Am. J. Hum. Genet. 59:1084–1090, 1996

Genomic Imprinting of the Human Serotonin-Receptor (*HTR2*) Gene Involved in Development of Retinoblastoma

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Summary

Epidemiological and genetic studies of retinoblastoma (RB) suggested that imprinted genes might be genetically linked to the RB gene. In this study, we found that the human serotonin-receptor, *HTR2*, gene, which had been mapped nearby the RB gene on chromosome 13, was expressed only in human fibroblasts with a maternal allele and not in cells without a maternal allele. The 5′ genomic region of the human *HTR2* gene was cloned by PCR-mediated method. Only the 5′ region of the gene was methylated in cells with the maternal gene, and it was not methylated in cells without the maternal gene. A polymorphism of *Pvu*II site of the gene was also found and useful for the segregation analysis in a family of a RB patient and for analysis of loss of heterozygosity on chromosome 13 in tumor and its parental origin. These results suggest that the human *HTR2* gene might be affected by genomic imprinting and that exclusive expression of the maternal *HTR2* gene may be associated with the delayed occurrence of RB, which had lost the maternal chromosome 13.

Introduction

Preferential mutation of the paternal allele of the retinoblastoma (RB) gene (Ejima et al. 1988; Dryja et al. 1989; Toguchida et al. 1989; Zhu et al. 1992; Kato et al. 1994*a*) and the delayed development of RB that have lost maternal chromosome 13 suggest that a growth-associated gene, which is affected by genomic imprinting, might exist in the vicinity of the RB gene (Kato et al. 1993, 1995). An imprinted gene on chromosome 13 was also predicted to be linked to the RB gene from the epidemiological and genetic analysis of RB (Naumova and Spienza 1994). Genomic imprinting, a phenomenon whereby specific paternal and maternal alleles are expressed unequally, has been reported for 16 genes (Barlow 1995). Some of these genes have been shown to be associated with human diseases and with the development of the mouse embryo (Barlow 1995). A candidate gene, *HTR2*, which has been mapped to the q14-q21 region on chromosome 13 (Hsieh et al. 1990; Sparkes et al. 1991; Bowcock 1993), encodes the receptor for a neurotransmitter, serotonin (5HT), and this gene has transforming activity that is dependent on 5HT (Julius et al. 1990).

RB is a pediatric malignant eye tumor. Loss of function of the RB gene is critical for the development of RB (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). About 30% of total RB patients are affected by bilateral multifocal tumors (Vogel and Rathenberg 1975) and categorized as hereditary who had inherited germ-line mutations of the RB gene. About 8.5% of new germline mutations can be detected by the karyotype analysis (Ejima et al. 1988). These mutations were interstitial deletions or rearrangements that involves 13q14 region. Somatic cells of these patients with an interstitial deletion of chromosome 13 harbor only the paternal or maternal chromosomal region that involves the RB gene and other genes in 13q14 region. These cells are also useful for analysis of the monoallelic expression of the genes that are inherited from only either parent. In this study, we cloned the 5′ region of the *HTR2* gene and also found a polymorphism of a *Pvu*II site, which was useful for segregation analysis of the family of RB patients and for loss of heterozygosity (LOH) in tumor cells and its parental origin. We also investigated that the methylation of the 5′ region and expression of the *HTR2* gene in the cells that harbor only either a paternal or maternal allele.

Patients, Material, and Methods

Patients and Samples

Tumor tissues and skin biopsies from RB patients and their parents were obtained at the National Cancer Center Hospital. Tumor cells were subjected directly to isolation of DNA. Fibroblasts expanded from skin biopsies

Received May 13, 1996; accepted for publication August 6, 1996.

