamartin and tuberin may be involved in a common biochemical pathway *in vivo* (19, 21). Indeed, direct interactions between tuberin and hamartin have been reported (21).

Because of the highly conserved nature of the *Tsc2* gene structure in vertebrates, the function and possible interaction of tuberin with hamartin may be conserved. Thus, we anticipated that studies of rat hamartin might provide some clues for elucidation of the molecular mechanisms of renal carcinogenesis in the Eker rat model. Therefore, we cloned and structurally characterized the rat *Tsc1* gene. Moreover, we identified somatic *Tsc1* mutations in one chemically induced rat RC.

MATERIALS AND METHODS

Tumor and Normal Tissue Samples. Three RCs induced by EHEN in F344 rats, four RCs induced by DEN in the F_2 progeny of a LEC/F344 cross, and one RC induced by DEN in the F_2 progeny of a LEC/WKAH cross were selected as described in our previous report (11). Seven RCs induced by EHEN in the F_1 progeny of a LEC/F344 cross were also analyzed. For this induction, 500 ppm of EHEN in drinking water was given for 2 weeks from 4 weeks of age, for 1 week from 9 weeks of age, and for 1 week from 12 weeks of age. The animals were sacrificed between 36 and 44 weeks of age, and tumor and normal tissue samples were taken and stored at −80°C until analysis.

DNA and RNA Isolation and Southern Blot and Northern Blot Analyses. DNAs were isolated from tumor samples by proteinase K digestion as described previously (22). Total RNAs were prepared with Isogen reagent (Nippon gene) according to the manufacturer's instructions. Southern and Northern blot analyses were performed as described previously (3). The membrane filters for hybrid cell panel analysis were kindly provided by Dr. G. Levan (University of Goteborg, Goteborg, Sweden; Refs. 23–25).

RT-PCR. For the initial isolation of partial rat *Tsc1* cDNA, first-strand cDNA was synthesized from 5 μg of rat (Long Evans strain) testis total RNA by RT using Superscript II (Life Technologies) and random primers in 20 μl of reaction mixture. A 1-μl aliquot of RT solution was used for PCR in 25 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1 mM $MgCl_2$, 200 mM deoxynucleotide triphosphates, 2 units of Taq DNA polymerase (Toyobo), and 100 pmol each of forward primer HTSF2 (5′-CTGGATCCCACAGAAGCCT-3′) and reverse primer HTSR2 (5′-CAGTCGACAGACTTGCTGG-3′). Temperature conditions were 92°C for 3

Received 9/21/98; accepted 12/18/98.

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[1] Supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research.

[2] To whom requests for reprints should be addressed, at the Department of Experimental Pathology, Cancer Institute, 1-37-1 Kami-ikebukuro, Toshima-ku, Tokyo 170-8455, Japan. Phone: 81-3-5394-3815; Fax: 81-3-5394-3815; E-mail: ohino@ims.u-tokyo.ac.jp.

[3] The abbreviations used are: RC, renal cell carcinoma; EHEN, *N*-ethyl-*N*-hydroxyethyl-nitrosamine; DEN, diethylnitrosamine; TSC, tuberous sclerosis; SSCP, single-strand conformational polymorphism; LOH, loss of heterozygosity; GAP, GTPase-activating protein; RT, reverse transcription; 5′-RACE, rapid amplification of the 5′ ends of cDNA; nt, nucleotide(s); aa, amino acid(s).

Table 1 *The oligonucleotide primer sets, $MgCl_2$ concentration, and annealing temperature for rat Tsc1 PCR-SSCP analysis*

