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Genomic analysis and functional expression of canine dopamine D2 receptor

Hyeonkoon Myeong [a,1], Doo-il Jeoung [1,a], Hyong-Kyu Kim [1,b], Yong-Seok Lee [b], Kyung Hee Kim [b], Ji Hong Ha [c], Chankyu Park [a,*], Bong-Kiun Kaang [b,*]

[a] *National Creative Research Initiative Center for Behavioral Genetics, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, South Korea*

[b] *Molecular Neurobiology Laboratory, Institute of Molecular Biology and Genetics, School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea*

[c] *Department of Genetic Engineering, Kyungpook National University, Taegu 702-701, South Korea*

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Abstract

Dopamine D2 receptor (DRD2) is one of the five dopamine receptors with seven transmembrane domains that are coupled to the G protein. We have cloned and characterized the genomic and cDNA sequences of the canine DRD2 gene, which are 12.7 and 2.7 kb in size, respectively. The genomic DNA is composed of seven exons and six introns, encoding a 443 amino acid protein with 95% amino acid identity to other mammalian D2 receptors. A length polymorphism was detected in intron 3 of the receptor gene. We also characterized alternatively spliced forms of DRD2 cDNAs, DRD2L and DRD2S. They showed a higher level of expression in midbrain and thalamus. The ratio between the long and short form is similar in RT-PCR reaction. In human and rodent, the same two spliced forms are known to be coupled to G_i-type heterotrimeric GTP binding protein, thereby opening an inwardly rectifying potassium channel, GIRK1. When the canine DRD2L and DRD2S were heterologously expressed in *Xenopus* oocytes, both forms activated GIRK1 potassium channels through coupling with G_i protein. This activation was dose-dependent, demonstrating its ligand specificity. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Alternative splicing; G protein-activated inwardly rectifying K^+ channel (GIRK); Polymorphism; *Xenopus* oocytes

1. Introduction

Dopamine D2 receptor (DRD2) is one of the five dopamine receptors involved in the dopaminergic pathways, which was shown to be associated with obesity and Tourette's syndrome (Blum et al., 1996; Comings et al., 1996). DRD2 has also been implicated in brain diseases such as schizophrenia (Wong et al., 1986; Seeman et al., 1989; Sarkar et al., 1991) and Parkinson's disease (Plante-Bordeneuve et al., 1997). DRD2 has been the target of antipsychotic drugs such as fluphenazine, chlorpromazine, thioridazine, and haloperidol (Goldstein and Deutch, 1992).

DRD2 is a member of the G protein coupled receptors and has been known to inhibit adenylyl cyclase activity and mitogenesis (Albert et al., 1990; Montmayeur and Borrelli, 1991). In mammals, DRD2 is alternatively spliced to generate two variants (Fishburn et al., 1995; Neve et al., 1991; Chio et al., 1990). Both forms of DRD2 were shown to inhibit adenylyl cyclase activity through heterotrimeric G_i proteins. These two receptor isoforms differ in their content of 29 amino acids, a portion of the third cytoplasmic loop. This region of DRD2 was shown to govern the interaction with different subtypes of $G_{i\alpha}$ proteins. Human and rodent DRD2L and DRD2S are known to be coupled to G_i-type heterotrimeric GTP binding protein (Dal Toso et al., 1989; Monsma et al., 1989; Montmayeur et al., 1993). The receptor-activated G_i or G_o proteins can

Abbreviations: bp, base pairs; cDNA, DNA complementary to RNA; DRD2, dopamine D2 receptor; GIRK1, G protein-activated inwardly rectifying K^+ channel type 1; kb, kilobases; PCR, polymerase chain reaction; RT, reverse transcriptase.

* Corresponding author. Fax.: +82-42-869-2610 (C. Park); tel.: +82-2-880-7525; fax: +82-2-874-1206 (B.-K. Kaang).
E-mail addresses: ckpark@mail.kaist.ac.kr (C. Park), kaang@snu.ac.kr (B.-K. Kaang)

[1] The first three authors contributed equally to this work.

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Table 1
Primer sequences used in this study