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0002-9297/96/5905-0017$02.00

of RB patients and their parents and a normal healthy individual were cultured in modified Eagle's minimum essential medium (Irvine) supplemented with 10% fetal bovine serum (HyClone) as described elsewhere (Ejima et al. 1988; Kato et al. 1993).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

cDNA of the *HTR2* and β-actin genes were amplified by RT-PCR as described by Kato et al. (1994*b*). Primers used were CAAACATCATGGCCGTCA and CACTCCGTCGCTATTGTC for the *HTR2* gene and CGTGGGCCGCTCTAGGCACCAG and TTGGCCTTAGGGTTCAGGGGGG for the β-actin gene.

Cloning of the 5′ Region of the Human HTR2 *Gene*

The 5′ region of the *HTR2* gene was cloned by a PCR-mediated method (Siebert et al. 1995) using a PromoterFinder DNA Walking kit (Clontech). Human genomic DNA was digested with *Pvu*II, ligated with adaptor, and amplified with adaptor primer 1 (AP1; CCATCCTAATACGACTCACTATAGGGC) and gene-specific primer 1 (GSP1; CATTCACCTTGATGTACCCACACTC). The amplified DNA was reamplified with AP2 (CTATAGGGCACGCGTGGT) and GSP2 (AACACTGAGGCTGGTGTACATGCTG). The amplified fragment was cloned into pCRII vector (Invitrogen), and the sequence was determined by the dideoxy chain-termination method with ΔTth polymerase (Toyobo). The genomic map of the 5′ region of the *HTR2* gene was determined by Southern blot analysis of *Pvu*II-digested DNA with the cloned sequence as probe.

Southern Blot Analysis

Southern blot analysis was accomplished as described by Kato et al. (1993). Genomic DNA was digested with appropriate endonucleases, fractionated in 0.7% agarose gel, and transferred to a nylon membrane filter (Hybond™ N; Amersham Japan). After hybridization with [^{32}P]-labeled probe, bands were detected by autoradiography.

Results

Expression of the HTR2 *Gene in RB Patients with Chromosomal Deletions and Rearrangements*

The expression of both the maternal and paternal RB genes has been detected in human somatic cells, although the expression of mutated alleles with a premature stop codon has not been detected in somatic cells of RB patients (Dunn et al. 1989; Kato et al. 1994*b*). In this study, to determine the dependence on parental origin of the expression of the human *HTR2* gene, we examined fibroblasts of hereditary RB patients (Ejima et al. 1988; Kato et al. 1994*a*) who had inherited either only the paternal or the maternal allele on chromosome 13 (as summarized in fig. 1). The expression of the *HTR2* gene was analyzed by the RT-PCR method. The PCR products were detected in cells from a normal healthy individual, in those from three RB patients with paternal deletion of chromosome 13 (RB32, RB162, and RB182), and in those from two patients with translocation of chromosome 13 with an other chromosome (RB9 and RB165), but they were not detected in cells from RB patients with maternal deletion on chromosome 13 (RB88 and RB141) (fig. 2).

Cloning of the 5′ Region of the Human HTR2 *Gene*

Methylation of the cytosine residue in the sequence CpG is a strong candidate for a marker of genomic imprinting (Barlow 1995). To analyze the methylation of the 5′ region of the *HTR2* gene, the upstream region of the *HTR2* gene was cloned by a PCR-mediated method (Siebert et al. 1995). Figure 4*A* shows a comparison of the sequences of the 5′ region of the *HTR2* gene from four mammalian species. Conserved regions included consensus sequences for binding sites for the transcription factors, bicoid (bcd; Driever and Nusslein-Volhard 1989), hunchback (hb; Stanojevic et al. 1989), s8 (de Jong et al. 1993), and δEF1 (Sekido et al. 1994) (fig. 4*A*). Sp1-like and TATA-like sequences were also found in the human sequence but not in the sequences of the other three species. Moreover, in the human 5′ region of the *HTR2* gene, five CpG sites and several short repeated sequences were identified (fig. 4*A*) that have been supposed to be characteristics of the imprinted genomic regions (Barlow 1995).

