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Isolation and characterization of the canine serotonin receptor 1B gene (*htr1B*)

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Abstract

The serotonin receptor 1B gene (*htr1B*) has been suggested to be implicated in mental disorders in both humans and other species. We have isolated a canine bacterial artificial chromosome (BAC) clone containing *htr1B*, revealed the coding and surrounding DNA sequence of canine *htr1B* and designed primer sets for genomic sequencing of the gene. A mutation scan in 10 dogs revealed five single nucleotide polymorphisms in the *htr1B* coding sequence. By random sequencing of subclones of the BAC a polymorphic microsatellite repeat was found. We found evidence for at least four extended haplotypes in six dogs of the same breed. The chromosomal localization of the gene was confirmed by fluorescence in situ hybridisation and radiation hybrid mapping. This work provides a starting point for mutation scans and association studies on dogs with behavioural problems.

Keywords: BAC clone; Microsatellite marker; SNP; FISH; Canine behaviour problem; Dog breed

1. Introduction

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) plays a central role in the molecular biology of the mind. Serotonin is involved in the regulation of sleep, fear, aggression, mood, and feeding behaviours (Gingrich and Hen, 2001). Neurons of the serotonergic system project throughout the central nervous system, and serotonin receptors can be found in primitive life forms such as flatworms (Venter et al., 1988). Currently, 14 different serotonin receptors have been identified (Hoyer et al., 1994).

One of the 14 serotonin receptors known to date is serotonin receptor 1B. Like most serotonin receptors it is a member of the family of G-protein coupled receptors. The protein has seven transmembrane regions and its activity is mediated by G-proteins that inhibit adenylate cyclase (Hamblin et al., 1992; Maroteaux et al., 1992). 5-HT_{1B} receptors can be found both pre- and postsynaptically on a variety of neurons in several brain areas, and they modulate the release of both serotonin and other neurotransmitters (Hartig, 1997; Hoyer et al., 2002). The gene encoding serotonin receptor 1B (*htr1B*) has no introns and in man 16 polymorphisms have been discovered in its coding and surrounding DNA sequence (Jin et al., 1992; Hamblin et al., 1992; Maroteaux et al., 1992; Sanders et al., 2002).

A bulk of studies has suggested involvement of *htr1B* in the aetiology of mental disorders (Sanders et al., 2002). Multiple linkage scans have identified a schizophrenia susceptibility gene in the human chromosomal region harbouring *htr1B* (HSA 6q13; see Sanders et al., 2002 for a review). Moreover, an association has been suggested between one of the polymorphisms in the human gene

Abbreviations: BAC, bacterial artificial chromosome; bp, base pairs; CFA, canine chromosome; EST, expressed sequence tag; FISH, fluorescence in situ hybridisation; 5-HT, serotonin; HSA, human chromosome; *htr1B*, gene encoding serotonin receptor 1B; LOD, logarithm of odds; ORF, open reading frame; PCR, polymerase chain reaction; RH, radiation hybrid; SNP, single nucleotide polymorphism; UTR, untranslated region.

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(G186C) and alcoholism, suicidality, and obsessive-compulsive disorder (Huang et al., 2003; Sanders et al., 2002). Knock-out mice lacking *htr1B* show several behavioural changes, including increased aggression (Saudou et al., 1994).

The present study on canine *htr1B* was performed in the context of a research project involving canine fear, aggression and impulsivity. Extreme aggressive behaviour in dogs is a major problem for victim, owner and dog (Hunthausen, 1997; Rusch et al., 2000). In order to reveal genetic factors involved in the aetiology of this behavioural problem, we are studying parts of the canine serotonergic system.

Information about canine *htr1B* is currently limited to its position on the canine map (Guyon et al., 2003) and partial nucleotide sequence of the coding region (Sgard et al., 1996). This paper reports on the isolation and characterization of a canine bacterial artificial chromosome (BAC) clone containing *htr1B*. We describe the complete coding sequence of the canine gene including non-coding flanking regions and five single nucleotide polymorphisms (SNPs), confirmation of its position in the dog genome, and isolation of a nearby microsatellite marker.

2. Materials and methods

2.1. Animals and DNA isolation

The Golden Retrievers, Dobermanns and Beagles used in this study were privately owned, apparently not closely related dogs, except for one sibling pair (Golden Retrievers 3031 and 3032). Information about their behavioural characteristics was not available. Canine and mouse (BALBc) genomic DNA was isolated from whole blood lymphocytes using the salt extraction method (Miller et al., 1988). Great Dane genomic DNA was extracted from spleen.