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)	Annealing temperature (°C)	$MgCl_2$ concentration (mM)
3	GTCATTTGTCACACTGTTCA	GCTCCAGGTCAAGAGAGAAC	256	55	1.0
4	GACACCCATTGAGGGAAAAG	GATATACGGACTGCCATGCT	194	55	0.75
5	GTAAACTTCATATGCTTCCC	TTTACAGAGGTATTTCCCTC	246	55	1.25
6	CTGAGCCTGACGTCGCCCTT	CTCTTGAGAAGCCTGATGAG	212	55	0.75
7	GAGCCGACTGTCGCTGGTTT	CAATCAATGGGCAGGGTTGT	219	55	0.75
8	GCCACACAACTTCACATCTA	CGGTATCCATAGATCACATT	190	55	1.0
9.1[a]	CTGGTGTGCTTAGATGGATT	CAGAGAGCTGGTGAGACACA	166	55	0.75
9.2[a]	GACCCTACCGAAGCCTCATA	CAAAGCGGAGGCAAACTCTG	211[b]	55	0.75
10	TACACTCACCCCTGCCTCCT	GTGGAGAAGGGATGCTGAGT	181	55	0.5
11	GCTCCCTTGCTGATTTCTGA	ATCAAGTCCCACCTAAGACC	174	55	1.0
12	GGCTCAGCCTTTCTATGTAT	GGTGTACTGGCATCTCAGGC	186	55	0.75
13	CCAGCGTTTTCACTGTCTTC	CTAGAGTTCAACGCGAAGAG	152	55	1.0
14	GTTAACCAGTATGGCCTGAG	ACACTTCATCACCTTGTAGC	192	55	1.0
15.1[a]	GTTTACAGGGTTCCCTTGTT	GGGAGTAAAGGCTTGCTTTG	262	55	1.0
15.2[a]	CACTCCTCCCTGGACAAACA	GACAAAGTGACAGGCAGTCT	252	55	0.75
15.3[a]	GATCATCTCTTTGAGGTGGC	ACAGTTAAAGGCAACAGCAC	217	55	0.75
16	GATGATCAGTAACTTCCTGT	GTGAGTGGGTCTGGATGTGA	117	55	0.75
17	GCCTATCTGCCACCTTCCCT	CACAGAGCAGTGGATGGGTG	242	60	0.5
18	GCCCATGTTGGGTAACAGCTAAA	GACACGGCTGACACCATGCCTTC	283	55	0.5
19	CTGTGCTCTGGTAACCGCTC	CAGACGTACAAGAAGTAATG	209	55	1.0
20	GCTGACCTTGCACTTTCTCA	AGTCTGTGTCTCTCAGTGCC	189	55	0.75
21	GCACTTGTCTGAAAGTACTG	CACTCACACCCAGACCTTTC	258	55	1.0
22	CTTCCGTATTCTGAACCACG	CAGAGTCCACCTTCCTTTCG	240	55	1.0
23.1[a]	GGTTACCGACTGCATTCTTC	CTGGAGTGGAAAGCTCACTG	206	55	1.0
23.2[a]	GCTGTGGAGGTAGAGTCACT	CAGAAAAGCTACTCATGGTC	252	55	1.0
23.3[a]	CCGTAATAAGAGTGAGAGCC	CTGTTCTGTGCCGACAAAGA	260	55	1.0

[a] Exon 9 was examined with two primer sets, exon 15 was examined with three primer sets, and exon 23 was examined with three primer sets.
[b] The product size of exon 9 latter half is for LEK, WKAH, BN, and F344 rats.

min for initial denaturation; 92°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min for the amplification (35 cycles); and 72°C for 3 min for the final extension (protocol I). Similarly, the 5′ part of the human *TSC1* cDNA was obtained by RT-PCR using forward primer HTSF1 (5′-GACCATGGCCCAACAAGCAA-3′), reverse primer HTR1 (5′-GGTGAGACACAGAATAGCCA-3′), and total RNA from the G401 cell line. To obtain the full coding region of rat *Tsc1* cDNA, first-strand cDNA was synthesized from rat (Long Evans) kidney total RNA using random primers, and PCR was performed using an Expand High Fidelity PCR kit (Boehringer Mannheim) according to manufacturer's instructions. Temperature conditions for PCR were 92°C for 3 min, 55°C for 1 min, and 72°C for 1.5 min for the initial polymerization; 92°C for 1 min, 62°C for 1 min, and 72°C for 1.5 min for the amplification (35 cycles); and 72°C for 3 min for the final extension (protocol II). The primers used were as follows: primers TSS1 (forward primer; 5′-ATCTAAAGAGCTTTCTGAGATCACCACT-3′) and TSA1 (reverse primer; 5′-GAGGTACCTCAGCTGTGTTCGTGATGAG-3′) were used for the 3′ part of the coding region; and primers TSS2 (forward primer; 5′-TGGAATTCACCATGGCCCAGCTAGCCAACA-3′) and TSA2 (reverse primer; 5′-AATGTCTTCCACCTTCGAGGGTCCAGTTCA-3′) were used for the 3′ part of the coding region. In each case, amplified products were subcloned into pBluescript II SK(+) (Stratagene) for sequencing and further analysis.

Screening of Cosmid and Phage Libraries. Rat (Wistar strain) cosmid and phage (Stratagene) genomic DNA libraries and a rat (Long Evans strain) kidney cDNA library (12) were screened with ^{32}P-labeled rat *Tsc1* and human *TSC1* cDNA fragments obtained by RT-PCR. Positive clones were analyzed by restriction enzyme digestion and Southern blot analysis using cDNA probes [RT-PCR fragments and human *TSC1* cDNA clone HA4782 (kindly provided by Dr. T. Nagase, Kazusa DNA Research Institute, Kisarazu, Japan; Ref. 26)]. For sequence analysis, various restriction fragments from cosmid and phage clones were subcloned into pBluescript II SK(+).

The 5′-RACE. The 5′-RACE based on inverse PCR was performed according to the method described by Maruyama *et al.* (27) with some modifications. Rat brain total RNA (3 μg) was used as a template for the RT reaction using the antisense primer TSRT6 (5′-AGGTGCTTATCATGTGGCTC-3′). After the hydrolysis of RNA with 0.5 N NaOH, cDNAs were precipitated and circularized in 40 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 20% polyethylene glycol 6000, and 50 units of T4 RNA ligase (Takara) at 16°C. Ligation mixture (0.3 μl) was subjected to PCR using primers TSRT1 (reverse primer; 5′-GGGACTCCTTGAAGATGGTT-3′) and TSRT3 (forward primer; 5′-GTGGGCCTATGCTTGTAAAC-3′). Temperature conditions for PCR were the same as those for protocol I. A 1-μl aliquot of the first PCR solution was used for the second PCR using primers TSRT4 (reverse primer; 5′-GCGAATTCGAGAGTAGCTCCCCAATGTT-3′) and TSRT5 (forward primer; 5′-ATCTCGAGGTTGGTGGATTATTACCTGGA-3′). Amplified products were subcloned into pBluescript II SK(+) after digestion with *Eco*RI and *Xho*I for sequence analysis.