Polymorphism of the PvuII *Site*

The 5′ region of the *HTR2* gene was analyzed by Southern blot method using the cloned sequence as probe (fig. 3). Two bands, 9.4 kb (allele 1) and 3.6 kb (allele 2), were detected when genomic DNA was digested with *Pvu*II. These two bands originated from polymorphism at the *Pvu*II site locating 3.6 kb from the *Pvu*II site at the 5′ end of the cloned sequence of the *HTR2* gene (fig. 4*B*).

LOH at the Locus of the HTR2 *Gene and Its Parental Origin*

In the family of RB228, the patient and her mother were heterozygous for both alleles and the father was homozygous for allele 2, and her tumor cells lost paternal allele 2 (fig. 3 and table 1). These results are consistent with our previous report that the paternal allele of the RB gene was lost in tumor of RB228 (Kato et al. 1994*a*). Moreover, tumor cells of unilateral RB patients (RB232 and RB270) lost one allele, and tumor cells of right eye of bilateral RB patient RB313 retained the heterozygosity. These results are also consistent with our previous report (Kato et al. 1993). Therefore, the

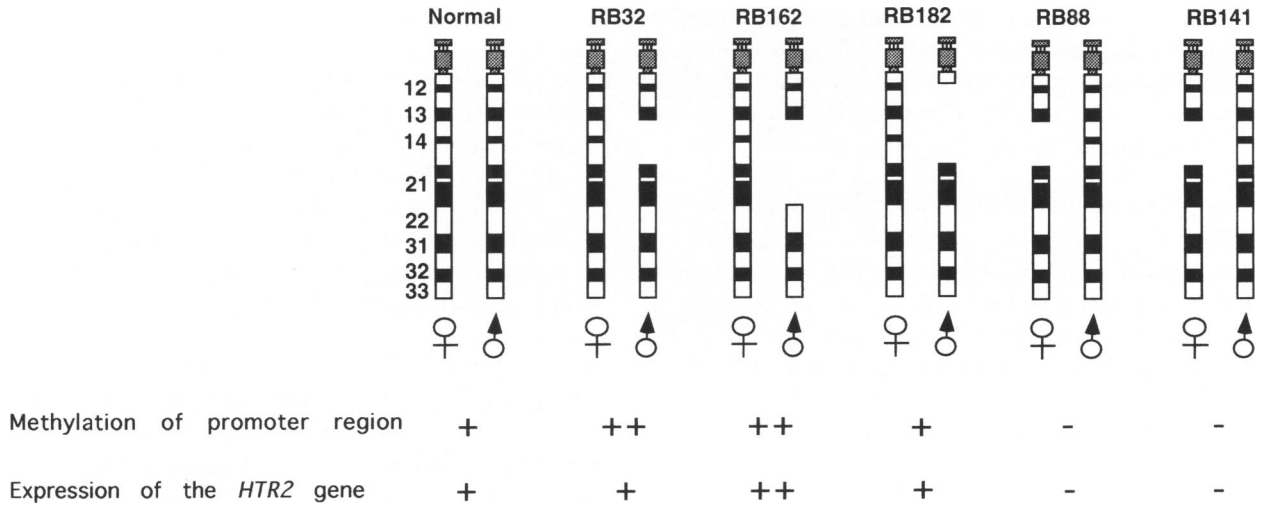

Figure 1 Schematic representation of the genomes of patients and a summary of the methylation of the 5′ region of the *HTR2* gene and the expression of this gene. The chromosomal abnormalities were as follows: paternal deletion of 13q14 in RB32; paternal deletion of 13q14-q21 in RB162; paternal deletion of 13q12-q14 in RB182; and maternal deletion of 13q14 in RB88 and RB141 (Ejima et al. 1988). These chromosomal deletions also resulted in deletions of the *HTR2* gene in the cells of RB patients. The methylation status and expression of the *HTR2* gene are summarized at the bottom of the figure. The expression of the *HTR2* gene was detected only in cells with a maternal *HTR2* gene.

HTR2 gene locus seems to be closely linked with the RB gene and might also be lost in RB accompanied with somatic loss of the RB gene.

