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Structure and Expression of a Murine Homologue of *sky* Receptor Tyrosine Kinase Gene[1]

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To identify new receptor tyrosine kinases (RTKs), we screened cDNAs from mouse mammary tumor cells and mouse brain. A homology search of the complete cDNA sequences obtained showed that one cDNA was a murine homologue of recently reported human *sky* [Ohashi, K. *et al.* (1994) *Oncogene* 9, 699-705]. Another cDNA obtained was also related to *sky* but had a 5′ upstream sequence similar to *brt* [Fujimoto, J. and Yamamoto, T. (1994) *Oncogene* 9, 693-698]. Analysis of the 5′ region of the *sky* genomic DNA revealed that *brt*-type and *sky*-type sequences are encoded by the *sky* gene in different exons. The upstream region of the *sky*-type coding exon is highly GC-rich and contains potential recognition sites for the Sp1 *trans*-acting factor, but lacks TATA and CAAT boxes, features commonly found in promoters of other RTKs. To examine whether this upstream region functions as a promoter, we fused it with chloramphenicol acetyltransferase (CAT) gene and transfected the construct into COS-7 cells. The results of the CAT assay showed that the *sky* upstream region retains a significant promoter activity. Furthermore, primer extension analysis revealed that the transcription starts at −240 nt upstream from the *sky* translation initiation codon. These observations suggest that the *brt*- and *sky*-types of mRNA are transcribed from a single *sky* gene by an alternative promoter usage.

Key words: brain, mammary tumor, promoter, receptor tyrosine kinase, *sky*.

Receptor tyrosine kinases (RTKs) play important roles in controlling growth and differentiation of a number of types of cells (*1*). RTKs are composed of three major regions, the extracellular region for binding to specific ligands, the transmembrane region, and the cytoplasmic region for signal transduction. The cytoplasmic region of a RTK contains a tyrosine kinase catalytic domain which is well conserved among RTKs over a wide variety of species (*2*). Recent advances in cloning techniques including PCR-based cloning (*3*) and expression cloning using anti-phosphotyrosine antibody (*4*) have facilitated rapid isolation of many novel RTK cDNAs. Among them were sequences related to the human and rat *sky* RTK cDNAs (*5, 6*), which encode two immunoglobulin-like domains and two fibronectin type III domains in the extracellular region and a protein tyrosine kinase domain in the intracellular region (*7-13*). In this study, we found two *sky*-related mouse cDNAs having different 5′ sequences. Based on this finding, we analyzed the mouse *sky* gene structure and characterized its promoter.

MATERIALS AND METHODS

Isolation of RTK cDNA Clones—Total RNA was extracted from primary culture of SHN mouse mammary tumor cells by the acid guanidinium thiocyanate-phenol-chloroform method (*14*). For cDNA synthesis, total RNA (1-5 μg) and a random hexamer (100 pmol; Takara Shuzo, Kyoto) in 5 μl of water were incubated at 65°C for 5 min then chilled on ice. The RNA was reverse-transcribed at 37°C for 90 min with SuperScript II (Gibco BRL) in a final volume of 20 μl according to the manufacturer's instructions. PCR was performed in 50 μl of a mixture containing 4 μM each of primers, 0.5-1 μl of cDNA template, 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 μg/ml gelatin, 0.2 mM each of dNTPs, and 0.025 units of *Taq* polymerase (Takara Shuzo) for 40 cycles (30 s at 94°C, 60 s at 40°C, 40 s at 72°C) using a DNA thermal cycler (Perkin Elmer Cetus). The following PCR primers, designed from the conserved tyrosine kinase domain (*2*), were used. TK5′ (sense primer): 5′-CAC[C/A]G[G/A]GA[C/T]NT[G/T/A]GC[T/A]GC[C/T/A][C/A]G[G/T/A]AA-3′ (corresponding to 1927 nt to 1949 nt from the first initiation codon of *tk19* cDNA sequence; DDBJ accession number D37777). TK3′ (antisense primer): 5′-TCCCA[C/G/A]AN[C/G/A]-[G/A]NNA[C/T]NCC[G/A][T/A]A[G/T/A][C/G]-[C/T/A]CCA-3′ (corresponding to 2141 nt to 2116 nt). The PCR products of around 200 bp were cloned into pBluescript II (Stratagene). After DNA sequence analysis, we identified a cDNA possibly encoding a novel RTK and named it *tk19* (#1 cDNA in Fig. 1). Based on the #1 cDNA sequence, we synthesized two sense primers, 5′-TTGTCG-ACGTGGCTGATTTTGGACTCTCTCG-3′ (corresponding to 1981 nt to 2003 nt with underlined additional nucleotides for a restriction site) and 5′-TTGTCGACTATTATCGTC-AGGGCTGTGC-3′ (corresponding to 2020 nt to 2042 nt),

[1] The sequence data reported in this paper have been submitted to the DDBJ database under the accession number D37777.