Primers[a]	Sequences	Uses
F1(282–306)	5′-atccactgaatctgtcctggtatga	Genomic DNA amplification
R1(504–482)	5′-aggctgacgatcaggtagttggt	Genomic DNA amplification
F2(407–432)	5′-ctcatcttcatcatcgtcttcggcaa	Genomic DNA amplification
R2(4788–4765)	5′-cctagctgggctcacctgtcaatg	Genomic DNA amplification
F3(4664–4679)	5′-gtggtaggtgagtggaa(g/a)tt(c/t)	Genomic DNA amplification
R3(7846–7826)	5′-cactctccgcctgttcantgg	Genomic DNA amplification
F4(7797–7825)	5′-ccgttatcatgaagtctaatgggagtttc	Genomic DNA amplification
R4(11 528–11 506)	5′-gctgttgacatagcccagccacg	Genomic DNA amplification
F5(9390–9409)	5′-tggagatggagatgctctcc	Genomic DNA amplification
R5(11 553–11 534)	5′-tggtgtagatgatggggttc	Genomic DNA amplification
F6(245–264)	5′-agagcctggccacccagtgg	Genomic DNA amplification
R6(11 635–11 614)	5′-ggaagcaggctgctgtgcaggt	Genomic DNA amplification
E1f (379–405)	5′-aagcagcatggcgtagtagttg	Inverse PCR around exon 1
E1r (457–476)	5′-ccgcgagaaggcgctgcaga	Inverse PCR around exon 1
E5f (7797–7815)	5′-ttagacttccatgataacgg	Inverse PCR around exon 5
E5r (7816–7835)	5′-tgggagtttcccagtgaaca	Inverse PCR around exon 5
E7f (11 460–11 479)	5′-atacactgtgagtgcaacat	Inverse PCR around exon 7
E7r (11 534–11 553)	5′-tggtgtagatgatggggttc	Inverse PCR around exon 7
pf1 (275–294)	5′-ggaagatctccgatggatccactgaacct	Subcloning into pSP64T
pr1 (11 607–11 587)	5′-ggaagatctcaggtcagcagtgcaggatc	Subcloning into pSP64T
pf(5623–5642)	5′-tcgggaggatgtctggccat	TG genotyping
pr(5812–5793)	5′-ttgggctgcagcctccttac	TG genotyping
E1f1(408–433)	5′-ctcatcttcatcatcgtcttcggcaa	RT-PCR
Altf(6623–6646)	5′-ccttctacgtgcccttcatcgtca	RT-PCR
Altr(11 635–11 615)	5′-ggaagcaggctgctgtgcaggt	RT-PCR
D2nf (275–294)	5′-ccgatggatccactgaacct	Northern blot
D2nr(5310–5291)	5′-gcatgggcatggccacagct	Northern blot
Actinf	5′-ccctccggaacgcaagtatt	Northern blot
Actinr	5′-ttgaccggcaagacagaaa	Northern blot

[a] Numbers in parentheses refer to the nucleotide positions of the canine DRD2 gene sequence.

open an inwardly rectifying potassium channel, GIRK1 (Dascal et al., 1993). In this paper, we present genomic and cDNA structures of the canine DRD2 gene with evidence of its functionality as measured by the activity of G_i-dependent potassium channels.

2. Materials and methods

2.1. Materials

Dopamine was obtained from Sigma Chemical Co. (St. Louis, USA). Primers used in this study were obtained from Genotech Co. (Taejon, South Korea).

2.2. Isolation and sequence analyses of genomic DNA for canine DRD2 gene

Blood (2–3 ml) was drawn from a Korean native dog (sapsaree) (Kim et al., 1998) and stored in EDTA-treated sterile tubes. Genomic DNA was isolated from the blood according to standard procedures described in Sambrook et al. (1989). The isolated genomic DNA was used as a template for PCR amplification of the canine DRD2 gene. PCR primer sets were initially designed using known conserved mammalian DRD2 sequences, and then canine-specific primers were designed from PCR results (Table 1). Primers F1 and R1 were used for the amplification of exon 1, and primers F2 and R2 for the amplification of exons 1 through 3, primers F3 and R3 for the amplification of exons 2 to 5, primers F4 and R4 for exons 5 to 7, and primers F5 and R5 for exons 6 and 7 of the canine DRD2 gene (Fig. 1B). To determine the boundary sequences of exons previously amplified, inverse PCR reactions (Sambrook et al., 1989) were carried out. The inverse PCR template was prepared by digesting canine genomic DNA with Pst I, followed by self-ligation. This ligated template was then PCR amplified using the primers listed in Table 1 to provide a DNA sequence around exons 1, 5, and 7. Finally, the intactness of the canine DRD2 genomic DNA was confirmed by PCR reaction using F6 and R6 primers (Table 1) (Fig. 1B). The PCR reactions were performed with Taq polymerase (LA Taq®, Takara Biochemicals, Japan) and consisted of one cycle of 95°C for 2 min, followed by 30 cycles of 95°C for 40 s or 20 s (F6 and R6 primers), 58°C for 40 s, and 72°C for 40 s or 68°C for 12 min (F6 and R6 primers). The final extension reaction was carried out at 72°C for 5 min or 68°C for 10 min (F6 and R6