Allele Frequencies

The distributions of genotypes and allele frequencies of 16 unrelated normal healthy individuals and 21 RB patients are summarized in table 2. No significant difference was observed between normal and RB populations.

Methylation Status of the 5′ Region of the HTR2 *Gene*

Figure 5 shows the results of the analysis of the methylation of the 5′ region of the *HTR2* gene. Genotypes

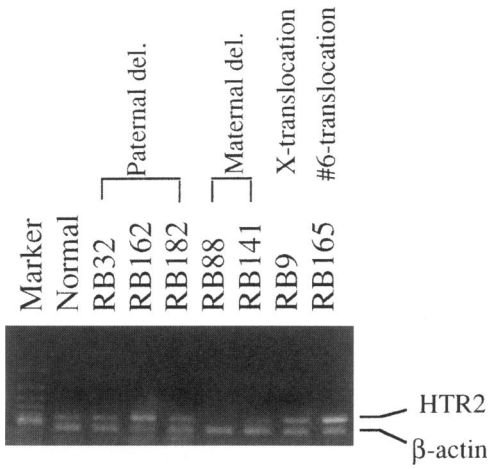

Figure 2 RT-PCR analysis of the expression of the *HTR2* gene. The expected product of PCR was detected in cells from a normal individual and from RB32, RB162, RB182, RB9, and RB165 (cells that retained the maternal allele on chromosome 13) but not in cells from RB141 and RB88 (cells that had lost the maternal allele on chromosome 13). RB9 was a case with translocation of paternal chromosome 13 to an inactive X chromosome, and RB165 is a case with translocation of paternal chromosome 13 to chromosome 6 (Ejima et al. 1988). These two cases with translocation of chromosome 13 retained both alleles of the *HTR2* gene.

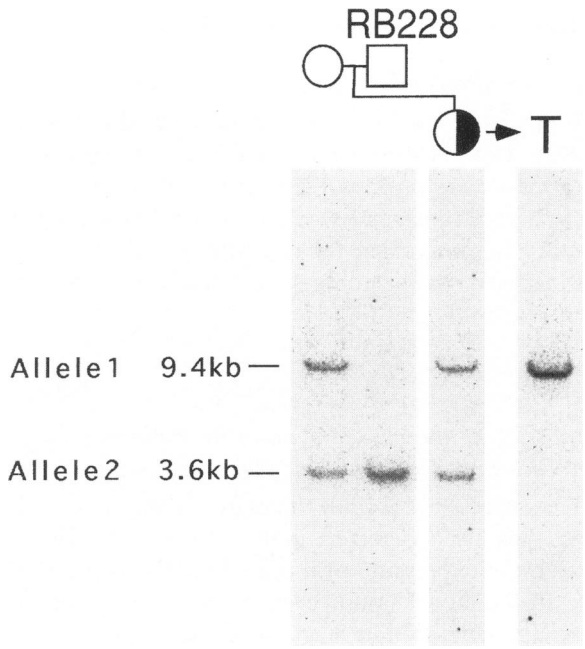

Figure 3 Example of the Southern blot analysis of the *HTR2* gene in a family of RB228. In the family of RB228, the patient (half-closed circle) and her mother (open circle) were heterozygous for both alleles, and the father (open square) was homozygous for allele 2 and her tumor cells (T) lost paternal allele 2.