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; RTK, receptor tyrosine kinase; RACE, rapid amplification of cDNA ends; nt, nucleotide.

and performed 3′ RACE (Rapid Amplification of cDNA Ends) (*15*) as described previously (*16*). Screening including colony hybridization resulted in the isolation of #2 cDNA (Fig. 1). This cDNA was used as a probe for the following cDNA library screening.

A mouse brain cDNA library prepared in lambda gt10 (Clontech, Palo Alto, CA) was screened with the #2 cDNA probe. The longest cDNA obtained (#3-1) is shown in Fig. 1. To obtain 5′ upstream region of the *tk19* cDNA, BALB/c mouse brain cDNA library was constructed using poly(A)$^+$ RNA purified with Oligotex dT-30 (Nippon-Roche, Tokyo) and a mixture of specific antisense primers (AS2: 5′-TTCTCGAGCATATGGCGTCTGCCCAC-3′ corresponding to 2172 nt to 2153 nt and AS3: 5′-TTCTCGAGTTCAGGCCTTTCTCGATTG-3′ corresponding to 1438 nt to 1419 nt) in a lambda gt10 cDNA cloning system (Amersham). The #3-2 cDNA was thus obtained.

The double-strand cDNA with an *Eco*RI adapter attached to both ends, which was used in the above library construction, served as a template for the following adapter-PCR reaction. A sense primer (*Eco*RI adapter primer: 5′-GAATTCGAGGATCCGGGTACCATGG-3′) and AS2 primer were used for the first round of PCR. The *Eco*RI adapter primer and an antisense primer (AS4: 5′-CTTCTGGGGATCCCAGTCGG-3′ corresponding to 1134 nt to 1115 nt) were used for the second round of PCR. PCR was performed for 30 cycles (30 s at 94°C, 60 s at 55°C, 3 min at 72°C) using 0.1 μM of each primer and 1 μl of ds cDNA or the first-round PCR product. This PCR yielded #4 cDNA (Fig. 1). To obtain the region further upstream, an antisense primer was synthesized (AS5: 5′-TTCTCGAGCTGCAGTTGAGCTTCACTG-3′ corresponding to 165 nt to 146 nt) and used for the third round of PCR. This PCR yielded two 5′ cDNAs encoding different N-terminal peptide sequences, *sky*-type (#5 cDNA) (*5*) and *brt*-type (#6 cDNA) (*7*). A longer 1,263-bp cDNA (#7) of *brt*-type was next obtained by PCR using *brt*-sense primer (5′-TTTCTAGATGGACGACAAACTGG-3′) and EXC (extracellular) antisense primer (5′-TTTCTAGAGGTGACCGATGTGCGGCTGTGGGGAGG-3′ corresponding to 1253 nt to 1234 nt).

Isolation of the sky Genomic DNA—To isolate the *sky* genomic DNA, cassette PCR (*17*) was performed according to the manufacturer's instructions (Takara Shuzo). One microgram of genomic DNA obtained from the BALB/c mouse liver was digested completely with *Sau*3AI and ligated to a *Sau*3AI cassette DNA. Nested PCR was done using C1 (cassette primer 1) and AS5 primers for the first-round PCR (30 s at 94°C, 2 min at 55°C, 3 min at 72°C for 30 cycles) and, C2 (cassette primer 2) and AS5 primers for the second-round PCR. The product was electrophoresed, blotted onto a Hybond-N+ membrane (Amersham) and analyzed by Southern blot using the #4 cDNA as a probe. A positive band of around 0.7 kb was cloned into pBluescript II and sequenced (G1 DNA).

A mouse genomic DNA library (EMBL-3 SP6/T7, Clontech) was screened with the above G1 DNA as a probe and three positive clones (genomic clone 1, 3, and 4) were obtained. Exon-intron organization was identified by examining the overlap with the cDNA sequence.

DNA Sequence Analysis—DNA was sequenced by the chain termination method (*18*) in a Hitachi SQ-3000 DNA sequencer or with [α-^{32}P]dCTP labeling and X-ray film exposure. To determine the sequences of cDNAs obtained after the adapter-PCR, at least three independent cDNA clones were used.

Analysis of sky mRNA Expression—Ten micrograms of total RNAs were electrophoresed on a 1.2% formaldehyde/agarose gel and blotted onto a Hybond-N membrane (Amersham). Hybridization was carried out at 65°C for 2 h in the Rapid hybridization buffer (Amersham) using the [α-^{32}P]dCTP-labeled #2 cDNA as a probe. The membrane was then washed with 0.2×SSC at 65°C for 30 min and exposed to X-OMAT film (Kodak) for 48 h.

