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Identification of a leader exon and a core promoter for the rat tuberous sclerosis 2 (*Tsc2*) gene and structural comparison with the human homolog

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Abstract. Hereditary renal carcinoma in the Eker rat is an excellent example of predisposition to a specific cancer being transmitted as a dominant trait. Recently, we identified a germline mutation of the tuberous sclerosis 2 (*Tsc2*) gene in the Eker rat. In the present study, we analyzed the upstream region of the *Tsc2* gene. A novel leader exon (exon 1a) in a CpG island was found, and core promoter activity was identified in a 242-bp region of this island. Exon 1a and the promoter region were conserved in the human TSC2 gene. In addition, a rat homolog of a gene found upstream of TSC2 in human has been identified, indicating that the genomic organization around *Tsc2*/TSC2 is conserved between the two species. Characterization of the 5′ region of *Tsc2* and TSC2 will facilitate studies of the regulation of the gene and its disregulation in tumorigenesis.

Introduction

Hereditary renal carcinoma (RC) in the rat was originally reported by Eker in 1954 (Eker and Mossige 1961). Recently, we and others have demonstrated that a germline insertion in the rat homolog of the human tuberous sclerosis 2 (TSC2) gene (European Chromosome 16 Tuberous Sclerosis Consortium 1993) gives rise to the dominantly inherited cancer in the Eker rat model (Hino et al. 1994; Young et al. 1994; Kobayashi et al. 1995a). We have recently described the entire cDNA and genomic structure of the rat *Tsc2* gene (Kobayashi et al. 1995b); there are 41 exons spanning ~35 kb of genomic DNA. The exon-intron structure and alternative splicing events of the *Tsc2* gene (involving exons 25 and 31) are largely conserved in human, mouse, and pufferfish (Kim et al. 1995; Xiao et al. 1995; Xu et al. 1995; Maheshwar et al. 1996; Olsson et al. 1996a).

The function of the TSC2 gene product (tuberin) is not yet understood, although it does contain a short amino acid sequence homology to Rap1 GAP (European Chr 16 Tuberous Sclerosis Consortium 1993; Kobayashi et al. 1995a, 1995b). Furthermore, Wienecke et al. reported that tuberin has weak GAP activity for Rap1a (Wienecke et al. 1995). Other potential functional domains include two transcriptional activation domains (AD1 and AD2) in the carboxyl terminus of the *Tsc2* gene product (Tsuchiya et al. 1996), a zinc-finger like region (our unpublished observation) and a potential src-homology 3 region (SH3) binding domain (Olsson et al. 1996a).

The detection of loss of heterozygosity (LOH) of the wild-type allele, even in the earliest preneoplastic lesions (Kubo et al. 1995), supports the hypothesis that a second somatic mutation is the rate-limiting step for renal carcinogenesis in the Eker rat model (Hino et al. 1993). LOH of the TSC2 locus is also observed in hamartomas from tuberous sclerosis patients, providing further evidence of the tumor suppressor nature of the TSC2 gene (Orimoto et al. 1996; Green et al. 1994; Henske et al. 1995; Carbonara et al. 1996).

The expression of the *Tsc2* gene seems to be ubiquitous in various adult human and rat tissues and cell lines (Kobayashi et al. 1995a, 1995b; Xiao et al. 1995). However, preferential expression of the *Tsc2* gene in the developing central nervous system and kidney of the rat (Geistand Gutmann 1995) and mouse (Olsson et al. 1996a) has been demonstrated by in situ hybridization. This suggests that some tissue- or stage-specific regulation may determine *Tsc2* gene expression. To help understand the gene function(s), it is essential to determine the expression profile of the *Tsc2* and TSC2 genes. Therefore, as the first step toward unraveling the underlying regulatory mechanisms, we have characterized the upstream regions of these genes.