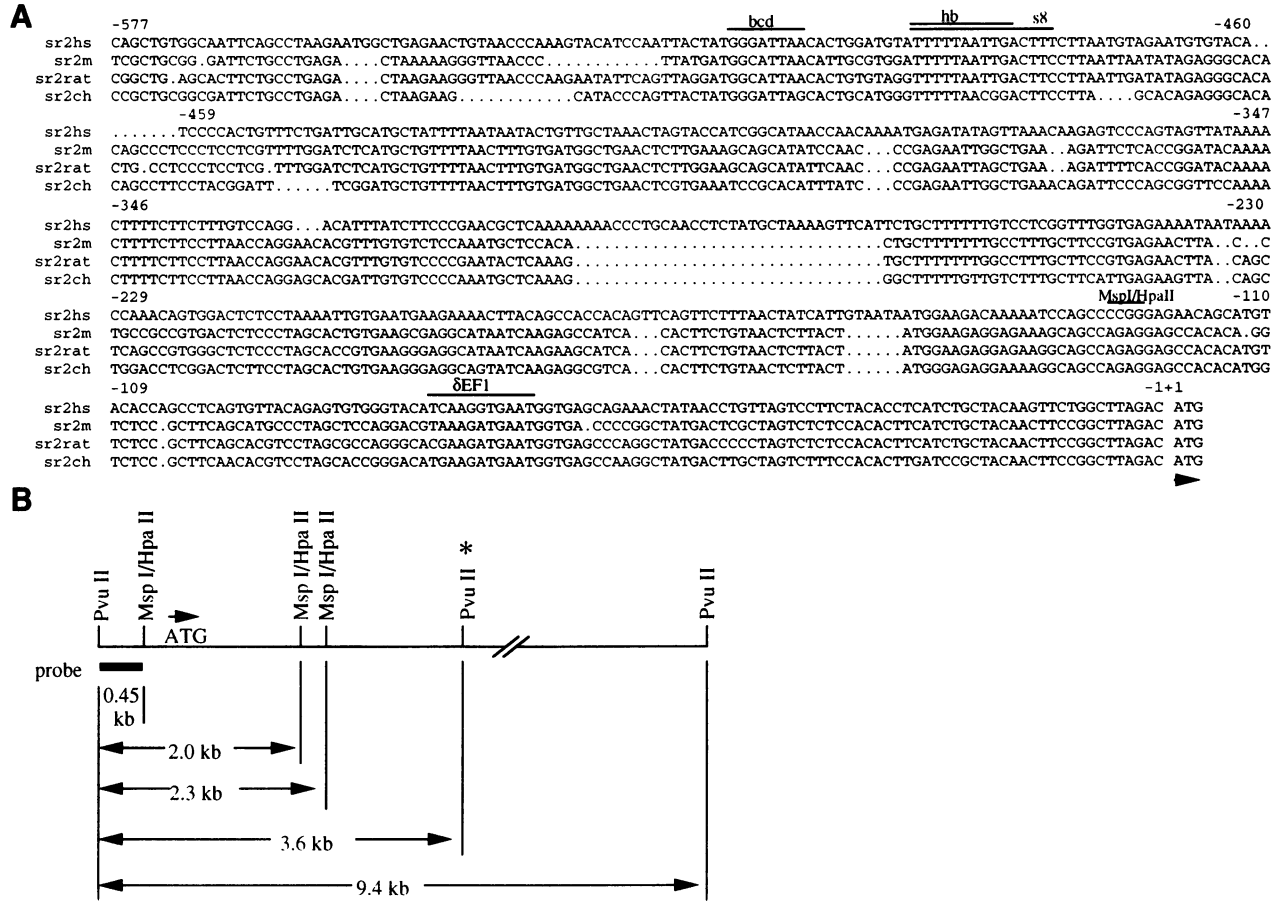

Figure 4 *A*, Sequence comparison of 5′ region of the *HTR2* gene from various mammalian species (sr2hs, human; sr2m, mouse; sr2rat, rat; sr2ch, Chinese hamster). The adenine of the initiation codon (ATG) is indicated by +1. In the highly conserved regions, the consensus sequences of binding sites for transcription factors, namely, bicoid (bcd; −513 to-506), hunchback (hb; −494 to −484), s8 (−494 to −480), and δEF1 (−76 to −66), were found (Driever and Nusslein-Volhard 1989; Stanojevic et al. 1989; de Jong et al. 1993; Sekido et al. 1994). Sp1-like (−137 to −122) and TATA-like (−379 to −376, −353 to −350, and −52 to −49) sequences were found in the human sequence, but these regions were not conserved in the other three species. The TATA sequence was found in the sequences from mouse and rat in regions different from that of this sequence in the human genome. No TATA sequence was found in the sequence from Chinese hamster. CpG sequences were found at five sites (−401 to −400, −314 to −313, −310 to −309, −253 to −252, and −125 to −124) in the human sequence. One *MspI/HpaII* site (CCGG; −126 to −123) was found in the human sequence (Sp1-like), and it was exploited for analysis of methylation of this site in DNA digested with the methylation-sensitive endonuclease *HpaII* (fig. 5). Several short direct repeats were found in the human sequence: four TGCTA repeats (−438 to −434, −418 to −414, −283 to −279, and −22 to −18); four GAATG repeats (−555 to −551, −470 to −466, −202 to −198, and −69 to −65); three AATGG repeats (−554 to −550, −148 to −144, and −68 to −64); two GAATGG repeats (−555 to −550 and −69 to −64); two AATG(T/A)AGAA repeats (−476 to −468 and −201 to −193); two ACCTC repeats (−290 to −286 and −30 to −26); and two CTTTT repeats (−346 to −342 and −265 to −261). The four-base unit AATG was repeated six times in the human sequence. *B*, Genomic map of the 5′ region of the human *HTR2* gene. The probe used for Southern blot analysis is indicated by a solid bar. The initiation codon is indicated by an arrow and ATG. When genomic DNA was digested with *PvuII*, fragments of 9.4 kb and 3.6 kb were observed (fig. 3). The *PvuII* site (indicated by an asterisk [*]) is polymorphic in the human or, at least Japanese, population. Two *MspI/HpaII* sites other than the site in the cloned fragment were detected.

