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Intragenic *Tsc2* Somatic Mutations as Knudson's Second Hit in Spontaneous and Chemically Induced Renal Carcinomas in the Eker Rat Model

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We searched for the rat homologue of the human tuberous sclerosis (*TSC2*) gene mutations in loss of heterozygosity (LOH)-negative Eker rat renal carcinomas (RCs) by polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) analysis using 45 primer sets covering all 41 coding exons and one leader exon including splicing donor/acceptor sites. We have identified intragenic somatic mutations in 7 of 21 spontaneous RCs, including one cell line (33%), and in 3 of 9 (33%) *N*-ethyl-*N*-nitrosourea (ENU)-induced LOH-negative RCs. Interestingly, five mutations in the spontaneous RCs were either deletion or duplication (5/7=71%). In contrast, all three in ENU-induced RCs were base substitutions (3/3=100%), as expected. Thus, a qualitative difference in the second hit might exist between spontaneous and ENU-induced mutations (e.g., deletion or duplication *versus* point mutation). By a direct cloning approach utilizing the restriction length difference caused by germline insertional mutation or reverse transcriptase-PCR analysis in two applicable cases, we could clearly show the presence of intragenic somatic mutations in the second copy (wild-type) of the *Tsc2* gene. This is the first demonstration at the DNA sequence level of the validity of Knudson's two-hits hypothesis in the *Tsc2* gene.

Key words: Tuberous sclerosis (*Tsc2*) gene — Hereditary cancer — Tumor suppressor gene — Eker rat — Two-hit

Hereditary renal carcinoma (RC) in the rat, originally reported by Eker in 1954, is an example of a Mendelian dominant predisposition to a specific cancer in an experimental animal.[1,2] Recently, we identified a new conserved linkage group on rat 10q and human 16p13.3 in which the Eker mutation was found to be tightly linked to the rat homologue of the human tuberous sclerosis (*TSC2*) gene,[3] and we and others identified a germline insertion in the *Tsc2* gene.[4,5] Our previous detection of loss of heterozygosity (LOH) of the wild-type *Tsc2* allele even in very early preneoplastic lesions, e.g., phenotypically altered renal tubules which appear around 5–8 weeks after birth,[6] and the effects of wild-type *Tsc2* gene transfer into Eker RC cells[7,8] led to the conclusion that a second somatic mutation involving the wild-type *Tsc2* gene is a rate-limiting step for renal carcinogenesis in the Eker rat, in line with a tumor suppressor/recessive nature for the *Tsc2* gene. In spontaneous RCs, ~60% show loss of the wild-type *Tsc2* allele.[9] However, ~40% of spontaneous RCs as well as *N*-ethyl-*N*-nitrosourea (ENU)-induced RCs retain the wild-type *Tsc2* allele.[9,10] In these RCs, the gene is expected to be inactivated by subtle mutation (e.g., point mutation) or via epigenetic mechanisms according to Knudson's two-hits hypothesis.[11]

The function of the *Tsc2* gene product [called "tuberin" in the human case; the rat Tsc2 product shows a 92% aa identity to its human counterpart[12,13]] is not yet understood, although it does contain a short region of amino acid sequence homology to ras family GTPase-activating proteins (Rap1-GAP) located downstream of the Eker insertion site.[12,13] Wienecke *et al.* reported that the human TSC2 product has weak Rap1 GAP activity.[14] We recently described transcriptional activation domains (AD1 and AD2) in the carboxyl terminus of the Tsc2 product, and the Eker insertional mutation disrupts their transcriptional activity.[15] Thus, examination of intragenic somatic mutations in the wild-type *Tsc2* gene should provide clues to gene function(s), as well as providing a test of the validity of Knudson's two-hits hypothesis.

In this study, we characterized, at the DNA-sequencing level, the second hit in the predisposing *Tsc2* gene in LOH-negative Eker rat RCs.