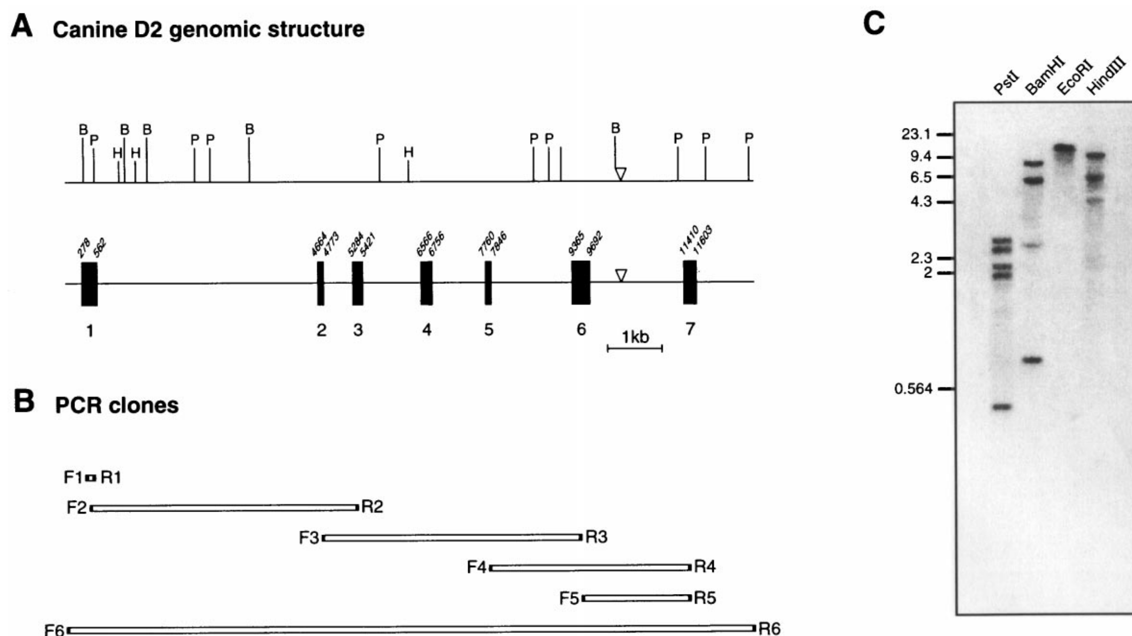


Fig. 1. Organization of the canine DRD2 gene. (**A**) Genomic organization and restriction map of the canine DRD2 gene. The solid boxes represent exons and lines denote introns and untranslated regions. Exons are numbered. The numbers on top of the boxes indicate the positions of the first and last nucleotides of exons. We found an insertion of SINE sequences in intron 6 creating a BamHI site in certain alleles. (**B**) Overlapping PCR genomic clones are schematically drawn as blank bars flanked with the sense and anitisense primers (see Materials and methods and Table 1). (**C**) Genomic Southern blot analysis of the canine DRD2 gene. Sapsaree genomic DNA (10 μg each) was digested with restriction enzymes, electrophoresed on a 0.7% agarose gel, blotted onto a membrane filter, and hybridized with the canine DRD2L cDNA. Positions of molecular weight markers are indicated in kilobases.

primers). PCR products were separated on an agarose gel and individually subcloned into the TA cloning vector, pTA2.1 (Invitrogen, Carlsbad, CA). The DNA sequence was determined by an ABI prism automatic sequencing machine (Perkin Elmer, Norwalk, CT). Southern blot hybridization was done as described in Sambrook et al. (1989) using a randomly primed ^{32}P-labeled canine DRD2L cDNA as probe.

Phylogenetic tree analysis of cDNA sequences of dopamine receptors was conducted by the Clustal method algorithm with weighted residue weight table provided by the MegAlign program software package (DNASTAR Inc., Madison, WI).

2.3. cDNA cloning and RT-PCR reaction

Total RNA was isolated from various tissues of a Korean sapsaree dog by using an RNAqueous isolation kit (Ambion, Austin, TX). mRNA was isolated from the hippocampus by using oligo-dT cellulose. Isolated total RNA or mRNA from the hippocampus was converted into cDNA using Superscript transcriptase II (Gibco-BRL, Gaithersberg, MD). The isolated cDNA from the hippocampus was PCR amplified using pf1 and pr1 (Table 1) as primers to obtain the full length of DRD2 cDNA. Pf1 contains the codons for the first six amino acids and pr1 contains the codons for the last four amino acids and the stop codon of the canine DRD2 cDNA. The PCR conditions were 30 cycles of 95°C for 20 s, 60°C for 40 s and 72°C for 2 min, followed by 5 min of extension reaction at 72°C. cDNA was subcloned into the Klenow-treated Bgl II site of the pSP64T vector by blunt-end ligation for in vitro transcription. The isolated cDNAs synthesized from total RNA from various canine tissues were amplified by PCR reaction using primers E1f1 and Altr (Table 1) to amplify a 1.2 kb coding sequence of DRD2 (Fig. 3B). Similarly, primers Altf and Altr (Table 1) were used to produce both 755 bp (D2L) and 668 bp fragments (D2S) of DRD2 (Fig. 3C). These PCR reactions consisted of one cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 40 s, 68°C for 40 s, and 72°C for 1 min with final extension at 72°C for 3 min. Bands were excised for sequencing by an ABI prism automatic sequencer (Perkin Elmer, Norwalk, CT).

2.4. Northern blot hybridization

mRNAs were prepared from various canine tissues according to the instruction manual of the manufacturer (Oligotex Direct mRNA Mini Kit, Qiagen, Hilden, Germany). Northern blots containing 2 μg poly(A)$^+$ mRNA from canine tissues were carried out as described in Sambrook et al. (1989). The probes were either the [α-^{32}P]-labeled PCR product (421 bp) of canine DRD2 containing exons 1, 2, and 3 or the [α-^{32}P]-labeled PCR

product (426 bp) of canine actin. The primers used for the generation of the probes are described in Table 1.