To analyze the relative abundance of the two types of *sky*/*brt* mRNAs by RT-PCR, *brt*-sense primer as described above, *sky*-sense primer (5′-GGACTGGCTTCTCTGCTGC-3′ corresponding to 55 nt to 73 nt), and Exon 4 antisense primer (5′-AGTGAGCCATACTGACTGAG-3′ corresponding to 353 nt to 372 nt) were used. To obtain standard DNAs for PCR quantification, we constructed two types of plasmids having the *brt*- or *sky*-type 5′ cDNA sequence from the mouse brain cDNA. The #7 cDNA, obtained by PCR using *brt*-sense and EXC antisense primers (Fig. 1) followed by cloning into the plasmid, served as a source of *brt*-type DNA. The 5′ region of this *brt*-type DNA was replaced with the *sky*-type 5′ DNA fragment cleaved from the #5 cDNA. One picogram each of the plasmids containing these DNA inserts was mixed and used as a standard for PCR analysis. PCR was performed using the brain cDNA preparations and *brt*-sense, *sky*-sense and Exon 4 primers (30 s at 94°C, 50 s at 60°C, 50 s at 72°C for 30 cycles). The PCR products were separated by electrophoresis on a 5% polyacrylamide gel and alkali-blotted onto a Hybond-N+ membrane. Southern blot analysis was done using the #4 cDNA as a probe. The *brt*- and *sky*-type PCR products were identified as 365-bp and 318-bp bands, respectively.

Assay for Promoter Activity—CMV promoter-enhancer fragment or the putative mouse *sky* promoter region was cloned into the *Hin*dIII site of pSV00CAT (Nippon Gene, Toyama) as described below, and the products were named pSVCMVCAT and pSV*sky*CAT, respectively. CMV promoter-enhancer DNA fragment was excised from pRc/CMV plasmid (Invitrogen) with *Sal*I and *Apa*I and cloned into pBluescript II. The CMV promoter-enhancer fragment was then excised from the resulting plasmid with *Hin*dIII. To construct the pSV*sky*CAT, a *Xba*I fragment of 3 kb containing the mouse *sky* exons 2A, 2B, 2C, and 3 was first cloned into pBluescript II. The putative *sky* promoter region (*Xba*I-*Apa*I, 870 bp) was then excised with *Apa*I and cloned into pBluescript II. The putative *sky* promoter region was finally excised from the resulting plasmid with *Hin*dIII. One microgram each of pSV00CAT, pSV*sky*CAT, or pSVCMVCAT was co-transfected with 0.25 μg of pCH110 (a β-galactosidase assay vector; Pharmacia) into COS-7 cells (RIKEN cell bank) cultured in a 35-mm well by using LipofectAMINE (Gibco BRL). After incubation for 72 h, cells were harvested and extracted in 100 μl of 250 mM Tris-HCl (pH 7.5). For normalization of the transfection efficiency, β-galactosidase activity in extracts was determined (*19*). Chloramphenicol acetyltransferase (CAT) reaction was performed using [^{14}C]1-deoxy chloramphenicol (sp. act., 2 GBq/mmol, 925 kBq/ml; Amersham) (*19*). Acetylated reaction products were separated on a silica gel plate (Kodak) and autoradiographed. After autoradiography, the portion of the silica gel plate corre-

sponding to the acetylated 1-deoxy chloramphenicol was cut out and the radioactivity was measured.

Analysis of the Transcription Start Site—Two antisense primers within the exon 2C, PX-1 (5′-GAGCAGCAGAGA-AGCCAGTC-3′, corresponding to 75 nt to 56 nt from the first nucleotide of the translation initiation codon) and PX-2 (5′-GGGGCTGCGGCTGGGCCACC-3′, corresponding to −171 nt to −190 nt) were used. Primer extension analysis was done using primers end-labeled with [γ-^{32}P]ATP. Forty micrograms of total RNA from the brain or the liver and 1 μl of the end-labeled primer in an annealing buffer containing 80% formamide, 0.4 M NaCl, 40 mM piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES, pH 6.4), 1 mM EDTA were heated for 5 min at 85°C then incubated for 16 h at 60°C. The annealed DNA was precipitated with ethanol and used for reverse transcription at 45°C as described above. Primer-extended products were run on an 8% polyacrylamide denaturing gel alongside the known sequencing ladders primed with the same oligonucleotide.