MATERIALS AND METHODS

Tissue samples, cell culture, and DNA and RNA isolation Tumors and corresponding normal tissues from ENU-treated or non-treated litters were stored at −80°C until use. ERC15 and ERC33 cells (gift of A. G. Knudson, Fox Chase Cancer Center) were cultured as de-

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scribed previously.[2] DNAs from tissues or cells were prepared by digestion with 2% sodium dodecyl sulfate/proteinase K followed by phenol extraction.[2] Total cellular RNAs were isolated by guanidium-isothiocyanate/phenol extraction and poly(A)$^+$RNAs were selected by using Oligotex beads (Nippon Roche, Tokyo). The *Tsc2* genotypes of tumors were determined by Southern blot analysis as described previously.[4]

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis PCR-SSCP was performed as described previously by Kubo *et al.*[16] and Orita *et al.*[17] with minor modifications. Briefly, amplifi-

Table I. Primers for PCR-SSCP Analysis

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
1a[a]	CCAGAGAGCTCAACTCAGAT	TGGATAAGAGAGGCCAACTC	235
1	AACTTTTCCTGGGGTGAATCA	CTGGGTTAGCAAGATAGAGG	235
2	CGGCCATTAAGAAGATCTCT	CCAGTAGCATAGCTGAGCAA	169
3	TTCTCAGTGTCCCCTTTTCG	CTTCATCCCACACAGCTCCT	163
4	GACAGCTGCATGGGAAGTG	CAAGGCCGGAGACACATAC	229
5	ACAGCCTCAGCTTTTCTCCT	GCTCTACGACTGAGCTGCAC	189
6	CGAGCTGACTTCAATGTTCT	AATGAAGCTGGGAGTTACTA	102
7	TAAGAGTTGGGCACCAGCAC	GCTCTCAGAAACAGCATGGG	197
8	CCTGGGGATCTATATCACCT	GTCTAAAGCCTCCTCCTGAC	226
9	GCTGGGACTCTGCTAATATC	CAGTCCACTGTGGTAGAGGA	201
10	ATACGGTCCTCTCTGCTCC	CCGTCTAGGACAATCGCAG	212
11	TGTGTTGAGAGGGTAAAGGA	AGAATCAGAAACCATGGCTC	248
12	CGGGAAGTTACTGACCTTAG	CCTGAGGTCATAAACAGACC	197
13	CTCTGCAGTGAGCCTGCTT	AGGCTTGAGACCTGAGTGC	146
14	TACCTCCCCATCTTGTGATT	ATCCACAGGTCTTTGTCCAT	232
15	AACTTACATGCTGGCCTCTT	ATTAGTCTTCTCACCCTCGC	173
16	CCTGGCTGCTGTCATAGAAT	GTACACCCATCAAGCACAGG	233
17	CTGACTGGCTGTTTTGCTC	CTATTGAACCGTCCACCCT	183
18	GCCTCACTTGTTTGTTGCAT	TAACAGAACCTAAGCGGAGC	216
19	CTCCATCGTCAAATCCTTCT	TGCAAAAGTCCACCATAAAC	207
20	GACGTCTCAGGGCTGTGTC	CATCCAGTGTCCCTTGGTC	211
21	GCAGACCACTGTAGCTTGTA	CTGTAAGCAGAACCACACAT	251
22	TAATGGCTGTCCTTGTGTCA	AAACTCTTAAGCTTGGAAGCC	200
23	ACCCGAGCTTCTGTCTCTT	GGCCCTGTCCATTACCTAT	181
24	CGGCTTTTGTCTCTGCTCC	AGAGTCCCACCCCCTTGAC	168
25	GGGACTTCCTTCCTCGCCTG	GGGACAAGCTCCAGATCCGT	201
26	GACCTCTAACCCTGGTATGG	CCAATCCTGTAGGTCCTCAT	220
27	CACATACAAGCAGGAGGTAGA	GCACAGTATACACAGAAGGGA	223
28	ACCGTGGTTGACTCTGTATG	TATGTGTCAGGAGATGGCTC	183
29	GGTCACCAGTTCTAAACTCC	ACTGCAACTCTCCAGACAGT	270
30	TGTGGCCTAACCTTTGAGGT	ATCTGCAGCTCAGCACTGC	275
31	GCTACTCGCGTGGTTACCTT	ACATGGTTTCCTCACAGGGA	128
32	GCAAGAAGCCCTGTCTGTGT	CAGCTTTCCCAACAACTTCC	191
33.1[b]	GCTTGTGCCTTCTAACAGTG	TGAGGGTTGGAAGGACAGGT	172
33.2[b]	ATTGAGCGGGCCATCTCCT	CGTCCAATGTCAGTCTTGTC	152
33.3[b]	CCTGAGTTACAGACCTTACA	GAATCAGAGATGGTATAGCC	239
33.4[b]	CGTATCACAGTCCCACCTG	GCAACTTGGGGTTTGTGCC	210
34	CCTTGACTTAGGTCCTGCC	CTGCCACGAGGGAATAGAC	217
35	GCCCTGAACTCACATAGGTT	AGTCCTATGCTGACTCCAGG	148
36	ATTCTCACGCCCTGGATTTT	GGCCTGCCTAGAGCAGTACA	237
37	TTTGGTATCCTGTCCTGGCA	TGCCTTGGTATCCAGCACAT	222
38	TTTAGCGCATGCTCATACC	CCTGACTCAGGCCAAAACT	170
39	TTGGCCTGAGTCAGGTATG	CCATAGTGAGCTCCACCGA	191
40	TCCTCGGTGGAACTCACTAT	TCACATCTCAGAAGCCCTGT	170
41	ACTTCTGGGCTCACTGCCAA	ACTCCAGTTGTGGCTCTGGT	227