2.5. Genotyping of polymorphic (TG) repeat

Genomic DNAs were isolated from bloods of Korean sapsaree dogs and other breeds of dog. For detection of polymorphic (TG), primers pf/pr (Table 1) were used. End labeling of the forward PCR primer pf (Table 1) with [γ-^{32}P ATP] was done according to standard procedures (Sambrook et al., 1989) using T4 polynucleotide kinase (New England Biolab, Beverly, MA). The PCR reaction consisted of one cycle of 95°C for 2 min followed by 30 cycles of 95°C for 40 s, 58°C for 40 s and 72°C for 1 min. The final extension was carried out at 72°C for 5 min. 6.5% PAGE gels were run for genotyping and subjected to autoradiography.

2.6. Functional expression and electrophysiology of DRD2s in Xenopus oocytes

cRNAs for canine DRD2L and DRD2S, mouse D2A (Montmayeur et al., 1991), and rat G protein-gated inwardly rectifying K^+ channel (GIRK) (Dascal et al., 1993) were prepared by in vitro transcription, using the cRNA Megascript kit (Ambion, Austin, TX). Microinjection of cRNAs into *Xenopus* oocytes (stages IV and V) and electrophysiological recording were done as described in Lee et al. (1999).

The oocytes were placed in a chamber perfused with Ca^{2+}-free ND96 (ND). To record the GIRK current, the solution was changed to high K^+ (hK) solution (Lee et al., 1999). Drugs dissolved in ND or hK solution were applied to the recording chamber via bath perfusion lines.

3. Results and discussion

3.1. Genomic structure, cDNA sequence, and expression pattern of the canine DRD2

We obtained an entire genomic sequence of the canine DRD2 from Sapsaree, a Korean dog breed (Kim et al., 1998), by employing a series of polymerase chain reactions and inverse PCRs. The sets of PCR primers were initially designed based on highly conserved sequences of various mammalian DRD2 sequences and later based on the sequences of canine PCR products. The size of the entire canine DRD2 genomic DNA was 12.7 kb, composed of seven exons and six introns (Fig. 1A). The overall genomic structures of canine and human DRD2 are quite similar, where the positions of the introns are identical; the largest exon is the sixth (328 bp), and the smallest the fifth (87 bp) (Grandy et al., 1989). Also, the first intron is the largest in human (6236 bp) and in dog (4101 bp). All six introns of canine and human DRD2 conform to the GT/AG consensus splice site rule. Nucleotide sequences of the exons and exon/intron junction regions are highly homologous between the canine and human DRD2 genes, although the DNA sequences in the introns are rather divergent between the two species (data not shown). Southern blot analysis of the genomic DNA showed that the sizes of all the strongly hybridizing bands match the expected restriction fragments of the gene (Fig. 1C).