RESULTS

Isolation of the tk19 cDNA—To isolate cDNAs encoding novel RTKs, we synthesized degenerate PCR primers designed from the conserved amino acid sequence in many tyrosine kinases (*2*) and amplified cDNAs from mouse mammary tumor cells. Sequence analysis of the obtained cDNAs revealed that one of them, *tk19*, was highly homologous to the corresponding regions of *tyro* 3 (*20*), clone 37 (*21*), *etk*-2 (*22*), and ES79 (*23*). After 3′ RACE we obtained a cDNA encoding the C-terminal region of the TK19 protein (Fig. 1, #2). The amino acid sequence deduced from the nucleotide sequence showed that 46% of the amino acids were identical to those of the corresponding C-terminal region of human AXL/UFO (*24, 25*). Based on a preliminary Northern blot analysis which showed that *tk19* was highly expressed in the brain, we cloned overlapping cDNA fragments encompassing the entire coding region of *tk19* from the brain cDNA (#3-1, #3-2, #4, and #5). After we had completed the sequence analysis of *tk19*, several groups reported the cDNA cloning of similar cDNAs, *i.e.*, *sky* (*5, 6*), *brt* (*7*), *tif* (*8*), *rse* (*9*), *tyro* 3 (*10, 11*), and *dtk* (*12, 13*). Since it seemed most likely that these cDNAs and *tk19* are all derived from the same gene, we decided to designate *tk19* as a murine homologue of *sky* to avoid further complication. The 880-amino acid sequence included a putative signal peptide, two immunoglobulin (Ig)-like domains (*26*) and two fibronectin type III (FN III) domains (*27*) in the extracellular region, the transmembrane region, and the cytoplasmic region, which contains a conserved sequence for protein tyrosine kinase with an ATP-binding site and a phosphatidylinositol 3-kinase binding site (*28*) (Fig. 1).

Expression of sky mRNA—Since we initially found the *sky* cDNA in mammary tumor cells, we examined its expression in the mammary gland and mammary tumors in addition to the brain and the testis. As other investigators have reported (*5-13*), high levels of expression of the

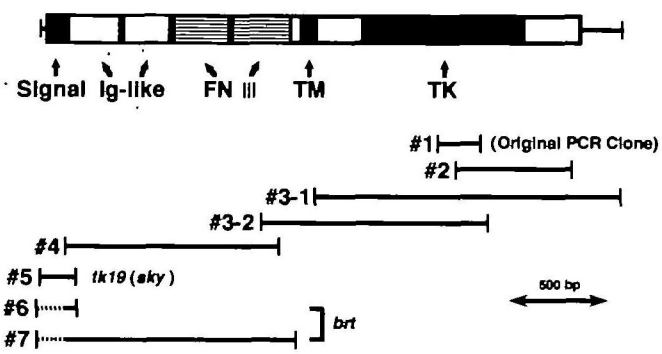

Fig. 1. **Schematic diagram of the *tk19* cDNA.** The coding region is shown by a box, and the 5′ and 3′ untranslated regions are shown by horizontal lines attached to the box. Signal, putative signal peptide sequence; Ig-like, immunoglobulin-like domain; FN III, fibronectin type III domain; TK, tyrosine kinase domain. The lines with numbers 1 to 7 indicate the location and the size of the isolated cDNAs. *sky*, a cDNA with the *sky*-type 5′ sequence; *brt*, cDNAs with the *brt*-type 5′ sequence. The location of the *brt*-type 5′ sequence is shown by broken lines in #6 and #7 cDNAs.

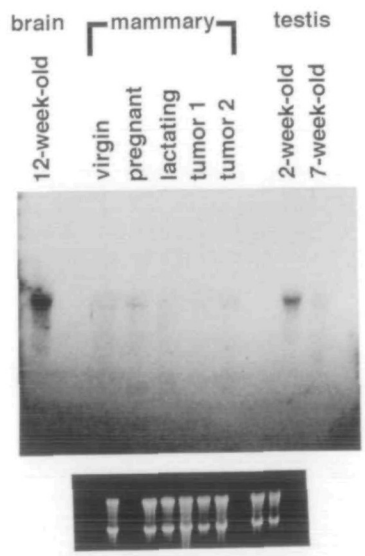

Fig. 2. **Analysis of *sky* mRNA expression.** Ten micrograms of total RNA isolated from the indicated mouse tissues were analyzed by Northern blot using the #2 cDNA as a probe. Ethidium bromide-stained 28S and 18S ribosomal RNAs on an agarose gel are shown at the bottom for comparison of the amount of total RNA applied. RNAs were obtained from 15-week-old virgin female, 16-day-pregnant, 7-day-lactating mammary glands, or from spontaneous mammary tumors of the SHN mouse.