Genomic PCR Analysis for the Determination of Exon-Intron Structures. Intron lengths were partially determined by PCR analysis using genomic DNA or cosmid DNA as templates and the Expand Long Template PCR System (Boehringer Mannheim) according to the manufacturer's instructions. For the amplification of introns between coding exons, primers for SSCP

Table 2 *Polymorphism detected by PCR-SSCP analysis in Tsc1 locus*

A. Exon	Position	Polymorphism LEC/LEK WKAH BN F344
3	26 bp before the start of the codon initiator	C→G
9	14 bp after the donor site of intron 9	20 nt deletion 5′-CTTTGGTCTCCTGTGTTTGCAACTTCAT-3′
	Codon 287	CAC(His)→CAG(Gln)
14	10 bp before the acceptor site of intron 13	G→C
15	4 bp before the acceptor site of intron 14	C→T
16	Codon 674	GTT(Val)→CTT(Leu)
23	Codon 1027	CGG(Arg)→TGG(Trp)
	Codon 1106	ATG(Met)→GTG(Val)
B. Exon	Position	Polymorphism LEK/WKAH BN
20	Codon 852	CTC(Leu)→CTT(Leu)

analysis were used. For introns between 5′ leader exons and exon 3, the following primers were used: (*a*) TS5U1, 5′-CAATTATGCTGTGTGGGCTC-3′ (reverse primer, on exon 3); (*b*) TS5U2, 5′-TGACCATGGAAGACACAAGG-3′ (forward primer, on exon 2); (*c*) TS5U3, 5′-GACGGTACATCAGTTTCCAG-3′ (reverse primer, on exon 2); and (*d*) TS5U, 5′-AGGGACTGTGAGGTAAACAG-3′ (forward primer, on exon 1). All amplified products were sequenced with the same primers for PCR, and exon-intron boundaries were confirmed.

PCR-SSCP Analysis. Details of the oligonucleotide primer sets, sizes of the PCR products, $MgCl_2$ concentrations, and PCR annealing temperatures for the *Tsc1* gene are summarized in Table 1. The primer sets for the *Tsc2* and *Vhl* genes were described previously (28, 29). PCR was carried out in a 10-μl volume including 50 ng of template genomic DNA, 1 mM each of the primers, 100 mM deoxynucleotide triphosphate, 0.5–1.25 mM $MgCl_2$, 2.5 μCi of [α-^{32}P]dCTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, and 2 units of Taq DNA polymerase (Toyobo). Temperature conditions for PCR were the same as those for protocol I (RT-PCR analysis). After PCR, samples were diluted with the loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue) and boiled for 5 min. A 1.5-μl aliquot of each diluted sample was run on a 6% polyacrylamide/10% glycerol gel at 4°C in 0.5× Tris-borate-EDTA buffer at 1400 V. Then gels were dried and autoradiographed with X-ray film. For sequencing, bands were dissected from the gels and extracted in 100 μl of distilled water, and fragments were reamplified by PCR with the same primers used for PCR-SSCP.

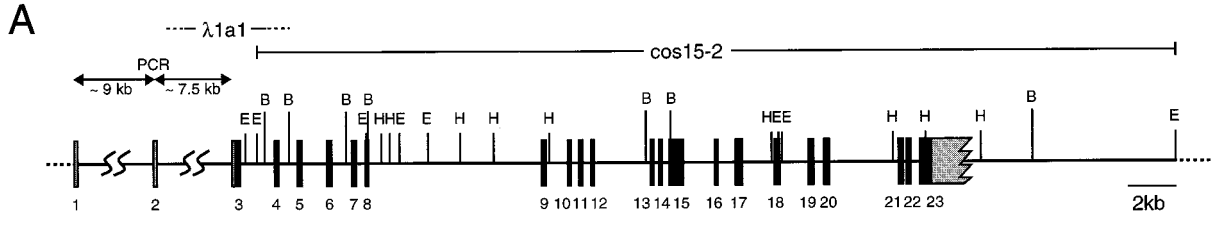
LOH Analysis. For detection of LOH in the *Tsc1* gene region in RCs from LEC/F344 F_1 progeny, a polymorphism in *Tsc1* intron 9 (Table 2) was used as a marker. PCR was performed for the PCR-SSCP analysis using a primer set for the latter half of exon 9. After 25, 30, or 35 amplification cycles, the PCR products were analyzed by 3% Agarose-1000 (Life Technologies) gel electrophoresis.

Sequence Analysis. For sequencing of the exon-intron boundaries and cDNAs, plasmid DNAs and genomic PCR fragments were subjected to the cycle sequence reaction using a dye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems), and sequences were analyzed with an ABI PRISM Model 310 genetic analyzer (Perkin-Elmer Applied Biosystems). For sequencing of PCR products from the SSCP analysis, a SequiTherm cycle sequence kit (Epicentre Technologies) was used with [α-^{32}P]dCTP.