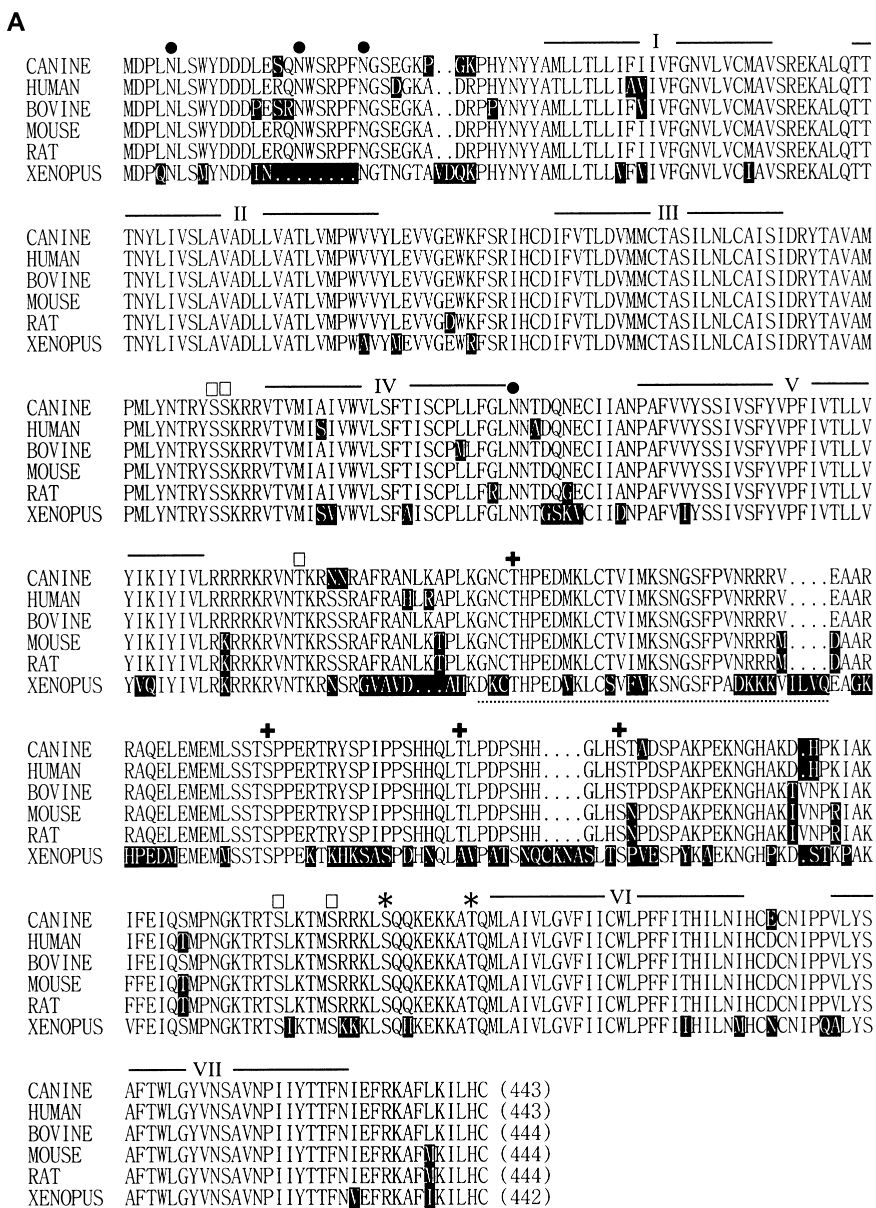
We determined the cDNA sequence of canine DRD2 by RT-PCR using mRNA purified from hippocampus of the canine brain. Two splice variants of DRD2 cDNAs, DRD2L (long) and DRD2S (short), were obtained. The cDNA of canine DRD2L is 2.7 kb in size and encodes 443 amino acids (Fig. 2A). DRD2S lacks 29 amino acids encoded by exon 5. The canine DRD2 has 73% and 94–96% amino acid sequence identity with the amphibian and mammalian DRD2s, respectively (Fig. 2A). The canine DRD2 shares features with other mammalian DRD2 family members, such as N-glycosylation and various phosphorylation sites. Alignment of the coding region reveals that the canine DRD2 sequence is much closer to the human DRD2 sequence than the rodent one (Fig. 2B).

We carried out Northern blot analysis to determine the tissue distribution of the canine DRD2 gene. Canine DRD2 showed the most abundant expression in midbrain, followed by cerebellum and thalamus (Fig. 3A). Consistent with the Northern blot data, RT-PCR analysis revealed strong expression in these same structures (Fig 4B). However, due to the greater sensitivity of RT-PCR, the expression of the canine DRD2 was also detected from cerebral cortex, hippocampus, and brain stem (medulla oblongata), but not in spleen. The expression pattern of the canine DRD2 is similar to that of rat. In situ hybridization analysis combined with RNA slot blot hybridization showed the most abundant expression of rat DRD2 in pituitary and striatum, followed by midbrain with a low level in hippocampus, cerebellum, and cortex (Neve et al., 1991). Rat RT-PCR data showed a similar expression pattern (O'Malley et al., 1990).

Both long (DRD2L) and short (DRD2S) forms of the receptor are found in approximately the same ratios; DRD2L seems to be more abundant than DRD2S (Fig. 3C). Therefore, there seems to be no tissue specificity to the expression of DRD2L and DRD2S isoforms, at least in the regions of the canine brain that we examined. This is consistent with the data from the rat brain (Neve et al., 1991).

3.2. Functional expression of DRD2 in Xenopus oocytes

We determined that the cDNA clones for the canine DRD2s, DRD2L and DRD2S, encode functional recep-



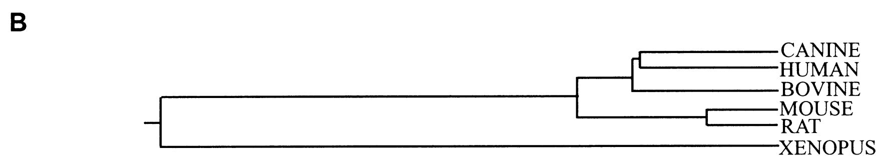


Fig. 2. (**A**) Predicted amino acid sequence of the canine DRD2 and its comparison with the bovine (Chio et al., 1990), human (Grandy et al., 1989; Dal Toso et al., 1989; Selbie et al., 1989; Stormann et al., 1990), mouse (Montmayeur et al., 1991), rat (Bunzow et al., 1988; Chio et al., 1990; Giros et al., 1989; Monsma et al., 1989; Rao et al., 1990; Miller et al., 1990), and *Xenopus* (Martens et al., 1991) DRD2 sequences. Seven putative transmembrane regions (I–VII) are indicated by overlines. Different amino acids are indicated by a black background. The 29 or 33 amino acids specific to the mammalian or *Xenopus* DRD2L, respectively, are indicated by a dotted underline. ● N-glycosylation signal sequences; + casein kinase-dependent phosphorylation sites; □ PKC-dependent phosphorylation sites; * cAMP-dependent kinase phosphorylation sites. (**B**) Phylogenetic tree based on the nucleotide sequences.