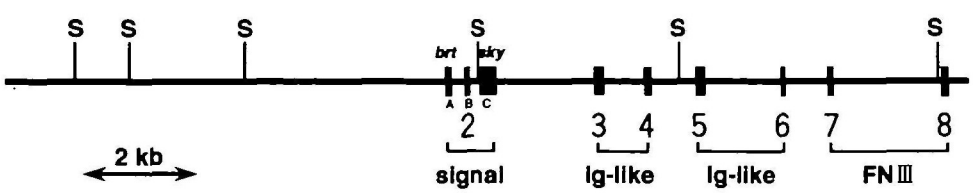

Fig. 3. ***Sac*I restriction map and location of exons in the mouse *sky* gene.** Nine exons are shown by solid boxes. S, *Sac*I restriction sites; Ig, immunoglobulin-like domain; FN III, fibronectin type III repeat; signal, putative signal peptide sequence.

4.4-kb *sky* mRNA were confirmed in the brain and the testis (Fig. 2). In the testis, the level of *sky* expression is higher at 2 weeks than at 7 weeks of age. In the virgin mammary gland, *sky* is expressed at a low level. The expression slightly increased in the pregnant mammary gland and then decreased in the lactating mammary gland. Overexpression of *sky* has not been seen in any of 16 spontaneous mammary tumors of the SHN mouse (two representative sets of data are shown in Fig. 2).

Structure of the sky Gene—During the course of the cDNA cloning, we found another type of cDNA that could possibly encode an alternative N-terminal region of *sky* (Fig. 1, #6 and #7 cDNAs). The 5′ region of these cDNA clones closely matched that of *brt* or *tyro* 3B (*7, 11*). Because the two different types of cDNAs shared a common 3′ downstream sequence, we hypothesized that the two mRNAs were produced by an alternative splicing. To confirm this, we analyzed the gene structure of *sky*. Using the cassette PCR technique, we cloned the genomic DNA around the diverging point of the two types of cDNAs. Computer analysis of the above genomic DNA sequence showed that the diverging point had a feature of exon-intron junctions (*29*). Using this genomic DNA as a probe, we then screened a mouse genomic library and obtained three positive clones (genomic clone 1, 3, and 4). Each clone had an insert of about 16 kb. Of the three clones, clones 1 and 4 hybridized with both #5 and #6 cDNA (*sky*- and *brt*-type) probes. The *Sac*I restriction map of the insert DNA of clone 4 and the lo[illegible]xons are shown in Fig. 3. Based on the cDNA sequences obtained in this study, we identified the presence of eight exons, 2A, 2C, 3, 4, 5, 6, 7, and 8, in the DNA sequence of clone 4. The two 5′ exons encode *brt*-type and *sky*-type 5′ mRNA, respectively. The numbering of the 5′ exons, 2A, 2B and 2C, follows the recent report by Biesecker *et al.* (*30*). The exons 2A (*brt*-type) and 2B [E2-29-type (*30*)] encode ambiguous signal peptides, whereas the exon 2C (*sky*-type) encodes a putative signal peptide sequence which conforms to the (−3), (−1) rule of Heijine (*31*). A comparison of the *sky* gene structure with that of the human *axl/ufo* gene (*32*) revealed that the exon arrangements closely resembled each other. The exon-intron junction sequences of the *sky* gene are shown in Fig. 4.

RT-PCR Analysis of Expression of the Exons 2A and 2C—To further examine which of the exons is mainly used for the *sky* mRNA in the brain, the expression of 5′ regions was examined using the RT-PCR method (Fig. 5). After PCR using sense primers specific for the exons 2A (*brt*-type) and 2C (*sky*-type) in combination with an antisense primer for the common exon 4, the products were separated

Exon no.	Exon size (bp)		Intron size (kb)		Exon no.
2A	(?)	.....ATTAAAG	gtaggc...(2.6)...		
2B	(?)	.....AGGGAGC	gaggca...(2.3)...	ctgcag GCCTGAA	3
2C	(334)	.....GCCGCAG	gtaggg...(1.8)...		
3	(184)	.....TACTCAG	gtgcag...(0.7)...	tcacag CCTAAAG	4
4	(101)	.....GTCGAAG	gtgagg...(0.8)...	ccctag GTGTGCC	5
5	(171)	.....GTGACAG	gtgagc...(1.4)...	ccccag GAGTGAC	6
6	(87)	.....CTTCAAG	gtaggg...(0.7)...	ccacag CACCGCC	7
7	(116)	.....TGTACAG	gtaggc...(2.0)...	ctgaag GTGGCAC	8
8	(178)	.....GGCCTAG	gtaaga...(?)...	? CGCCAGC	9

Fig. 4. **Nucleotide sequences at the splice junctions in the mouse *sky* gene.** Nucleotides in the exons are shown in upper-case letters, and those in the introns are shown in lower-case letters.