Materials and methods

DNA and RNA isolation and Northern blot analysis. Rat genomic DNA was isolated by proteinase K digestion followed by phenol extraction as previously described (Hino et al. 1993). Total RNAs were isolated from adult rat tissues and cultured cells by guanidinium-isothiocyanate/phenol-chloroform extraction. Poly(A)$^+$ RNAs were selected with oligo (dT) latex beads (Nippon Roche). Northern blot analysis was performed as previously described (Kobayashi et al. 1995a).

5′-RACE. Rat 5′-RACE was performed as previously described (Kobayashi et al. 1995b). Briefly, 1st strand cDNA was synthesized from 4 μg of brain or testis total RNA by use of the primer RTSC36 (5′-TCCAAAGTGCCTCCACTGC-3′), and then a dG-tail was added with terminal deoxynucleotidl transferase. PCR was carried out with primers $(dC)_{12}$ and RTSC37 (5′-CACAGATCTGCCCTATCATC-3′). Portions of the amplified products were re-amplified by a second round of PCR with an internal primer 5NE2 (5′-ACCCAGGAAAGGGCGCAC-3′) and a $(dC)_{12}$ primer. Human 5′-RACE was carried out using Human Fetal Brain Marathon-Ready™ cDNA (Clontech) and the TSC2 primers MP49 (5′-CCTCTGCAGACCTGGGATTTGGCCTCGGTGTTC-3′) and MP59 (5′-AATCTTTGCTTGTTGGTTTGGCCATGGTGGACC-3′), according to the manufacturer's specifications.

RT-PCR and genomic PCR. Rat first-strand cDNAs were synthesized from 4 μg of total RNAs with random primers and Superscript reverse transcriptase (Gibco-BRL). One-twentieth of the reaction mixtures were subjected to PCR with an exon 1a specific forward primer, 5NE1 (5′-

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GGTGTGCGCCCTTTCCTG-3′) and an exon 1 reverse primer, RTSC94 (5′-TGAACTTCTCCTTCAAACCTG-3′). The rat intron between exon 1a and exon 1 was amplified with primers 5NE1 and RTSC94. Human first-strand cDNA was obtained from 1 μg of RNA with the Promega Reverse Transcription System according to supplied protocols. One-twentieth of the reaction volume was used in subsequent PCR reactions with the forward primers 0eF (5′-TGCGCCTTTCCTCCGCGT-3′), 0iF (5′-ACAGAACTACAACTCCCAGCA-3′), 1iF (5′-TGTTGCTCAGATGTC-CCCATT-3′), together with the exon 3 reverse primer MP63 (5′-GCTCCGGCTGCAACAGATC-3′). The 0eF-MP63 amplified product was purified with Geneclean (Stratech) and subjected to a second round of amplification.

Primer extension analysis. Primer extension was carried out according to standard procedures (Sembrook et al. 1989). Briefly, hybridization of the ^{32}P-labeled 5NE2 primer and 10 μg of adult rat brain poly(A)$^+$ RNA or control *E. coli* tRNA was carried out in 30 μl of hybridization buffer (40 mM piperazine-N,N′-bis[2-ethanesulfonic acid], pH6.4; 1 mM EDTA, pH8.0; 0.4 M NaCl; 80% formamide) at 30°C overnight. After ethanol precipitation, the extension reaction was carried out with Superscript in 20 μl of the reaction mixture containing 50 mg/ml of actinomycin D. After ribonuclease digestion, products were separated on 8% polyacrylamide/7M urea gels and subjected to autoradiography.

Construction of chloramphenicol acetyltransferase (CAT) reporter plasmids. A ~1.6-kb *Xba*I-*Bam*HI fragment containing the CAT coding region and SV40 poly(A) signal from pCAT-BASIC (Promega) was subcloned into *Xba*I/*Bam*HI-digested pBluescript SK(+) to construct pBT-CAT. A ~1.2-kb fragment spanning the 5′ part of the rat *Tsc2* gene was prepared from a *Tsc2* mini-gene plasmid (Kobayashi et al. 1997) by *Nco*I digestion and treatment with T4 DNA polymerase followed by *Not*I digestion. This *Not*I-*Nco*I fragment was subcloned into the *Not*I-*Xba*I site of pBT-CAT; the translational initiation codon of *Tsc2* gene was intact and was fused in-frame to the CAT initiation codon. Subfragments of the ~1.2-kb *Not*I-*Nco*I fragment were prepared by *Pvu*II, *Pvu*II/*Pst*I, or *Pvu*II/*Sac*I digestion. These fragments were subcloned into the *Xba*I site of pBT-CAT after treatment with T4 DNA polymerase. Ligation sites and orientation of all subclones were confirmed by sequencing.