tors by expressing them in *Xenopus* oocytes. To test if the canine DRD2s activate G_i proteins like the other mammalian DRD2s, we expressed GIRK1 and the receptors by microinjecting in vitro transcribed cRNAs into *Xenopus* oocyte. Voltage-clamp experiments using two electrodes showed that replacement of calcium-free ND96 solution by high potassium (hK) solution evoked an inward current (I_{hK}). The amplitude of I_{hK} in oocytes

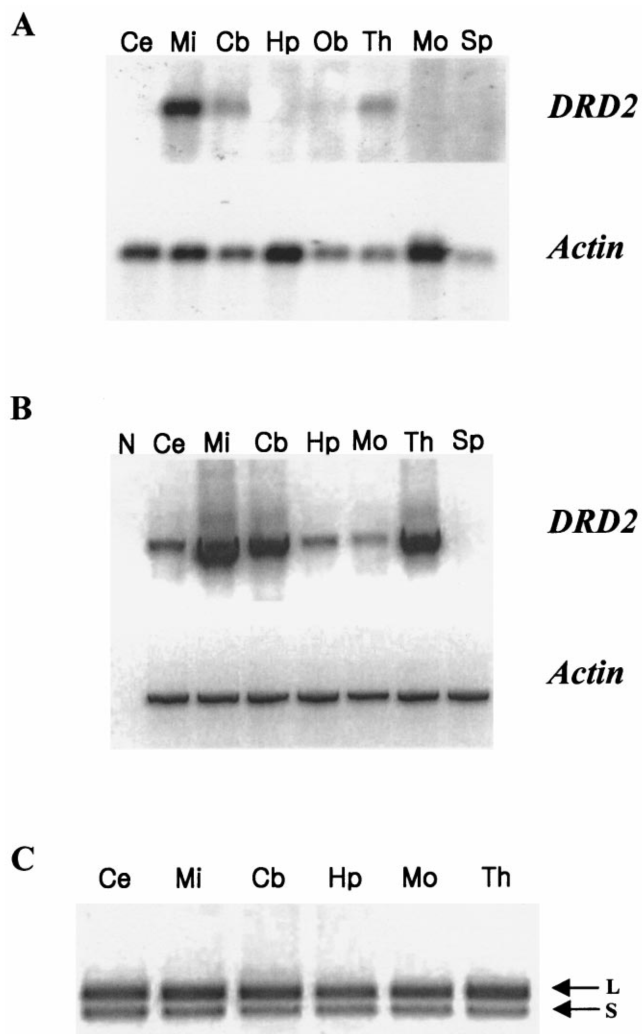


Fig. 3. Tissue distribution and alternative splicing forms of mRNA of the canine DRD2. (**A**) Northern blot containing total RNA (20 μg each) from various sapsaree tissues hybridized to DRD2 exons 1–3 and actin. The mRNA bands of DRD2 and actin on the blot are approximately 2.8 and 3.0 kb in size, respectively. (**B**) RT-PCR analysis of expression pattern of DRD2. N denotes negative control reaction using water instead of cDNA as template. The PCR products of DRD2 and actin are about 1.2 kb and 450 bp in size, respectively. (**C**) Alternative splicing forms of canine DRD2. L and S denote long (D2L) and short (D2S) isoforms, respectively. The PCR fragments of D2L and D2S are 755 and 688 bp, respectively. Samples were diluted appropriately to present equal amounts of PCR products. Ce, cerebrum (temporal lobe); Mi, midbrain; Cb, cerebellum; Hp, hippocampus; Ob, olfactory bulb; Mo, medulla oblongata; Th, thalamus; Sp, spleen. Heart β-actin was used as an internal control.