RESULTS

Structural Analysis of the Rat Tsc1 Gene. To clone rat *Tsc1* cDNA and genomic DNA, we initially performed RT-PCR analysis using primers designed from the human *TSC1* cDNA sequence (20). A ~1.2-kb fragment was successfully amplified with a primer set (HTSF2 and HTSR2) from rat testis mRNA (data not shown). Sequence analysis of the amplified portion of this fragment revealed a derivation from rat *Tsc1* mRNA, showing homology with nt 1030–2229 of the human *TSC1* cDNA sequence (data not shown). Subsequently, cosmid and phage clones containing rat *Tsc1* genomic DNA regions were isolated by library screening, and their exonic sequences were determined (Fig. 1*A*). Also, a partial rat *Tsc1* cDNA fragment corresponding to nt 739-1722 of human *TSC1* cDNA was isolated from a kidney cDNA library. After the identification of sequences corresponding to exons 3 and 23 of the human *TSC1* gene in a cosmid clone, the full coding region of rat *Tsc1* cDNA was obtained by RT-PCR (Figs. 1*A* and 2*A*; GenBank/EMBL/DDBJ accession number AB011821). The 5′-RACE was also performed to analyze the 5′-untranslated region of *Tsc1* mRNA (Figs. 1*A* and 2*B*; GenBank accession number AB016165).

The exon-intron organization of the rat *Tsc1* gene demonstrated essential similarity with the human *TSC1* gene, except for the junction



B

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..........................  (-184) AGGGACTGTGAGGTA..(Exon  1: >41 nt)..GGGAGGAGACGGTGG (-144) gtgagtacgggggtgtttcc.....
.....gtatctggttttccctccag (-143) TGACCATGGAAGACA..(Exon  2:  63 nt)..CTGTCCCCAGAACAG ( -81) gtaaggcagacatttagtct.....
.....ttcatatttccctttgcag ( -80) TCTGGTAGTGGCCCT..(Exon  3: 186 nt)..AGTCCCTCAATTCTG ( 106) gtaagtagtgacttgttctc.....
.....tgattgctctttttgtttag ( 107) AACGTGGGCCTATGC..(Exon  4: 104 nt)..GAGCCACATGATAAG ( 210) gtgagtgctggtgggatagc.....
.....ttgatgttttatattttcag ( 211) CACCTCTTGGACAAA..(Exon  5: 153 nt)..CTGAAATGTCTCAAG ( 363) gtaggatatttgttaaggtt.....
.....gtcgcccttcccttctgcag ( 364) ATGGACACTGATGTT..(Exon  6: 145 nt)..GCCTGAAGAAACCAG ( 508) gtataactccaaggtcctca.....
.....tcgctggtttccttccttag ( 509) GCCATGTGACAGAAG..(Exon  7: 155 nt)..GAAGAAGTGGTCAAG ( 663) gtgagttcaaaccaacaacc.....
.....gtcaacccgaactcttctag ( 664) CCAATGATGGAGCAT..(Exon  8:  74 nt)..ACTGGACCCTCGAAG ( 737) gtacagaaactaaattagtg.....
.....acttatgttccctttttctag ( 738) GTGGAAGACATTAGA..(Exon  9: 176 nt)..CACAGAATAGCTATG ( 913) gtaaaaagctgctttggtct.....
.....tcccttccctttcatttcag ( 914) GGGGCGCTACTTCCA..(Exon 10: 116 nt)..CCTGAGCCGCTGCAA (1029) gtacggtgccactcagcatc.....
.....atttctgatctgacttgtag (1030) GCTAGTCTTTGGAGC..(Exon 11: 115 nt)..TTGGTACTACCACTG (1144) gtatgtgggtcttaggtggg.....
.....ttgtccttgtgtctgagcag (1145) GTGGAAAAGGAACTC..(Exon 12: 119 nt)..ACACCCCCCAGGAAG (1263) gtgagctccaggcctgagat.....
.....ttcactgtcttccatttcag (1264) GAAGAAAGAGCAGAT..(Exon 13:  70 nt)..GTGACCGCGGATTAG (1333) gtaagctccccggggaatgg.....
.....gtctgtctgtctctctgcag (1334) AGGATCTACCTGGAA..(Exon 14: 105 nt)..AAGATAAGGAAGAAG (1438) gtaacatccctgggtggcta.....
.....tttccccactctcccttcag (1439) CTGCGATATCTAAAG..(Exon 15: 559 nt)..CAAGGAACTGAGCAG (1997) gtgagccaggccgtgctgtt.....
.....cctgtgaaatcttttcctag (1998) GTTGTCCTTGCCCAG..(Exon 16:  44 nt)..GGACCCACTTTGGAG (2041) gtaaacgctgcttcggttcg.....
.....tccctctgctttacaatcag (2042) GCTCTCCCCCCTCAG..(Exon 17: 167 nt)..CACAACGCAGCGATG (2208) gtgagcagccagggcgagcc.....
.....agtgttttctgcctttgctag (2209) AAAGATCAGTTGAAG..(Exon 18: 183 nt)..AGTCAGGAGTTACAG (2391) gtactggctgctataccgg.....
.....cggccgctggtgtttggcag (2392) ACGAAGCTGGAGGAC..(Exon 19: 111 nt)..CAGGTCTCTCAGAAG (2502) gtaagagcagagccccccc.....
.....ttctcacttccgcctggcag (2503) CTCTCCAATAGTGAG..(Exon 20: 123 nt)..CCTGACACCACCAAG (2625) gtatgtggccagggcactga.....
.....gactccagacctttttatag (2626) GAAGTAGAAATGATG..(Exon 21: 188 nt)..CAAGAGCCAGGCGAG (2813) gtgactcactggaaaggtct.....
.....aaccacgccctgtctttcag (2814) TGGACAGCTGCTGGC..(Exon 22: 162 nt)..GGCAGCTGAAGAGAG (2975) gtaagtgcagccacgtcggg.....
.....cttccctgcgtctctttacag (2976) GCTTGACTGTTGTAC..(Exon 23:>517 nt)..CACGAACACAGCTGA (3492) AGAGGGTCTGTCAGTGTTAA.....
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Fig. 1. Structure of the rat *Tsc1* gene. *A*, exon-intron organization of the rat *Tsc1* gene. Exons are shown as boxes with numbers beneath them. In each box, *filled* and *shaded areas* indicate coding and noncoding regions, respectively. The notched 3′ end of exon 23 denotes the incomplete determination of the 3′-untranslated region. Regions covered by a cosmid clone (*cos15-2*) and by a phage clone (*λ1a1*) are indicated. Both ends of λ1a1 were not completely analyzed. Two intronic regions amplified by long PCR are shown by *arrows* with sizes. Restriction endonuclease sites are as follows: *B*, *Bam*HI; *E*, *Eco*RI; and *H*, *Hin*dIII. *B*, sequences of exon-intron boundaries of the rat *Tsc1* gene. Exon and intron sequences are shown by *uppercase* and *lowercase letters*, respectively. The number and length (in nt) of each exon are shown in parentheses in the middle of the figure. In exon 23, the size of the coding region is indicated. The nt numbers for the 5′ and 3′ends of exonic sequences (except for the 3′ end of exon 23) were also shown in parentheses at both sides of each exonic sequence. In exon 23, the nt number for the 3′ end of a termination codon (*TGA*) is indicated. The adenine of the first translational initiation codon (ATG) was defined as nt number 1. The 15-mer sequence deleted in a cDNA clone and a termination codon is *underlined*.