a) A 5′-leader exon.
b) Exon 33 was examined using 4 primer sets.

cation was carried out in a 10 μl reaction mixture (including 50 ng of tumor DNA, 10 pmole of each primer, 1 m*M* $MgCl_2$ and 2.5 μCi of [^{32}P]dCTP). Mixtures were denatured at 93°C for 5 min followed by 35 cycles of 93°C for 1 min, 55°C for 1 min and 72°C for 1.5 min. In the last cycle the 72°C step was extended to 4.5 min. After amplification, samples were boiled for 3 min after addition of 240 μl of loading buffer (95% formamide, 20 m*M* EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue). Then, 1.5 μl aliquots of each sample were loaded onto 6% polyacrylamide/10% glycerol gels for electrophoresis (at 4°C). After drying, gels were exposed to X-ray films at −50°C. For sequence analysis, aberrantly shifted bands were dissected out from gels and extracted with 100 μl of distilled water. Then, DNA fragments were reamplified by PCR with the same primers as used for SSCP and were subcloned into pBluescript SK(+) (Stratagene, La Jolla, CA). Multiple subclones or mixtures of subclones were sequenced for each case. The primer sets and sizes of the PCR products for the exons described in this study are summarized in Table I.

Analysis of genomic DNA clones Genomic DNA (60 μg) from the LK12g RT1 RC was digested completely with *Eco*R I and separated by 0.7% SeaPlaque GTG agarose (FMC Bioproducts, Rockland, ME) gel electrophoresis. Gel slices containing *Eco*R I fragments sized approximately 9 kb to 23 kb were dissected out. After having been released by the action of Gelase (Epicentre Technologies, Madison, WI), *Eco*R I fragments were cloned into the lambda DASH-II vector (Stratagene, La Jolla, CA) to construct a size-selected library. About 10^5 clones were generated from the prepared *Eco*R I fragments. This size-selected library was screened with a ^{32}P-labeled probe covering the 3′ part of the rat *Tsc2* cDNA.[13] Phage DNAs from positive plaques were analyzed by restriction enzyme digestion, together with phage clones containing wild-type (17 kb) or mutant-type (22 kb) *Eco*R I fragments of the Eker rat *Tsc2* gene.[4]