of a normal healthy individual and a patient RB9 were homozygous for allele 2. RB32, RB141, RB182, and RB88 were hemizygous for allele 2. RB162 was hemizygous for allele 1. RB165 was heterozygous for both alleles. When genomic DNA was digested with *PvuII* and *MspI*, only a 0.45-kb fragment was detected in all cases investigated. However, a 2.3-kb fragment, which was resistant to an endonuclease *HpaII* because of methylation of the cytosine residue of CpG, was detected in cells from a normal healthy individual and from five RB patients with paternal deletion of chromosome 13 and translocations of the paternal chromosome (RB32, RB162, RB182, RB9, and RB165), but it was not detected in cells from RB patients with maternal deletion (RB88 and RB141).

Discussion

The 5′ region of the *HTR2* gene was cloned, and a two-allele polymorphism of the *PvuII* site was found.

Table 1

Genotype of the *HTR2* Gene in RB Patients and Their Tumors and Parents

Patient/Tumor	Genotypes	
RB228 (unilateral)	1, 2	
Tumor	1	Loss of paternal allele 2
Mother (healthy)	1, 2	
Father (healthy)	2, 2	
RB232 (unilateral)	1, 2	
Tumor	2	Loss of allele 1
RB270 (unilateral)	1, 2	
Tumor	1	Loss of allele 2
RB313 (bilateral)	1, 2	
Tumor (right eye)	1, 2	

The polymorphism was useful for segregation analysis in a family of a RB patient and for LOH analysis in tumors. These results are completely consistent with our previous results (Kato et al. 1993, 1994*a*), suggesting that the *HTR2* gene may be tightly linked with the RB gene. Although several polymorphisms in the RB gene (Wiggs et al. 1988; Yandell and Dryja 1989) and on chromosome 13 (Cavenee et al. 1984; Dryja et al. 1984; Nakamura et al. 1988) were reported, there were some patients who showed homozygosity at all the polymorphic loci. Therefore, the polymorphism found in the *HTR2* gene may also be useful for linkage study of RB patients and LOH analysis of RB.