Fig. 2. Structural conservation between rat *Tsc1* and human *TSC1* genes. *A*, aa sequence alignment of rat and human hamartins. The aa sequence of rat hamartin (*Rat*) using single-letter codes is aligned with that of human hamartin (*Human*). The number of the first aa residue of each line is indicated on the *left*. An *asterisk* between two sequences indicates the identity of each residue between rat and human. Putative transmembrane and coiled-coil regions are denoted by a *box* and *underlined*, respectively. A GSPPS sequence deleted by an alternative splicing event is indicated by *dots* above the sequence. *B*, comparison of the 5′-untranslated region of rat *Tsc1* and human *TSC1* cDNAs. The nt sequence of the 5′-untranslated region of rat *Tsc1* cDNA (*Rat*) is aligned with that of human *TSC1* cDNA (*Human*). The number of the first nt residue of each line (defining the 5′ end of each sequence as number 10 is indicated on the *left*. Translational initiation codons (ATG) are *underlined*. An *asterisk* between two sequences indicates the identity of each residue between rat and human. Sites of conserved exon-exon junctions are indicated by *arrowheads* with the numbers of the two junctional exons.

of exons 11 and 12 (intron 12; Fig. 1, *A* and *B*). The rat exon 11 sequence (115 nt) was found to be 3 nt longer than that of the human sequence at its 3′ end. Complimentarily, the rat exon 12 sequence (119 nt) was 3 nt shorter than that of the human sequence at its 5′ end. The GT/AG rule was kept at all intronic splicing donor and acceptor sites (Fig. 1*B*). The deduced aa sequence of the rat Tsc1 product consisted of 1163 residues and showed ~86% identity with that of human hamartin (Fig. 2*A*). A putative transmembrane region (aa 127–144) and a potential coiled-coil region (aa 719–998) were conserved between rat and human (Fig. 2*A)*. In the COOH-terminal region encoded by exon 23, a 3-aa deletion and two single aa insertions were found in rat hamartin as compared with human hamartin (Fig. 2*A*). During RT-PCR analysis, we identified a cDNA fragment lacking nt 2042–2056 (15 bp) at the exon 16-exon 17 junction (data not shown). This 15-bp sequence was pyrimidine rich and ended with an AG dinucleotide (Fig. 1*B*). These results indicate that the second splicing acceptor site exists in the 5′ part of exon 17. The use of this second acceptor site causes a 5-aa (Gly-Ser-Pro-Pro-Ser) deletion (Fig. 2*A*).