Transfection and CAT assay. HeLa cells were cultured in DMEM supplemented with 10% FCS. Aliquots of 2 × 10^5 cells were seeded in 60-mm dishes and cultured for 24 hours. Plasmids were transfected by the calcium phosphate method as described (Sambrook et al. 1989). For each transfection, 2 μg of the CAT reporter plasmid and 1 μg of pCMVβ (Clontech) were co-transfected. Cells were harvested at 48 h after transfection and assayed for CAT activity by standard thin-layer chromatography method (Sambrook et al. 1989) with [^{14}C]chloramphenicol (Amersham). Quantitation of acetylated and nonacetylated forms of [^{14}C]chloramphenicol was performed with a BAS2000 Bioimage Analyzer (Fuji Film). CAT activities were normalized to β-galactosidase activity, measured according to the manufacturer's instructions (Clontech) with *o*-nitrophenyl-b-galactopyranoside as a substrate. Three independent transfection experiments with two different sets of plasmid DNA preparations were carried out.

Sequence analysis. All PCR-amplified products were cloned into either pBluescript (Stratagene) or pCRII (Invitrogen). Sequencing of plasmid subclones and cosmid clones was performed with a T7 sequencing kit (Pharmacia) and a SequiTherm cycle sequencing kit (Epicentre Technologies), respectively. Homology and transcription factor binding site searches were carried out with the FASTA or BLAST systems and the TFMATRIX database, respectively.

Results

Identification of a leader exon in the rat Tsc2 gene. We and others previously reported the primary structure of rat *Tsc2* cDNA (Kobayashi et al. 1995b; Xiao et al. 1995). However, subsequent 5′-RACE analysis revealed that there is an additional leader sequence in the rat *Tsc2* mRNA (Fig. 1A and 1B).

The longest leader sequence of the rat *Tsc2* cDNA was 119

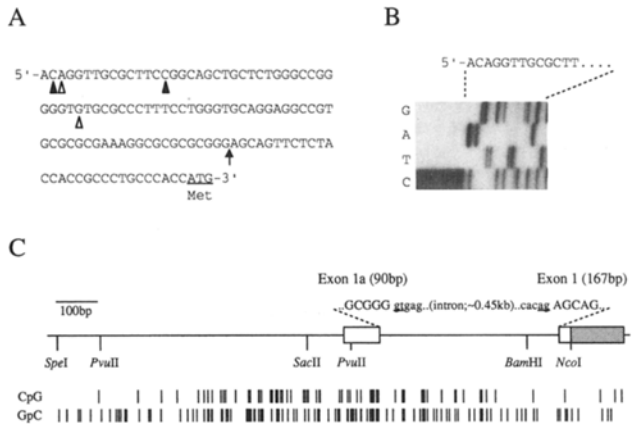

Fig. 1. Identification of a novel leader exon of the rat *Tsc2* gene. **A.** The longest leader of the *Tsc2* cDNA sequence obtained by 5′-RACE analysis. The translational initiation codon (ATG) is underlined. The 5′-terminal nucleotides found in other 5′-RACE products are marked by open arrowheads. The exon boundary is denoted by an arrow. Two transcriptional start sites identified by primer extension analysis are marked by closed arrowheads (see Fig. 3). **B.** The results of sequence analysis of the 5′-RACE subclone with the longest 5′-end. **C.** A schematic representation of the leader exon (exon 1a), coding exon 1 and 5′-upstream region of the *Tsc2* gene. Exon 1a (90 bp) and exon 1 (167 bp) are shown as two boxes. Open and shaded regions indicate the 5′-UTR and the protein coding region, respectively. Sequences for exon-intron (lower case letters) boundaries are shown above. Splicing donor and acceptor site consensus sequences are underlined. Positions of CpG and GpC dinucleotide sequences are denoted by vertical bars at the bottom.