The expression of the *HTR2* gene was detected only in cells with a maternal allele (figs. 1 and 2), suggesting that only the maternal *HTR2* gene might be expressed in human fibroblasts. Likewise, the 5′ region of the *HTR2* gene was methylated only in cells with a maternal

Table 2

Distribution of Genotypes and Allele Frequencies of *HTR2* Gene among RB Patients and Normal Individuals

	No. of Genotypes			No. of Alleles (Frequency)	
	1, 1	1, 2	2, 2	1	2
Normal individuals (16)	2	6	8	10 (.313)	22 (.687)
RB patients (21)	1	9	11	11 (.262)	31 (.738)

NOTE.—Difference between allele frequencies in normal individuals and RB patients is not statistically significant.

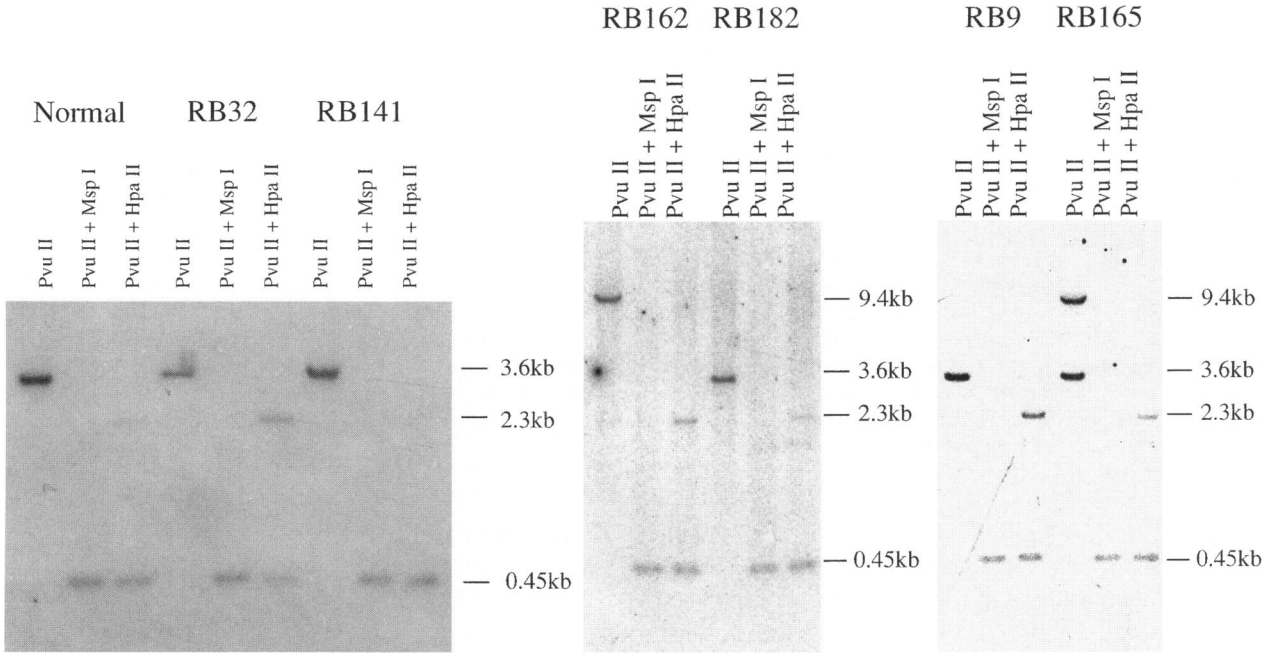

Figure 5 Examples of Southern blot analysis of the 5′ region of the *HTR2* gene. As mentioned in the legend to figure 4, one *Pvu*II site exhibited polymorphism. The normal individual used in this study and RB9 were homozygous for the allele that yielded the 3.6-kb fragment; RB32, RB141, RB88, and RB182 were hemizygous for the allele that yielded the 3.6-kb fragment; RB162 was hemizygous for the allele that yielded the 9.4-kb fragment; and RB165 was heterozygous. When the genomic DNA was digested with *Pvu*II and *Msp*I, only a 0.45-kb fragment was detected in all samples used in this study. By contrast, when DNA was digested with *Pvu*II and *Hpa*II, a 2.3-kb fragment was detected in normal individual, RB32, RB162, RB182, RB9, and RB165, but not in RB141.