In man, *TSC1* mRNA has a long 3′-untranslated region (~4.5 kb; Ref. 19). Although we have not yet fully accomplished sequence analysis of the 3′-untranslated region of rat *Tsc1* cDNA, substantial homology of the sequenced region of exon 23 with the 3′-untranslated region of human *TSC1* cDNA was identified (data not shown). In addition, the mRNA size (~8 kb) of rat *Tsc1* was similar to that of human *TSC1* mRNA detected by Northern blot analysis (see below). These results indicate that rat *Tsc1* mRNA also has a long 3′-untranslated region. The longest 5′-untranslated region, which was obtained by 5′-RACE, was 184 bp in length and showed an 84.5% sequence identity with that of human *TSC1* cDNA (Fig. 2*B*). Thus, the 5′-untranslated region of *Tsc1*/*TSC1* mRNA was also conserved.

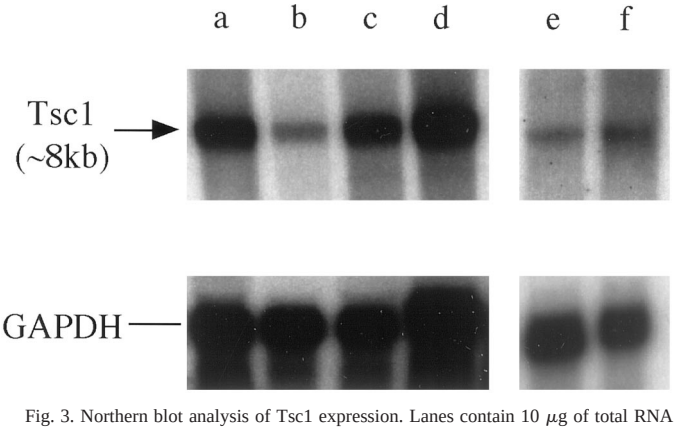

Fig. 3. Northern blot analysis of Tsc1 expression. Lanes contain 10 μg of total RNA from rat organs. Probes are 5′ ~2.4-kb region of rat Tsc1 cDNA. An ~8-kb band similar in size to that of human TSC1 mRNA was detected. The *bottom panels* show the results with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes using the same blot. *a*, brain 1; *b*, liver; *c*, spleen; *d*, kidney; *e*, heart; *f*, brain 2.

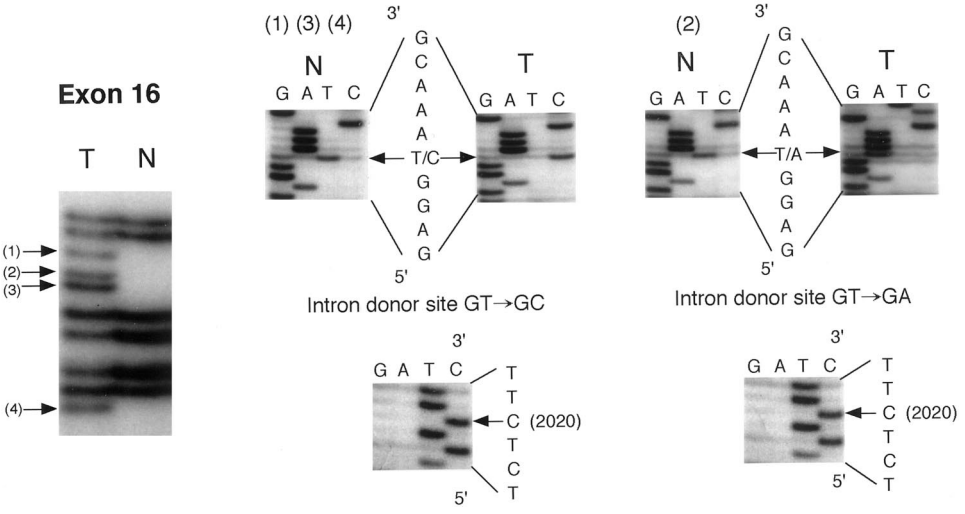

Fig. 4. Detection of mutations in the *Tsc1* gene of sample 9 [EHEN-induced (F344xLEC) F_1 rat RC]. SSCP changes and the DNA sequence showing the SSCP change are shown. Mutant alleles and mutations are indicated by *arrows*. The sequence at nt 2020 of the four shifted bands was C, and both mutations were derived from the LEC allele. *N*, normal; *T*, tumor.

Chromosomal Localization and Expression of the Rat Tsc1 Gene. The chromosomal localization of the rat *Tsc1* gene was determined by Southern blot analysis of a human/rat somatic cell hybrid panel. The appearance of rat-specific bands coincided with the presence of rat chromosome 3 in each cell clone (data not shown).

The expression of *Tsc1* was examined in several rat tissues by Northern blot analysis. A ~8-kb band similar in size to that of human *TSC1* mRNA was detected in the brain, liver, spleen, kidney, and heart (Fig. 3).

Mutational Analysis of the Tsc1 Gene in Rat RCs. To investigate the possible involvement of *Tsc1* gene alteration in chemically induced renal carcinogenesis in rats, we searched for mutations in 21 coding exons (exons 3–23) of *Tsc1* by PCR-SSCP analysis in 15 RCs induced by EHEN or DEN. Of these RCs, eight were used in our previous PCR-SSCP analysis of the *Tsc2* and *Vhl* genes (11). The remaining seven RCs were newly established RCs. In this study, an analysis of *Tsc2* and *Vhl* as well as *Tsc1* gene mutations was carried out.