nucleotides, 36 nucleotides longer than that reported by Xiao and colleagues (1995). It does not contain a translational initiation codon. Sequencing a genomic cosmid clone spanning the 5′ region of the rat *Tsc2* gene (cosTsc2-1) (Kobayashi et al. 1995b) revealed that this leader sequence is separated by a 0.45-kb intron, 90 nucleotides from its 5′-terminus (Fig. 1A and 1C). The new exon (named exon 1a) has a relatively high GC content (67/90, ~75%) and comparison with the recently reported mouse *Tsc2* cDNA (Kim et al. 1995) suggests conservation across rodents (data not shown). The size of the coding exon 1 was defined as 167 nucleotides, including 29 nucleotides of the leader sequence (Kobayashi et al. 1995b). So far, we have not observed intronic sequence upstream to the coding exon 1 in 5′RACE subclones with RTSC37 and $(dC)_{12}$.

By RT-PCR analysis with an exon 1a-specific primer, we examined the expression of exon 1a in adult rat brain, liver, spleen, kidney, and testis. Amplified products (130 bp) were detected in all tissues examined (data not shown). By sequencing of subclones from this RT-PCR, we identified another splicing variant between exon 1a and coding exon 1. In one subclone, the 5′ terminal AGCAG sequence of coding exon 1 (Fig. 1A) was deleted (data not shown); the 3′ terminal AG of this deleted sequence may be used as a second splicing acceptor site. This second acceptor site usage seems to be a rare event since all subclones from 5′-RACE products and the majority of subclones of the RT-PCR contained the AGCAG sequence.

Structural analysis of the upstream region of the rat Tsc2 gene. We sequenced the 1.2-kb *Not*I-*Nco*I genomic DNA fragment, which extends from 710 bp upstream of exon 1a through to the translational initiation codon in exon 1 (Fig. 1C, EMBL/GenBank/DDBJ accession no. D84251). Primer extension analysis with poly(A)$^+$ RNA from adult rat brain as a template identified two major transcriptional start sites in exon 1a (Fig. 1A and 2). The most 5′ of these two sites was the second "C" of exon 1a as defined by 5′-RACE. In the upstream region, there are no typical

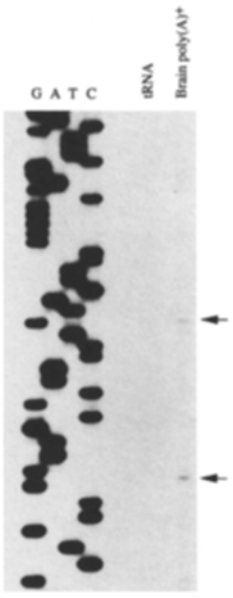

Fig. 2. Primer extension analysis of the rat *Tsc2* gene. As a size marker, the sequence ladder of rat *Tsc2* genomic DNA with the same primer used in the primer extension reaction was loaded on the left. Two major bands obtained with brain poly(A)$^+$ RNA template but not with *E.coli* tRNA are denoted by arrows (see Fig. 1A).

CCAAT and TATA boxes. An Sp1 binding core sequence (5′-GGGCGG-3′) (Kadonaga et al. 1986) and two potential cEts binding sites (Nye et al. 1992) were identified (Fig. 4). Many 5′-CG-3′ dinucleotides were identified immediately upstream of exon 1a (Fig. 1C); this CG rich region around exon 1a is characteristic of a CpG island (Bird 1986).