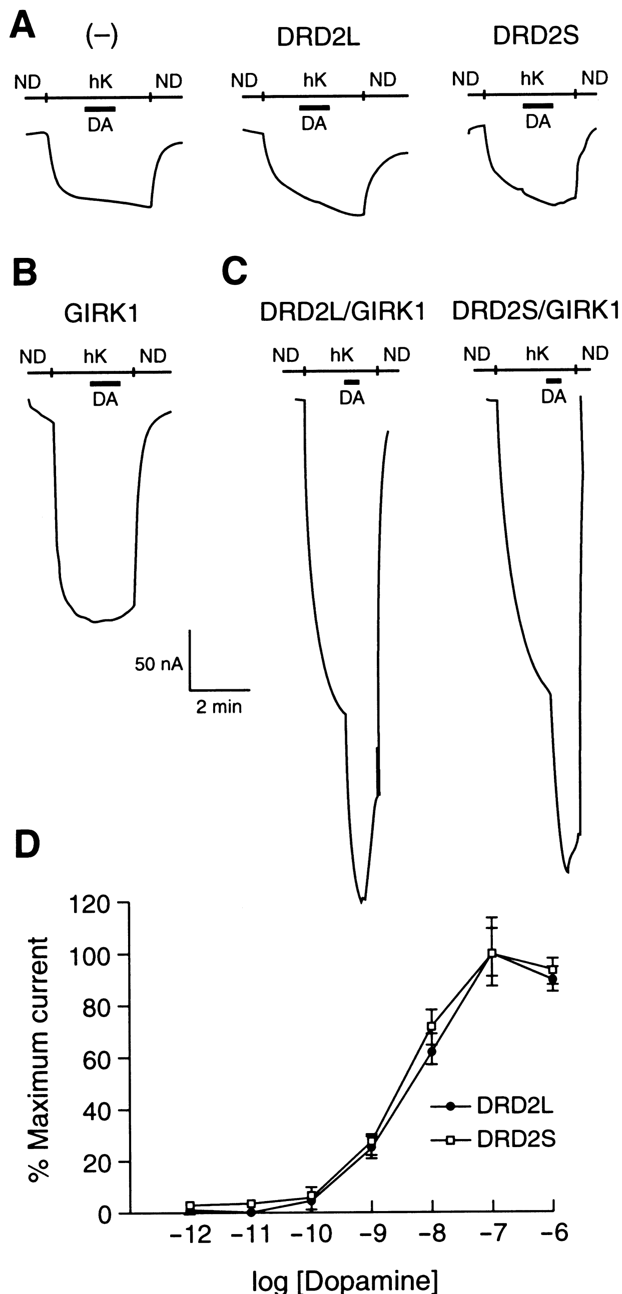


Fig. 4. Functional expression of the canine DRD2s in *Xenopus* oocytes and their coupling to the G_i protein. Oocytes were microinjected with cRNAs for DRD2L (250 pg), DRD2S (250 pg), and GIRK1 (2.5 ng). All the current traces were recorded at a holding potential of −70 mV. Dopamine (DA) was applied in the bath solution as indicated by bars. (**A**) Control oocytes uninjected (–) or expressing the receptors alone (DRD2L or DRD2S). (**B**) Control oocyte expressing GIRK1 only. (**C**) Activation of GIRK1 channel by DRD2s. Only the oocytes expressing both the receptors (DRD2L or DRD2S) and GIRK1 channel produced an inward current by treatment with 100 nM dopamine. (**D**) Dose-response curves of the canine DRD2s. The GIRK1-specific inward current was elicited with various concentrations of dopamine. The peak amplitude of the GIRK current was measured and normalized to the maximum current value. Each data point represents the average value of at least three separate recordings from different oocytes. The error bars indicate the standard error (S.E.).

microinjected with DRD2L (51.3 ± 8.3 nA, $n=4$) or DRD2S (56.7 ± 1.7 nA, $n=3$) alone was not significantly different from that in uninjected naive oocytes (52.5 ± 10.1 nA, $n=4$) (Fig. 4A). The amplitude of I_{hK} was larger in oocytes microinjected with GIRK1 alone (141.7 ± 9.3 nA, $n=3$) (Fig. 4B).

When oocytes injected with canine DRD2L or DRD2S and GIRK1 cRNAs were treated with dopamine, an additional increase in the inward current was

Table 2
Allele frequencies of the TG repeat sequences

Breeds	No. of individuals	Major allele	Frequency (%)[a]
Sapsaree	73	1	66
Yorkshire terrier	8	5	88
Labrador retriever	13	4	77
German shepherd	6	4	67
Others[b]	9	4	39

[a] Frequencies represent the major alleles in each dog breed.
[b] These include two bulldogs and one each of: English cocker spaniel, beagle, golden retriever, Korean Jindo dog, Chinese sharpei, and rotweiler.

detected in a dose-dependent and saturable manner with $EC_{50}=5$ nM (Fig. 4C and D). Similarly, in a control oocyte experiment, mouse D2A (or DRD2L) also activated this type of inward current (data not shown). By contrast, uninjected oocytes and oocytes injected with GIRK1 cRNA alone or canine DRD2s alone were not affected by 100 nM dopamine (Fig. 4A and B), indicating that the additional increase in I_{hK} is the GIRK1 current specifically activated by DRD2s. The inward current through GIRK1 reaches a maximal level with 100 nM dopamine in the oocytes expressing canine DRD2L (138 ± 17.7 nA, $n=5$) or DRD2S (150 ± 14.1 nA, $n=4$). The dose-response curves for canine DRD2L and DRD2S are very similar to each other (Fig. 4D). These data illustrate that the cloned canine DRD2L and DRD2S encode functional DRD2s with pharmacological characteristics very similar to those of other reported mammalian DRD2s (Dal Toso et al., 1989; Monsma et al., 1989; Montmayeur et al., 1993; Guiramand et al., 1995).

In addition, activation of the GIRK1 channel by the canine DRD2s appears to be mediated by G_i protein, but not by G_o protein because these receptors did not produce the transient endogenous Ca^{2+}-activated Cl^- current that is known to be stimulated by G_o protein (data not shown). Therefore, our results demonstrate that these canine receptors are coupled only to G_i heterotrimeric GTP-binding proteins like other mammalian DRD2s.