With regard to the *Tsc1* gene, we identified two mutations in 1 EHEN-induced RC (sample 9) as well as nine polymorphisms in 15 RCs (Fig. 4; Table 2). The two mutations were in the splicing donor site of intron 16, giving rise to a GT to GA transition and a GT to GC transversion. In exon 16, a polymorphic sequence at nt 2020 (G *versus* C) was found (Table 2), and the rat in which sample 9 RC developed was a heterozygote for this polymorphism. Autoradiography of SSCP of exon 16 showed several discrete shifted bands in sample 9 that were not seen in the normal tissue sample from the same rat or other RC samples (Fig. 3). When these shifted bands were sequenced, both mutations were found with C at nt 2020, whereas the normal band contains either C or G. Thus, the two mutations were generated on the same allele. In sample 9, no mutations were detected in either the *Vhl* or *Tsc2* gene (see below). In intron 9, a polymorphic 20-bp deletion was identified in the LEC allele used here (Table 2). We used this polymorphism as a marker for LOH detection in applicable cases of RC from LEC/F344 F_1 and F_2 and LEC/WKAH F_2 progeny. However, LOH was not detected in any of the RCs (data not shown).

No mutations in the *Vhl* gene were identified by our PCR-SSCP analysis in the seven newly established RCs (data not shown). In contrast, five *Tsc2* mutations were found in three samples (Table 3). These results regarding the absence of mutations in *Vhl* and the presence of mutations in the *Tsc2* gene in chemically induced RCs were in line with those from our previous report (11).

DISCUSSION

In this report, we isolated and characterized the rat *Tsc1* gene and performed a structural comparison with human *TSC1* as an initial step toward the analysis of the biochemical pathways in which tuberin and hamartin may be involved in rat renal carcinogenesis.

The high degree of structural similarity observed suggests functional conservation of hamartin in these species. The predicted coiled-coil region, in particular, appears to be highly conserved and thus may be functionally important, possibly for multiple protein-protein binding. The tuberin-binding site in human hamartin reported by Slegtenhorst *et al.* (20) contains 70 amino-terminal residues of this coiled-coil region, and the authors also mentioned homophilic interactions through this coiled-coil region. We have in fact identified a rat tuberin-binding site in a fragment containing the amino-terminal half of the coiled-coil region of rat hamartin,[4] and the available information therefore indicates that it is a key element in the *in vivo* function of the two proteins.

In addition to higher homology of the coding region, rat *Tsc1* also demonstrated other characteristics in common with its similar human counterpart. The 5′- and 3′-untranslated regions of mRNA are conserved and may be functionally important in the regulation of gene expression at the posttranscriptional level. Our Northern blot analysis showed ~8-kb *Tsc1* mRNA to be highly expressed in the brain as well as in the heart and kidney. The lower expression of *Tsc1* found in the liver is similar to that in the human case (20). Analysis of the expression profile of *Tsc1* mRNA and protein at the histological level should clarify the existence of posttranscriptional regulation of gene expression.

The localization of rat *Tsc1* on chromosome 3 is reasonable because other homologues of genes on human chromosome 9q32–q34, such as the c-*Abl* proto-oncogene and the gene for adenylate kinase 1 (*AK1*), have also been mapped to rat chromosome 3 (30).

As seen in human TSC, *Tsc1* and *Tsc2* deficiency might cause a similar phenotype in the rat and thus facilitate RC development. As one approach to investigate this possibility, we searched for *Tsc1* mutations or deletions in chemically induced RCs; some of these RCs contained somatic *Tsc2* mutations that we have identified previously (11). The two *Tsc1* splice donor site mutations found in sample 9 appeared to be on the same allele, as revealed by the presence of the same polymorphic sequence in their neighborhood. These splice do-

[4] S. Hasegawa and O. Hino, unpublished observations.