allele (figs. 1 and 5), suggesting that only the maternal 5′ region of the *HTR2* gene might be methylated. RB9 and RB165 were cases with translocation of chromosome 13 and each retained both alleles of the *HTR2* gene. In the case of RB9, paternal chromosome 13 was translocated to an inactive X chromosome (Ejima et al. 1988). Since an inactivated X chromosome is highly methylated, the *HTR2* gene on the translocated chromosome 13 might be expected to be methylated. In any case, it seems possible that the methylation of the *HTR2* gene might be positively associated with the expression of the gene. The *HTR2* gene was mapped to mouse chromosome 14 (Hsieh et al. 1990; Liu et al. 1991; Sparkes et al. 1991). Mouse chromosome 14 is also suggested to be affected by genomic imprinting (Cattanach and Beechey 1990). Genomic imprinting in tumorigenesis has been suggested in mice that inherited a paternally derived, mutated RB gene (Harrison et al. 1995). Naumova and Spienza (1994) also predicted that an imprinted gene might be genetically linked to the RB gene, from their epidemiological and genetic analysis. Therefore, the genomic region that contains the RB and *HTR2* genes might be affected by genomic imprinting. Exclusive expression of the maternal *HTR2* gene might be associated with delayed development of RB tumors that have lost the maternal allele on chromosome 13 (Kato et al. 1993, 1995), although we cannot ignore the possibility that other unidentified imprinted genes in the same region may be associated with delayed development of RB with maternal allele loss. Preferential mutations on the paternal RB gene in human RB tumors (Ejima et al. 1988; Dryja et al. 1989; Zhu et al. 1992; Kato et al. 1994*a*) and in osteosarcomas (Toguchida et al. 1989) might also due to differences in methylation of genomic regions between maternal and paternal chromosomes.

In the conserved domains of the 5′ region of the *HTR2* gene, we found binding sites for transcription factors, bcd (Driever and Nusslein-Volhard 1989), hb (Stanojevic et al. 1989), s8 (de Jong et al. 1993), and δEF1 (Sekido et al. 1994). bcd, hb, and s8 are transcription factors with a homeodomain. While bcd is a maternal gene of *Drosophila melanogaster* (Driever and Nusslein-Volhard 1989), it is unclear whether this gene is associated with genomic imprinting. δEF1 is a repressor of E2-box–mediated transactivation (Sekido et al. 1994). Methylation of this region might interfere with the action of transcriptional repressors. It seems unlikely that methylation of cytosine residues in this region directly affect the binding of these transcription factors, because the CpG sequence is not found in the binding sites for these transcription factors. Alternatively, methylation might be associated with chromatin structure, and it might indirectly affect the binding and/or activation activity of transcription factors (Banerjee and Smallwood 1995). Although we cannot ignore the possible importance of other binding sites for transcription factors further upstream of the cloned region, and although transcription factor(s) directly associated with genomic imprinting remain(s) to be elucidate, it seems possible that the methylation of the 5′ region might be closely associated with the expression of the *HTR2* gene.

In order to know the details of the genomic imprinting of the *HTR2* gene, genetic analysis using experimental animals such as mouse may be necessary. However, the exclusive expression and methylation of the human maternal *HTR2* gene found in this study would be one of important clues for understanding the mechanisms of cancer development and genomic imprinting.

Note added in proof.—The nucleotide-sequence data reported in this paper will appear in the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank nucleotide-sequence databases with the following accession number: D87030.

Acknowledgments

The authors are very grateful to Ms. K. Sudo for her technical assistance. This work was supported in part by grants from the Ministry of Health and Welfare and from the Ministry of Education, Science, and Culture, Japan.

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