RT-PCR analysis First-strand cDNAs were synthesized from 2 μg of poly(A)$^+$ RNAs from ERC15 or ERC33 using the RTSC24 (5′-AATGCTGCCTCAAAGTCC-TC-3′) primer in a 20 μl reaction volume. Then 1 μl aliquots of the resultant mixtures were subjected to PCR using an internal reverse primer, RTSC23 (5′-TG-GCACATGAGCCTCTCCT-3′) and a forward primer, RTSC96 (5′-AAACCTTCTAGGTACCCATCT-3′). For mutant-allele specific RT-PCR, 1 μg of poly(A)$^+$ RNAs and the MTA6 primer (5′-AGCAGAAGGCAA-TTTTCGAGT-3′) were used in a reverse transcription reaction, and MTA2 (5′-TCCTCCTGAAGCTGAAG-AGT-3′, reverse) and RTSC18 (5′-GTCTAATGCCCT-TATGGCTG-3′, forward) primers were used in PCR. Sequences of PCR products were determined after subcloning into pBluescript SK(+).

RESULTS

Intragenic somatic mutations A total of 20 spontaneous primary tumors, 9 ENU-induced primary tumors[9, 10] and

Table II. *Tsc2* Mutations Identified by PCR-SSCP Analysis

Tumor	Exon	Codon[a]	Mutation[b]
Spontaneous tumors			
ERC 15	9	305–311	20 nt duplication (frame shift) 5′-CTGGGGAGCTCACCGACTCTATTCGCTC-3′
LK8B RT1	9	302–308	19 nt deletion (frame shift) 5′-ATGGCGCTCTGGGGAGCTCACCGACTC-3′
LK19b RT2	15	564–568	14 nt deletion (frame shift) 5′-GGCAGTCCTCGGGCTCCTGGTC-3′
LK19b RT3	19	719	1 nt deletion (frame shift) 5′-GCTACAAAG-3′
LK12g RT1	40	1732–1736	14 nt deletion (frame shift) 5′-CCGATCCAACCCCACGGACATC-3′
BN12a RT	intron 20		GA transition GT (splice donor site)→AT
LK20b RT	9	320	TC transition CTG (Leu)→CCG (Pro)
ENU induced tumors			
LU5C/LK6c RT1	21	790	CA transversion TAC (Tyr)→TAA (Stop)
LU5C/LK6D RT1	36	1602	TA transversion TGT (Cys)→TGA (Stop)
LU5C/LK6C RT2	intron 35		TC transition GT (splice donor site)→GC

a) Codon numbers are from the sequence including two alternatively spliced exons (exons 25 and 31) and the alternative splicing acceptor site of the intron 25/exon 26 boundary (CAG).
b) For the duplication and deletions, affected nucleotides in the normal sequence are underlined. For point mutations, sequences before and after the base-substitution are underlined.