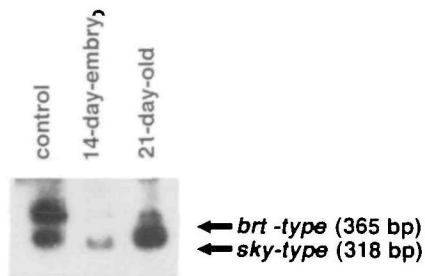

Fig. 5. **RT-PCR analysis of *brt*- and *sky*-type mRNA expression in the mouse brain.** Primers used were *brt*-sense and Exon 4 antisense primers for *brt*-type cDNA, and *sky*-sense and Exon 4 antisense primers for *sky*-type cDNA amplification. Total brain RNAs from the indicated age were reverse-transcribed into cDNAs and used as templates. After PCR the products were analyzed by Southern blot with the #4 cDNA as a probe. As controls for PCR, *brt*-type and *sky*-type cDNAs were used. The *brt*- and *sky*-type products were detectable as 365-bp and 318-bp bands, respectively.

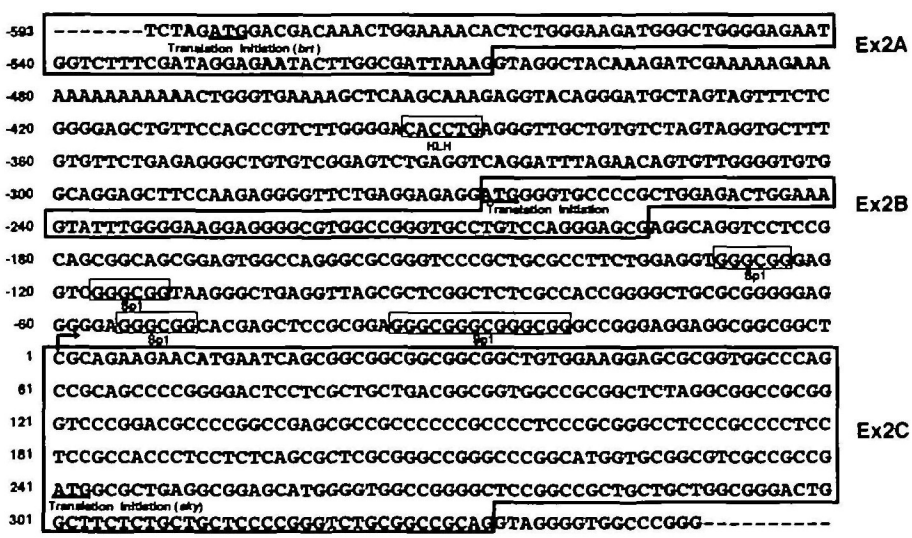

Fig. 6. **Nucleotide sequence of the 5′ region of the mouse *sky* gene.** The nucleotide of the major transcription start site determined by primer extension analysis (shown by a broken arrow) is assigned the number +1. Nucleotides 5′ to this position are assigned negative numbers. The exons 2A, 2B, and 2C, and conserved motifs for Sp1 and bHLH binding sites are boxed.

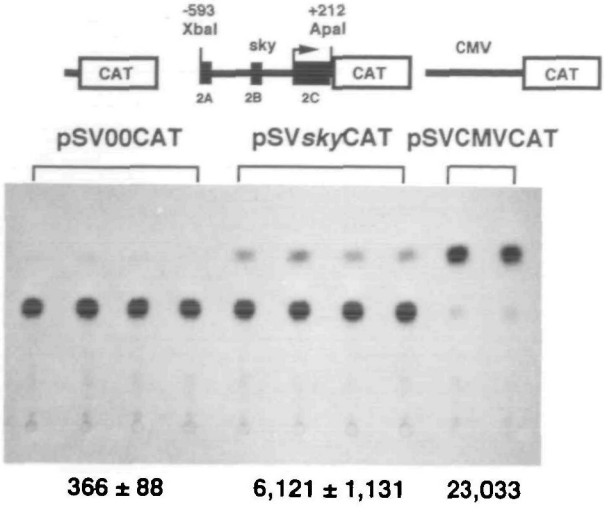

Fig. 7. **CAT activity in COS-7 cells.** Schematic representation of the plasmids used for transfection is shown at the top. CAT, pSV00CAT plasmid. *sky*, the putative promoter region of the *sky* gene. The major transcription start site is shown as a broken arrow (as the nucleotide number +1). Black boxes indicate exons. CMV, the enhancer-promoter sequence from the human cytomegalovirus. COS-7 cells were co-transfected transiently with each of the above plasmids and pCH110. CAT activity was measured 3 days after transfection. CAT assay was performed on extracts normalized for β-galactosidase activity. Mean cpm±SE of the acetylated products are shown at the bottom.