Table 3 *Mutations of Tsc1 and Tsc2 gene in rat RCs*

No.	Strain	Sex	Age (wk)	Tumor size (mm)	Histology	Treatment	Mutations[a]
1	F344	M	47	10	RC	0.1% EHEN (1 wk × 3) + 0.01% $KBrO_3$	
2	F344	M	47	25	RC	0.1% EHEN (1 wk × 3) + 0.01% $KBrO_3$	Tsc2: exon 21 TGT→TGA Cys^{800}→Stop
3	F344	M	47	20	RC	0.1% EHEN (1 wk × 3) + 0.01% $KBrO_3$	Tsc2: exon 10 TAT→TAA Tyr^{336}→Stop Tsc2: intron 11 donor site GT→GC
4	(LEC × WKAH)F_2	F	77	7	RC	DEN (100 mg/kg × 2)	Tsc2: exon 19 CTC→CGC Leu^{713}→Arg
5	(LEC × F344)F_2	F	76	50	RC	DEN (100 mg/kg × 2)	Tsc2: exon 33 TTG→TAG Leu^{1384}→Stop
6	(LEC × F344)F_2	M	77	10	RC	DEN (100 mg/kg × 2)	
7	(LEC × F344)F_2	M	76	25	RC	DEN (100 mg/kg × 2)	Tsc2: exon 20 TTG→TAG Leu^{780}→Stop Tsc2: intron 27 acceptor site AG→AT
8	(LEC × F344)F_2	M	76	8	RC	DEN (100 mg/kg × 2)	
9	(F344 × LEC)F_1	M	36	6	RC	0.05% EHEN (2 wk + 1 wk × 2)	Tsc1: intron 16 donor site GT→GA Tsc1: intron 16 donor site GT→GC
10	(F344 × LEC)F_1	M	41	1.5	RC	0.05% EHEN (2 wk + 1 wk × 2)	Tsc2: exon 1 TTG→TAG Leu^{45}→Stop Tsc2: exon 9 AAC→AGC Asn^{314}→Ser Tsc2: exon 34 CTC→CGC Leu^{1508}→Stop
11	(F344 × LEC)F_1	M	41	12	RC	0.05% EHEN (2 wk + 1 wk × 2)	
12	(F344 × LEC)F_1	M	42	8	RC	0.05% EHEN (2 wk + 1 wk × 2)	Tsc2: exon 26 TTG→TAG Leu^{1020}→Stop
13	(F344 × LEC)F_1	M	43	3	RC	0.05% EHEN (2 wk + 1 wk × 2)	
14	(F344 × LEC)F_1	M	44	4	RC	0.05% EHEN (2 wk + 1 wk × 2)	
15	(F344 × LEC)F_1	M	43	2	RC	0.05% EHEN (2 wk + 1 wk × 2)	Tsc2: LOH Tsc2: intron 34 donor site GT→GC

[a] *Tsc2* mutations of samples 1–8 were reported previously (11). *Vh1* mutations were not detected by our PCR-SSCP analysis.

nor site mutations may affect splicing. *Tsc2* mutations were not detected in sample 9. Several possibilities could explain the occurrence of these double mutations. One possibility is that two (or more) independently arising RCs were included in sample 9, and *Tsc1* mutations were formed separately in different RCs during the early stages of renal carcinogenesis. Advanced carcinomas are known to be monoclonal, but Novelli *et al.* (31) reported mixed karyotype adenomas in familial adenomatous polyposis and suggested cell to cell cooperation in adenoma formation before monoclonality due to a dominantly growing clone. Merritt *et al.* (32) also observed that intestinal adenomas in the Min mouse, an experimental model for familial adenomatous polyposis, have a polyclonal structure. The other possibility is that the *Tsc1* mutations occurred separately within the same tumor after the initiation of RC development. In either case, the finding of *Tsc1* mutations suggests the involvement of the *Tsc1* alteration in RC development in the rat.

The mutations analyzed in this study and reported previously are summarized in Table 3. The 15 chemically induced RCs showed a chromophilic type histology with a tubular or papillary pattern, not a clear cell type histology. They showed the same histopathological features, comparing the one with *Tsc1* mutation *versus* others with *Tsc2* mutations.

The frequency of *Tsc2* mutations (8 of 15) was higher than that of *Tsc1* mutations (1 of 15) in chemically induced RCs ($P < 0.05$). We are now trying to ascertain whether chemical renal carcinogenesis is possible in transgenic rats constructed with extra copies of the wild-type *Tsc2* gene. This experiment may also provide evidence of the involvement of *Tsc1* alterations in RC development in the rat.

In man, loss of the *TSC1* locus has been detected in hamartomas from TSC patients, suggesting that two hits of *TSC1* may cause their development (33). A similar case is applicable for *TSC2*/*Tsc2* in human TSC hamartomas and RCs in Eker rats or *Tsc2* knockout mice.[5] Thus far, however, we have not found a loss of the *Tsc1* locus in sample 9 or in other RCs. We know that our SSCP analysis may have limitations. One possible reason, other than an insufficient sensitivity of SSCP analysis or a dominant negative effect on Tsc1 function, is that these tumors could contain mutations in other extra-exonic regions that are critical for *Tsc1* gene expression or could have deletions in primer annealing sites. Another possible reason is a reduction of expression of the wild-type *Tsc1* allele by some epigenetic mechanism, such as methylation of the promoter region. Detection of two hits of *Tsc1* in RCs will strengthen the case for the involvement of *Tsc1* alterations in renal carcinogenesis. Phenotypic analysis of *Tsc1* knockout mice should also clarify this possibility and the *in vivo* interactions of hamartin and tuberin.

In summary, we have isolated and characterized the rat homologue of the human *TSC1* gene. Structural analysis of rat *Tsc1* rendered mutational analysis of RC samples possible, and we identified two mutations in one EHEN-induced RC by PCR-SSCP analysis. Biochemical studies using the *Tsc1* cDNA cloned here as well as further mutational analysis of rat RCs should provide clues to the functions of hamartin and tuberin and the molecular mechanisms of tumor development associated with *TSC1*/*Tsc1* or *TSC2*/*Tsc2* mutations.

ACKNOWLEDGMENTS

We thank Dr. T. Nagase (Kazusa DNA Research Institute, Kisarazu, Japan) for providing the human TSC1 cDNA clone HA4782, Dr. S. Mashima for help in this work, and Drs. H. Sugano, T. Kitagawa, and A. G. Knudson for encouragement throughout this work.

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