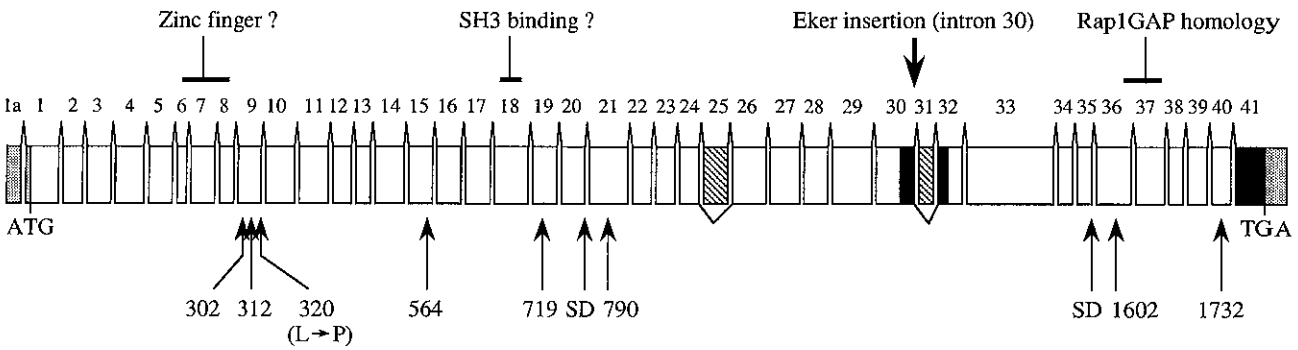

Fig. 1. Schematic representation of intragenic *Tsc2* mutations found in this study. Each numbered box indicates an exon of the rat *Tsc2* gene. Sites of translation initiation (ATG) and termination (TGA) codons are shown below. Two striped boxes, filled and shaded areas indicate alternatively spliced axons, transcriptional activating domains and 5′- and 3′-untranslated regions, respectively. Codon numbers corresponding to the predicted truncation sites of normal Tsc2 protein caused by mutations, one missense case (codon 320; leucine to proline) and two splicing donor sites (SD) affected by a point mutation are shown below with arrows. Structural characteristics of the Tsc2 protein are shown above.

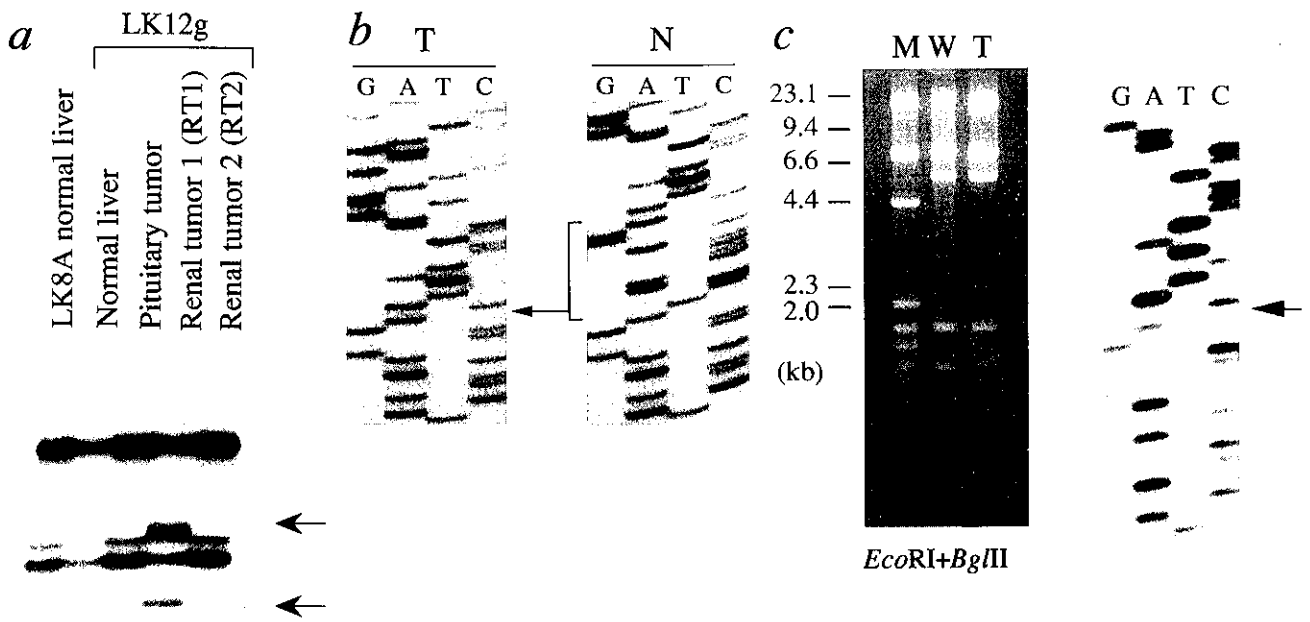

Fig. 2. Analysis of a mutation in LK12g RT1. a, SSCP analysis of exon 40. Tumor samples from two rats (LK8A and LK12g) are shown. Arrows indicate aberrantly shifted bands in the LK12g RT1 case. b, Sequence analysis of a shifted band (T) and the normal band (N) in LK12g RT1. A 14 nt deletion in exon 40 is denoted by the parenthesis and arrow. c, Analysis of a genomic DNA clone from LK12g RT1. The left panel shows the *Eco*R I/*Bgl* II double digestion pattern with phage clones containing a genomic *Eco*R I fragment covering the 3′ part of the *Tsc2* gene. Each clone contains a fragment from LK12g RT1 (clone 1, lane T), a mutant-type allele (lane M) or a wild-type allele (lane W) control from the Eker rat. The right panel shows the sequence ladder around the deletion point (arrow) in exon 40 in clone 1.