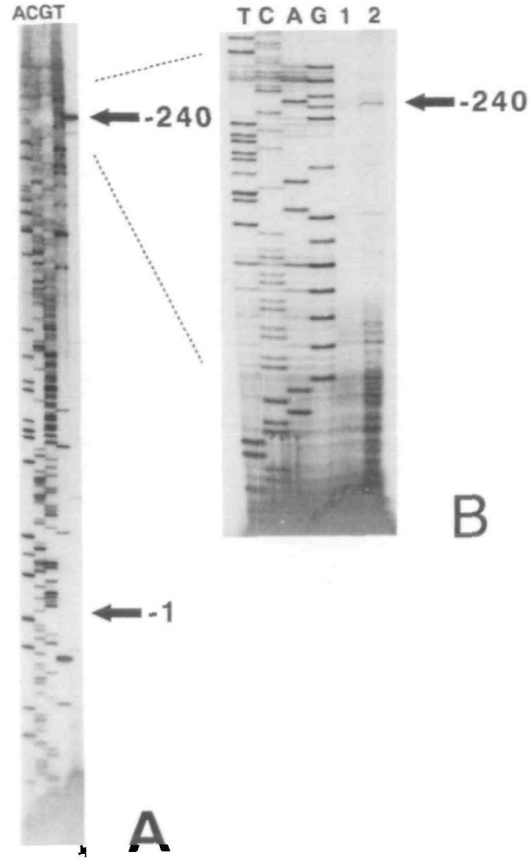

Fig. 8. **Primer extension analysis of *sky*.** (A) Primer extension analysis was done using an antisense primer, PX1 (75 nt to 56 nt from the translation initiation site), and total RNA from the brain as described in "MATERIALS AND METHODS." The nucleotide sequence ladder of the *sky* gene primed with the PX1 is shown on the left side as a size marker. A major band (arrow) is seen around −240 nt from the translation initiation site. (B) Primer extension analysis was done using an upstream primer, PX2 (−171 nt to −190 nt) and total RNA from the liver (lane 1) or the brain (lane 2). The nucleotide sequence ladder was made with the PX2 primer. Note the presence of a brain-specific major band at −240 nt from the translation initiation site (arrow).

by polyacrylamide gel electrophoresis and analyzed by Southern blot. The results showed that the exon 2C-derived PCR product (*sky*-type, 318 bp) was the major one throughout the stages of mouse brain development. PCR using the same amounts of control cDNAs in the same amplification mixture showed that both primers are equally effective in amplifying these cDNAs. Exon 2C is also expressed as a major product in the testis (data not shown).

Analysis of the sky Promoter Region—To localize the sequence that regulates the expression of exon 2C, we determined the DNA sequence of the upstream region of this exon. As shown in Fig. 6, a GC-rich region is recognizable upstream of the exon. A homology search of this region identified consensus sequences for transcription factor binding sites. In particular, binding sites for transcription factor Sp1 (GGGCGG) (*33*) are present at −21 nt to −34 nt, −50 nt to −55 nt, −112 nt to −117 nt, and −124 nt to −129 nt, and a basic helix-loop-helix (bHLH) protein binding site (CANNTG) (*34*) is present at −388 nt to −393 nt. However, TATA and CAAT box consensus elements are absent in the region. These features are commonly found in other RTK genes such as *axl/ufo* (*32*), the NGF receptor (*35*), the CSF-1 receptor (*36*), c-*kit* (*37*), and the insulin receptor (*38*). Since the expression of the *sky* mRNA has been reported in the human kidney (*5, 8, 9, 12*) and the mouse kidney (*7, 10, 11*), and the promoter activity of the TATA box-absent insulin receptor gene has been demonstrated in the kidney-derived COS cells (*38*), we examined the promoter activity of the 5′-flanking region of the *sky* exon 2C using COS-7 cells. As shown in Fig. 7, extracts from COS-7 cells transfected with pSV00CAT (without promoter) showed little CAT activity, whereas those from COS-7 cells transfected with pSV*sky*CAT showed a low but significant CAT activity. Extracts from COS-7 cells transfected with pSVCMVCAT, a positive control, showed high CAT activity.

Determination of the Transcription Start Site of the sky Gene—Since exon 2C was expressed as a major transcript and the presence of a possible promoter in the 5′ region of exon 2C was suggested, we examined the transcription start site of the *sky* gene by primer extension analysis using a primer, PX1, derived from the coding region on exon 2C. For primer extension, total RNA from the brain was annealed to the ^{32}P-labeled PX1 and reverse transcribed. As a result, a major extended band located at around −240 nt from the first translation initiation codon and a few minor bands were detected (Fig. 8A). To determine the transcription start site more precisely, we next used an upstream antisense primer, PX2, and total RNAs from the liver (low level expression of the *sky* mRNA) and the brain. After primer extension, we found a major band which is specific to the brain (lane 2, Fig. 8B). The size of the band well matched the result of primer extension analysis shown

in Fig. 8A. A band of the same size was seen when total RNA from the testis was used (data not shown). These results suggest that the major transcription start site of the *sky* gene is −240 nt relative to the translation initiation site (see Fig. 6).