Identification of the core promoter in the rat Tsc2 gene. CAT assays were performed to determine whether the 710 bp upstream region contains the core promoter. A significant level of CAT activity was observed with either the full-length ~1.2-kb *Not*I-*Nco*I fragment (pBT-PR-CAT) or the ~0.6-kb *Pvu*II subfragment, which contains two major transcriptional start sites of the *Tsc2* gene (pBT-PPF-CAT) (Fig. 3). When this ~0.6-kb *Pvu*II fragment was divided into a 5′ 397-bp *Pvu*II-*Sac*I fragment (pBT-PSA-CAT) and a 3′ 242-bp *Pst*I-*Pvu*II fragment (pBT-PPS-CAT), only the latter exhibited promoter activity. The 242-bp sequence contains two major transcriptional start sites defined by primer extension analysis and the preceding 222 nucleotides of upstream sequence (Fig. 4).

Structural comparison of exon 1a and the promoter region with the human TSC2 gene. 5′-RACE with human fetal brain Marathon-Ready™ cDNA extended the known 5′ end of the TSC2 cDNA (2) by 17 nucleotides (data not shown). Of these nucleotides, 11 were identical to the genomic sequence distal to the previously identified exon 1; however, the remaining 6 nucleotides diverged from the flanking distal genomic sequence. CW24, the human genomic subclone upstream of exon 1, was sequenced (Fig. 4). The 6 divergent nucleotides were identified at the 3′ end of a region homologous to the rat exon 1a. The genomic sequences at points of divergence corresponded to the 3′ splice acceptor and 5′ splice donor consensus sequences (Shapiro and Senapathy 1988). The size of predicted new intron was 521 nucleotides. To confirm the presence of exon 1a in the human TSC2 gene, RT-PCR analysis was carried out with cDNA from human lymphoblastoid cells. A band of predicted size was amplified with a primer predicted to lie within exon 1a (0eF) but not with a primer predicted to lie upstream of exon 1a (0iF) or in the new intron (1iF) (data not shown). Sequencing of the PCR products confirmed the presence of the human exon 1a.

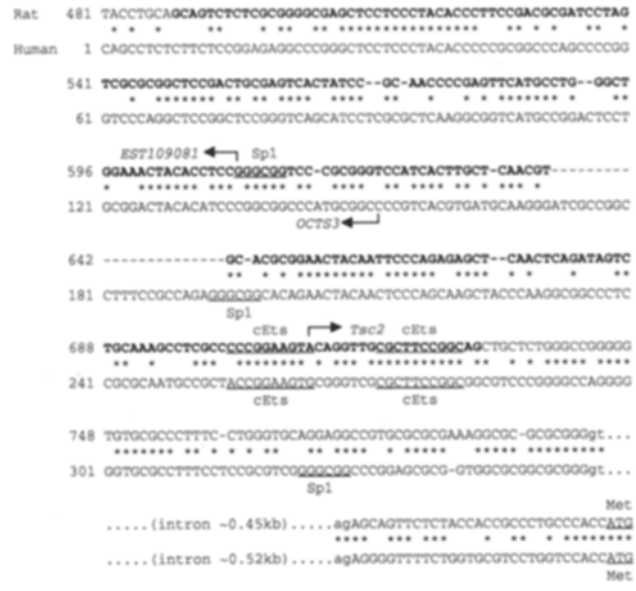

Fig. 4. Structural comparison of the upstream region and exon 1a between rat and human Tsc2 genes. Sequence comparison of a ~350-bp region containing exon 1a and the upstream sequence as well as the 5′ terminal part of coding exon 1 is shown. Identical nucleotides between rat and human sequence are marked with asterisks. 5′ and 3′ splicing consensus sites are typed by lower case letters. Potential transcription factor binding sites and translational start methionine codons are underlined. The region showing core promoter activity (see Fig. 4) in rat sequence is denoted by bold-typed letters. Transcriptional orientations of rat *Tsc2* gene and adjacent genes (human OCTS3 and the rat gene corresponding to EST109081) are shown by arrows from nucleotides corresponding to 5′ terminus of each cDNA sequence.