3.3. Polymorphism associated with the canine DRD2 gene

We found a microsatellite with TG repeats in the third intron of the canine DRD2 gene (Table 2). Its size

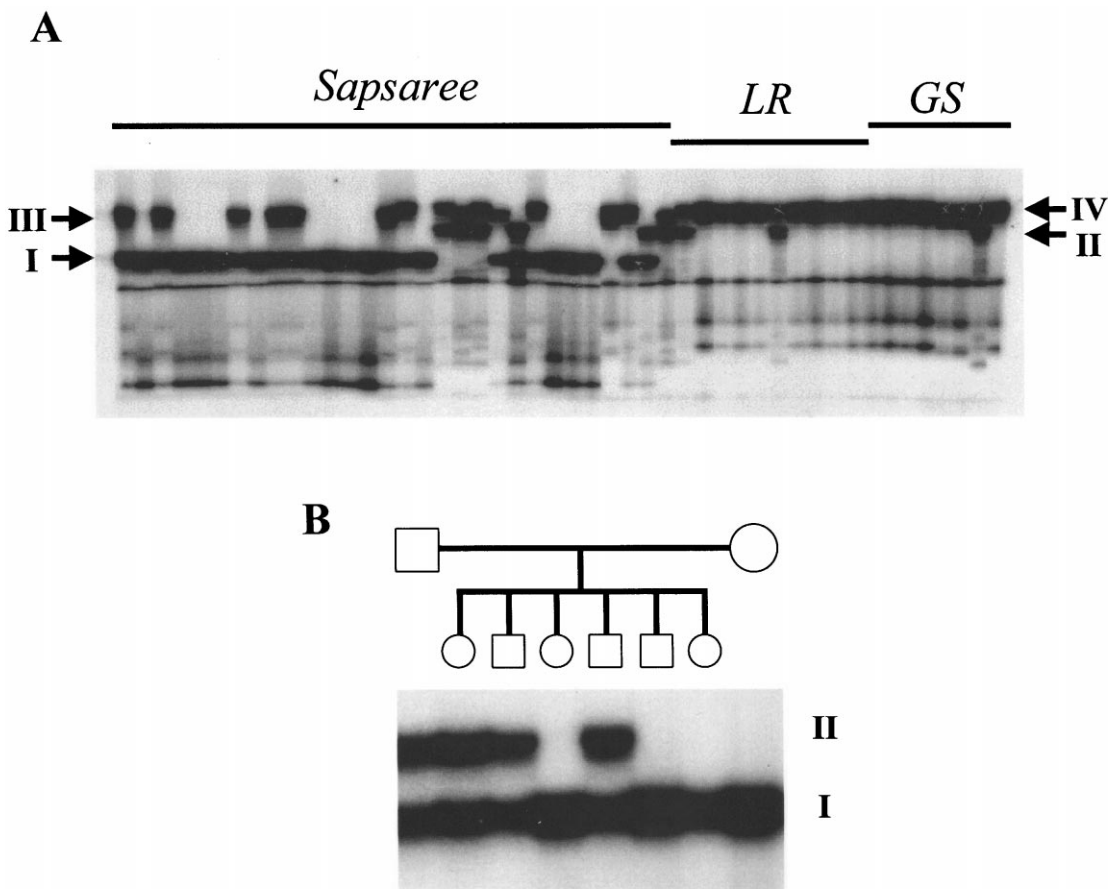


Fig. 5. Polymorphic (TG) microsatellite and cosegregation. (**A**) Genotyping; *LR* and *GS* denote Labrador retriever and German shepherd, respectively. (**B**) Mendelian cosegregation of Poly (TG) microsatellite in Korean sapsaree pedigree. Roman numerals denote alleles.

is variable such that there are five different alleles of 73 genotyped individuals of the sapsaree population. The polymorphic information content (PIC) of the marker in the sapsaree population is calculated to be 0.51. Allele frequencies are variable in different dog breeds; allele 1, the smallest allele, is the most abundant in sapsaree, whereas the most abundant in both German shepherd and Labrador retriever is allele 4 (Fig. 5A, Table 2). This polymorphic marker showed Mendelian cosegregation pattern in a pedigree analysis (Fig. 5B). The functional consequence of the TG repeat polymorphism, however, remains to be determined.

The finding of polymorphism in the canine DRD2 gene and the molecular and pharmacological similarity between human and canine DRD2s will be useful in carrying out neurological and behavioral association studies with the dog as a model animal.

Acknowledgements

The nucleotide sequences of genomic DNA, long form cDNA, and short form cDNA of the canine DRD2 have been submitted to GenBank under accession numbers AF293962, AF293963, and AF293964, respectively. The mouse DRD2 cDNA clone was kindly provided by Dr. J.H. Paik, (Yonsei University, South Korea). This work was supported by Biotech 2000 to B.-K.K. and J.H.H. and by Creative Research Initiative Program to C. Park. Y.-S.L. and K.H.K. were supported by BK21 Research Fellowship.

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