one tumor cell line derived from a spontaneous tumor (ERC15)[2] were examined using 45 primer sets covering 41 coding[13] and 1 leader exon (Kobayashi *et al.*, submitted) and splicing donor/acceptor sites (Table I). The mutations found are summarized in Table II and Fig. 1. Four deletions [19 nt in exon 9, 14 nt in exon 15, 1 nt in exon 19 and 14 nt in exon 40 (Fig. 2, a and b)] and one duplication (20 nt in exon 9), were detected in spontane-

ous RCs (LK8B RT1, LK19b RT2, LK19b RT3, LK12g RT1) and ERC15, respectively. These mutations cause frameshifts predicted to truncate the normal Tsc2 product at codons 302, 564, 719, 1732 and 312, respectively.[13] Interestingly, two tumors from a single rat (LK19b) possessed different deletions. The deletion found in LK19b RT2 was not present in RT3, and *vice versa*, revealing the multi-centric origin of renal tumors. Five cases (BN12a RT, LK20b RT, LU5C/LK6c RT1, LU5C/LK6D RT1 and LU5C/LK6C RT2) of a single base substitution were also identified: two nonsense mutations at codons 790 (exon 21; LU5C/LK6c RT1) and 1602 (exon 36; LU5C/LK6D RT1), one missense mutation substituting proline for leucine at codon 320 (exon 9; LK20b RT) and two splicing donor site mutations in introns 20 (BN12a RT) and 35 (LU5C/LK6C RT2).

Detection of Knudson's second hit in the wild-type *Tsc2* allele According to Knudson's two-hits hypothesis, intragenic mutations should occur in the wild-type *Tsc2* allele. Therefore, as a next step, we examined the allelic location in two applicable cases (LK12g RT1 and ERC15).

In the Eker rat, the germline insertion in intron 30 of the *Tsc2* gene creates RFLPs between the two *Tsc2*