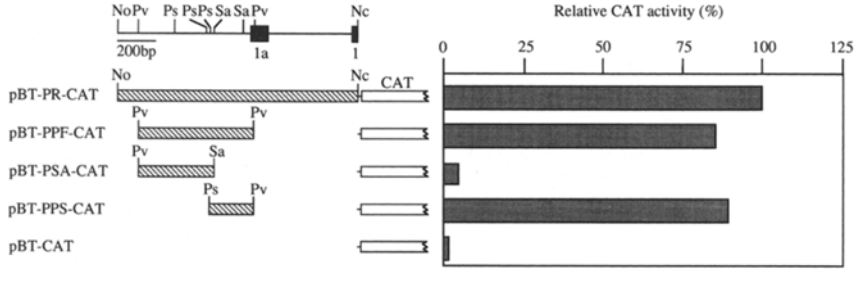

Fig. 3. Detection of promoter activity for the rat *Tsc2* gene. On the left, CAT reporter constructs are schematically represented. Shaded boxes indicate the *Tsc2* gene fragments derived from the ~1.2-kb *Not*I-*Nco*I fragment shown above. Exons 1a and 1 are shown by solid black boxes. Restriction sites are as follows: No, *Not*I; Pv, *Pvu*II; Ps, *Pst*I; Sa, *Sac*I, Nc, *Nco*I. The CAT codingregion is denoted by the open boxes. On the right, relative CAT activities obtained by introduction of each plasmid into HeLa cells are shown. Results are averages of three independent experiments. The CAT activity obtained by introduction of pBT-PR-CAT was assigned the value of 100%.

Comparison between human and rat of a ~350-bp region containing exon 1a and the upstream sequence revealed several regions of conservation and divergence (Fig. 4). This human ~350-nucleotide region was also rich in CpG dinucleotide sequences and has no typical CCAAT and TATA boxes. Approximately 60% nucleotide sequence identity was found in this region between the two species. The Sp1 binding site core sequence found in rat was not conserved; however, two other Sp1 sites were found in the human sequence (Fig. 4). The two cEts binding sites were conserved between species (Fig. 4).

Close proximity of the OCTS3 homolog upstream of the Tsc2 gene. Previously, a novel gene, OCTS3, was identified upstream of the human TSC2 gene mapped in a head-to-head orientation (European Chr 16 Tuberous Sclerosis Consortium 1993; Aspinwall et al. 1997). Database searches revealed that the 5′ sequence of a rat EST cDNA clone from PC12 cells (EST109081, accession no. H33256) (Lee et al. 1995) matched (127/129 bp) a region upstream of the *Tsc2* gene (data not shown). This sequence showed ~60% nucleotide sequence identity with human OCTS3 sequence (Fig. 4). The transcriptional orientation of the gene corresponding to EST109081 was opposite to the *Tsc2* gene. The nucleotides corresponding to the 5′ termini of EST109081 and exon 1a of rat *Tsc2* gene were separated by only 99 bp (Fig. 4). In the human case, the 5′ terminus of the OCTS3 cDNA started 16 nucleotides upstream of the EST109081 cDNA. Using the *PstI-PvuII* fragment containing the OCTS3 homologous sequence, we identified a ~1-kb mRNA in normal kidney and in Eker rat RC cell RNAs by Northern blot analysis (data not shown). This is consistent with the size of the human OCTS3 transcript (European Chr 16 Tuberous Sclerosis Consortium 1993; Aspinwall et al. 1997).