DISCUSSION

In this study we cloned a new receptor tyrosine kinase cDNA, *tk19*, and found that the *tk19* mRNA was expressed mainly in the mouse brain and testis. The amino acid sequence deduced from the cDNA sequence of *tk19* revealed that TK19 was identical to RSE/DTK (*9, 13*) and closely related to TYRO 3 (*10, 11*). These murine homologues of human SKY (*5*) belong to a family of RTKs having two immunoglobulin-like domains and two fibronectin type III domains in the extracellular region, the transmembrane domain and a protein kinase domain in the cytoplasmic region. This family consists of SKY/BRT/RSE/TYRO 3/DTK, AXL/UFO/ARK, EYK, and MER (*5-7, 9-13, 24, 25, 39-42*). In this paper we adopted the name *sky* in place of *tk19* to avoid further complication in naming.

We found two types of 5′ sequences, *brt*-type and *sky*-type, in the *sky* cDNA. Recently, Schulz *et al.* (*11*) also reported the presence of these sequences in the mouse *tyro* 3 cDNA. To elucidate whether the two types of 5′ sequences of the *sky* mRNA are transcribed from the same gene or from different genes, we isolated genomic DNA clones of *sky*. Subsequent genomic DNA sequence analysis revealed that both sequences are present in the *sky* gene as different exons. A similar observation on the structure of the 5′ region of the *sky* gene has recently been published by Biesecker *et al.* (*30*) while this manuscript was in preparation. To further examine the possibility of alternative promoter usage, which has been reported for the colony stimulating factor-1 receptor gene (*36*), we sequenced the 5′-flanking region of exon 2C. The results showed that this region is highly GC-rich, contains Sp1 binding sites (*33*) and a basic helix-loop-helix (bHLH) protein-binding site (*34*), but lacks TATA and CAAT boxes. These features are commonly found in other RTK genes such as *axl/ufo* (*32*), the NGF receptor (*35*), the CSF-1 receptor (*36*), c-*kit* (*37*), and the insulin receptor (*38*). To determine the promoter activity of this 5′ region, we constructed the plasmid pSV*sky*CAT and transfected it into COS-7 cells. The extracts from transfected cells showed a distinct CAT activity. These results strongly suggest that the 5′ region of exon 2C functions as a promoter for the *sky* gene.

Initially we cloned the *sky* cDNA from mouse mammary tumor cells and expected that it would be overexpressed in mammary tumor cells. However, Northern blot analysis of total RNA from more than 16 spontaneous mammary tumors of the SHN mouse showed no evidence of overexpression of the *sky* mRNA as compared with the expression levels in virgin, pregnant, and lactating mammary glands. Mark *et al.* reported a high level of expression of *sky* in the human breast (*9*), and Taylor *et al.* (*43*) reported the overexpression of *sky* in mammary tumors of the transgenic mice that express *wnt*-1, *fgf*-3, or both. These reports suggested that *sky* may be related to the development and/or progression of normal and abnormal mammary glands. In this study, however, we were unable to detect the overexpression of *sky* in mammary tumors. Thus, SKY may not be involved in the spontaneous mammary tumorigenesis in the SHN mouse.

As for the functions of *sky* family genes, *axl/ufo* and *eyk* are known as oncogenes (*24, 44*). TYRO 3 has also been shown to be oncogenic, since Rat-2 fibroblasts transfected with the gene continued to grow in soft agar (*10*). The activation of these oncogenes may be induced by a homophilic interaction between the extracellular regions of the RTKs. This hypothesis came from the resemblance of the extracellular regions of SKY and that of neural cell adhesion molecule (*45*). DTRK (*46*), a *trk* family RTK having an adhesion molecule-like structure, and ARK (*47*) have been shown to be involved in the homophilic interaction. Likewise, protein tyrosine phosphatases, with extracellular regions similar to SKY, have been reported to mediate the homophilic binding (*48, 49*). These results suggest the possibility that the homophilic interaction between the SKY molecules may contribute to cell-cell interactions in the brain and the testis. Alternatively, the ligand-receptor interaction, as seen in many RTKs, may be operative. In this context, the recent identification of protein S or Gas6 as the ligand for SKY (*50-52*) is particularly important. Which of these mechanisms, *i.e.*, homophilic binding or ligand binding, is functional in different tissues awaits further investigation.

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