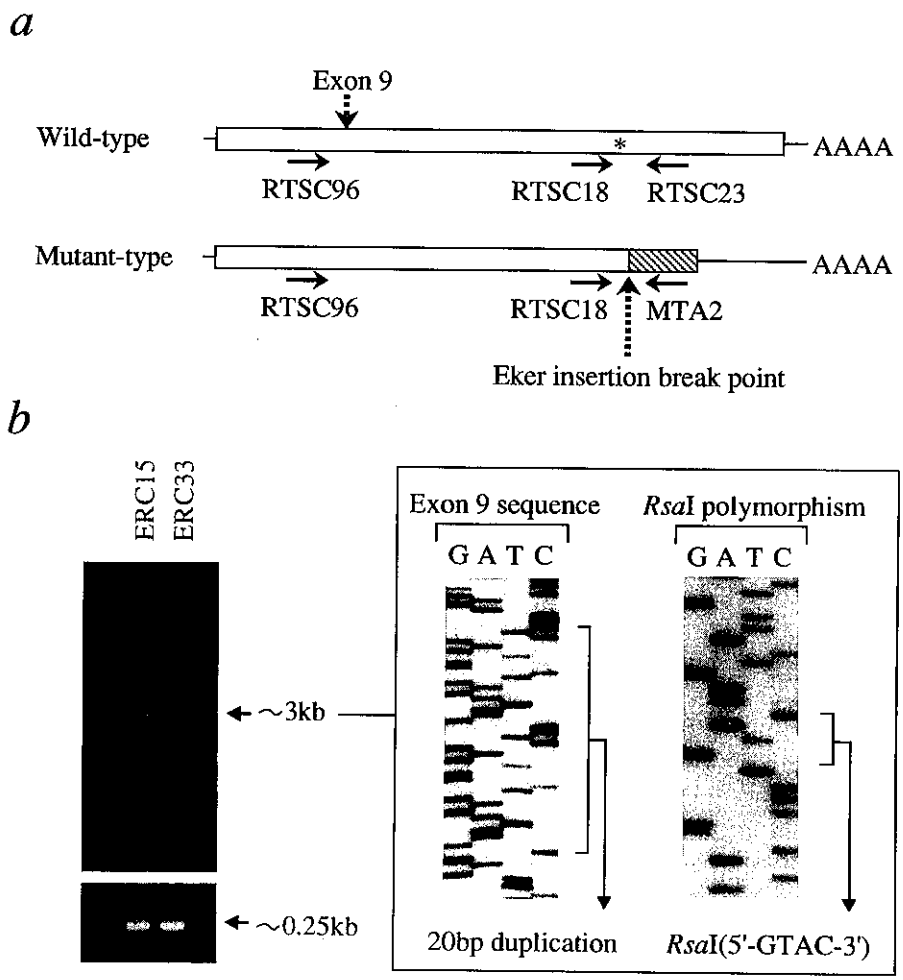

Fig. 3. Detection of the mutation on the wild-type *Tsc2* allele in ERC15. a, Primers used for amplification of *Tsc2* cDNA fragments. The wild-type and mutant-type cDNAs for the ERC 15 case are schematically represented. AAAA and the open and striped boxes indicate the poly (A) tail, the normal *Tsc2* open reading frame and the aberrant portion created by the Eker insertion, respectively. Locations of primers are shown by arrows with names. The asterisk denotes the *RsaI* recognition sequence at the polymorphic site in exon 30. b, The 20 bp duplication in the wild-type *Tsc2* cDNA in ERC15. Two panels on the left show the results of RT-PCR using the RTSC23/RTSC96 primer set for wild-type cDNA amplification (upper panel) and the MTA2/RTSC18 primer set for mutant-type cDNA amplification (lower panel). ERC33, lacking the wild-type *Tsc2* allele, was used as a control. In the right box, the results of sequence analysis of the 20 bp duplication and the polymorphic site in a 3kb fragment subclone amplified from ERC15 are shown.

alleles in the 3′ region in heterozygous carriers.[4] To take advantage of this, we cloned a wild-type polymorphic *Eco*R I fragment (~17 kb) containing exon 40 from LK12g RT1 DNA to examine whether the 14 nt deletion involved is located in the wild-type allele (Fig. 2c). Exon 40 sequencing of the isolated clone showed a 14 nt deletion identical to that found on SSCP analysis (Fig. 2c). Thus, we concluded that the 14 nt deletion found in LK12g RT1 is located on the wild-type *Tsc2* allele, and hence represents a second hit in accordance with Knudson's hypothesis.

For the second case (ERC15), we used RT-PCR to examine whether the 20 nt duplication found was present in the wild-type *Tsc2* allele (Fig. 3a). Primer sets encompassing the wild-type *Tsc2* cDNA sequence including the germline breakpoint fail to amplify efficiently fragments from mutant-type *Tsc2* mRNA templates affected by a germline insertion.[4] Use of such a primer set covering exon 9 resulted in the expected ~3 kb fragments being amplified from the ERC15 mRNA template, whereas no amplification occurred from ERC33 which had lost the wild-type *Tsc2* allele (Fig. 3b). Subclones from these ~3 kb fragments all contained a 20 nt duplication identical to that found by PCR-SSCP analysis (Fig. 3b). The two *Tsc2* alleles in ERC15 show a polymorphism in exon 30 which we reported previously (data not shown)[18]; the wild-type allele has a *Rsa*I recognition sequence at codons 1249–1250, whereas the mutant-type allele does not. All of the above subclones had this *Rsa*I recognition sequence (Fig. 3b). From these observations we can conclude that the 20 nt duplication is located within the wild-type *Tsc2* allele in ERC15 and, hence, could also be considered as a second hit.