Discussion

Structural characterization of the upstream region of the rat *Tsc2* gene, the gene predisposing to hereditary renal cancer in the Eker rat model, revealed a novel leader exon, called exon 1a. This exon was found to be evolutionarily conserved in the human TSC2 gene. There is a homologous sequence to exon 1a in the mouse *Tsc2* cDNA (Kim et al. 1995), indicating that exon 1a is conserved in mammals, suggesting some functional role. Exon 1a is relatively GC rich, especially in its 3′-terminal portion, and formation of a secondary structure contributing to post-transcriptional regulation of *Tsc2* and TSC2 may occur as suggested for murine *TP53* or human BRCA1 (Mosner et al. 1995; Xu et al. 1995). Although we have not detected intronic sequence between exon 1a and exon 1 in our 5′-RACE and RT-PCR analysis, the possibility of existence of other short exon or minor transcriptional start site(s) in this intron should not be excluded. Transcriptional start site(s) in exon 1a other than those detected here also should not be excluded, since we used a primer in exon 1a.

The structural characteristics of the core promoter region resemble those of promoters of various housekeeping genes or universally expressed tumor suppressor genes (Kuzmin et al. 1995 and references therein); this is consistent with rat *Tsc2* gene expression in various adult tissues or cell lines (Kobayashi et al. 1995b; Xiao et al. 1995). We identified various conserved sequences upstream of TSC2 gene, including two cEts binding sites. These conserved sequences may be important for expression of the *Tsc2*/TSC2 gene. Future expression study with mutant constructs and determination of transcriptional factors interacting with these sequences will uncover the importance of those sequences. The finding of high mRNA levels in the developing central nervous system (Green et al. 1994) suggests that stage- or tissue-specific regulation could determine some aspects of the *Tsc2* gene expression. Sequence(s) important for such specific expression may be localized in regions other than the 242-bp core promoter region. Notably, a *Tsc2* mini-gene consisting of a genomic fragment containing a 710-bp upstream region has been found to rescue animals from the lethality of the homozygous *Tsc2* Eker mutation and suppress renal carcinomas in the Eker rat in transgene experiments (Kobayashi et al. 1997). This suggests that the minimal regulatory region for the functional *Tsc2* gene expression may be localized in this 710-bp genomic fragment.

In the Eker rat, a somatic mutation (or "2nd hit") in the wild-type *Tsc2* allele is a rate-limiting step for renal carcinogenesis (Kubo et al. 1994, 1995; Hino et al. 1993; Orimoto et al. 1996; Kobayashi et al. 1997). In RCs that show neither LOH nor intragenic mutation of *Tsc2* gene (Kobayashi et al. 1997), mutations in the promoter or other regulatory regions, resulting in significantly reduced expression, may occur as the 2nd hit, as reported for germline mutations of the human retinoblastoma gene (RB1) (Sakei et al. 1991). Detailed analysis of the promoter and other regions critical for *Tsc2* expression, along with an extensive search for mutations in those sequences, will address this possibility. Studies of the methylation status of the CpG island in the core promoter of *Tsc2*/TSC2 may also help resolve some mechanism of tumor development as suggested in other tumor suppressor genes (Jones 1996).

Rat Chr 10q12, a region syntenic with human Chr 16p13.3, has a dense population of genes (Hino et al. 1994). In the human, two genes, OCTS3 and PKD1, closely flank the TSC2 gene (European Chr 16 Tuberous Sclerosis Consortium 1993; Maheshwar et al. 1996). Similarly, in the rat, a homolog of the human PKD1 gene lies 3′ to the *Tsc2* gene (Kobayashi et al. 1995a), with only a 64-bp interval separating the two poly(A) signals (Olsson et al. 1996b and T. Kobayashi et al., unpublished data, accession no. D85767). In this study, a rat homolog of the OCTS3 gene (*Octs3*), corresponding to EST109081, was identified in the upstream region of the *Tsc2* gene. Therefore, the genomic organization around the *Tsc2* gene is evolutionarily conserved in rat, human, and possibly in mouse (Olsson et al. 1996b). Although we have not yet determined the exact positions of the transcriptional start site(s) and promoter region of the *Octs3* gene, they may be localized around exon 1a of the *Tsc2* gene to form a "bidirectional promoter" (Campbell et al. 1994). If this is the case, the expression of *Tsc2* and *Octs3* may be regulated, at least in part, by the same or coordinated mechanisms.

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