DISCUSSION

Our previous studies suggest that a second somatic mutation involving the wild-type *Tsc2* gene (second hit) is a rate-limiting step for renal carcinogenesis in the Eker rat and that the *Tsc2* gene is a tumor suppressor.[2, 6, 7, 9, 19] Supporting those studies, here we detected intragenic somatic *Tsc2* mutations in addition to the germline mutation in 9 LOH-negative primary RCs and the ERC15 cell line from the Eker rat. In so doing, we clearly demonstrated the presence of intragenic somatic mutations in the wild-type *Tsc2* allele in two cases. This is the first demonstration at the DNA sequence level of the validity of Knudson's two-hits hypothesis in the *Tsc2* gene.

Our analysis revealed that the Eker rat RCs have various somatic *Tsc2* mutations in addition to the germline insertion. Except for three cases, including one missense and two splicing donor site mutations (LU5C/LK6c RT1, BN12a RT and LU5C/LK6C RT2), a protein truncation was predicted by each mutation found in this study. We have recently reported the existence of transcriptional activating domains (AD1 and AD2).[15] The mutation found in exon 40 in LK12g RT1 predicts a deletion in AD2, encoded by exon 41,[13] and this might be important for the function of the Tsc2 product. Although the effects of two splicing donor site mutations are not wholly predictable, they should include a deletion or insertion of a protein sequence, or instability of the *Tsc2* transcripts. The missense case, a leucine-to-proline substitution found in LK20b RT may cause an aberrant secondary structure of the Tsc2 product. We did not find any missense mutations in the Rap1-GAP homology region of the Tsc2 product (exons 36 and 37).[12, 13] The Tsc2 product also contains two potential zinc finger-like structures (exons 6–8) (unpublished) and an Src-homology 3 region (SH3) binding domain (exon 18)[20] (Fig. 1). We did not find any missense mutations in these regions in this study.

It is noteworthy that ENU-induced RCs exhibited only point mutations at various sites. This supports our hypothesis that ENU enhances renal carcinogenesis in the Eker rat by causing base substitutions (point mutations) in the wild-type *Tsc2* allele,[10] although only three tumors were characterized. As analysis of missense mutations can determine functionally important amino acid residues in proteins, our ENU-induced tumors should provide clues for elucidation of the functional role of the Tsc2 product. The Eker rat is also susceptible to radiation carcinogenesis,[2] and determination of radiation-induced second hits in the *Tsc2* gene may therefore be feasible. Thus, the Eker rat is a promising animal model for analyzing genetic events in hereditary and induced cancer.

Although four tumors could not be examined for all of the 42 exons due to the limited amount of DNA available, we failed to identify any *Tsc2* mutation in 16 of 30 tumors (53%) even when the examination was complete. Thus, our SSCP analysis may have limitations. One possible reason, other than insufficient sensitivity of SSCP analysis itself, is that these tumors could contain mutations in other extra-exonic regions which are critical for *Tsc2* gene expression, or have deletions in primer annealing sites. Another possible reason is reduction of expression of the wild-type *Tsc2* allele by some epigenetic mechanism such as methylation of the promoter region.[21] The latter is feasible, as the core promoter region of the *Tsc2* gene is localized on a CpG island (Kobayashi *et al.*, unpublished data). Further analysis may reveal the inactivating mechanisms in RCs which did not show mutations in our SSCP analysis.

It is well-known that tuberous sclerosis shows a variety of phenotypes even within the same family. Second somatic mutations are not yet well-characterized in human cases, though it was recently reported that hamartomas and angiomyolipomas developing in tuberous sclerosis

patients may demonstrate LOH at the *TSC2* locus.[22, 23] The Eker rat bears a single gene mutation with a dominant predisposition and develops four different tumors, kidney, spleen, uterus and pituitary, although the predilection for extra-renal tumors is not as complete as with RCs.[18, 24, 25] Further investigation, including a comparative mutational analysis of rat and man,[26–29] should provide insights into species-specific differences in tumorigenesis and/or cell-type specific carcinogenesis.

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