2.2. BAC library screening

Primers 1 and 2 (Table 1) were based on the coding sequence of *htr1B* in the mouse (GenBank accession number: M85151). These primers amplified a 997-bp fragment of *htr1B* in a PCR using 500 ng genomic mouse DNA, 0.5 μM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase in 1× Gibco-BRL buffer in a 100-μl reaction volume. This 997-bp probe (*htr1B*-997) was labelled with [α-^{32}P] dATP using a megaprime DNA labelling kit (Amersham, Piscataway, NY). The nucleotide sequence of *htr1B*-997 is 91% identical to the corresponding region in human *HTR1B*, whereas it is only 67% identical to the closely related murine *htr1D*. We therefore did not expect the probe to hybridise with other members of the family of serotonin receptor genes.

The canine genomic BAC library RPCI-81, derived from a Dobermann (Li et al., 1999), was screened with a pool of different probes including *htr1B*-997. Filters were prehybridised (1 h) and hybridised (overnight) in Church Buffer at 65 °C, washed, and exposed to film at −70 °C (see http://bacpac.chori.org/highdensity.htm for details). Positive colonies were picked, grown overnight in 3-ml Luria-Bertani medium with chloramphenicol (20 μg/ml) and 1 μl of the overnight bacterial culture was spotted onto a Hybond N$^+$ membrane (Amersham). These colonies were hybridised with probe *htr1B*-997 to ensure that they contained *htr1B*. Positive colonies were picked, grown, and the alkaline lysis method as described on the BacPac website (http://bacpac.chori.org/bacpacmini.htm) was used for BAC DNA isolation.

2.3. Southern blot

In order to select a BAC clone containing *htr1B*, 10 μg genomic Golden Retriever DNA, 10 μg genomic Great

Table 1
Primer pairs used in this study (T_A = annealing temperature)

Primer numbers	Sequence (5′–3′)	Position[a]	Length of product (bp)	T_A (°C)
1	CCT GGA AAG TCC TGC TGG	128 to 145[b]	997	60
2	GCT TGT TTG AAG TCC TCA TTG G	1103 to 1124[b]		
3	GGC GAG GAG AGA CAT GGA A	−13 to 6	317	62
4	TCA CCA GGA TGG AGA CGA G	286 to 304		
5	CTC ATC ACC TTG GCC ACC AC	169 to 188	752	60
6	CTA GCG GCC ATG AGT TTC TTC	900 to 920		
7	ACA TCC TCT ACA CCG TGT ACT C	611 to 632	680	57
8	GCC AGA AGA CAG AGC CTC A	1272 to 1290		
9	CTT CTC AGG CAT CAT TCT CC	not available	139	57
10	CGT GGA GCC TGC TTC TT			

[a] Unless stated otherwise, positions are based on the coding sequence of canine *htr1B* published in this paper, where the A of the ATG start codon is designated number 1.
[b] The position of this primer is based on the coding sequence of murine *htr1B* (accession number M85151).

Dane DNA and 500 ng BAC clone DNA were digested with *Eco*RI. In addition, genomic DNA was digested with *Bgl*II, *Bam*HI and *Pst*I. The resulting fragments were separated on a 0.7% agarose gel and transferred to Hybond N^+ membrane (Amersham).

Prehybridisation (1 h) and hybridisation (overnight) were performed at 65 °C in 0.5 M sodium phosphate buffer (pH 7.4)/7% SDS/0.1% EDTA using probe *htr1B*-997. The blots were washed three times for 5 min and 1 time for 15 min (genomic DNA) or three times for 15 min (BAC DNA) with 40 mM sodium phosphate buffer/0.1% SDS at 65 °C, and exposed to film for 3 days at −70 °C (genomic DNA) or 1.15 h at 20 °C (BAC DNA). The BAC clone with the strongest signal (BAC 18L8) was selected.

2.4. Sequence analysis of canine htr1B

The majority of the canine *htr1B* sequence was determined by sequencing subcloned fragments of BAC 18L8. BAC 18L8 DNA (1 μg) was digested with *Sau*3AI, the resulting fragments were purified and ligated into *Bam*HI digested pZErO™-1 vector with T4 DNA ligase (New England Biolabs), transformed into TOP10F′ bacterial cells and plated on low salt LB plates with 50 μg/ml zeocin. Zeocin-resistant colonies were transferred to Hybond N^+ membranes, and DNA from these colonies was hybridised with the *htr1B*-997 probe. DNA was isolated from the positive colonies with a Promega DNA purification system and sequenced with T7 (5′-TAA TAC GAC TCA CTA TAG GG -3′) and SP6 (5′-ATT TAG GTG ACA CTA TAG -3′) primers using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA.).

The borders of the gene were sequenced by direct BAC DNA sequencing. Twenty picomoles of a reverse (5′-ATC ACC AGG ATG GAG ACG AGC A-3′) or a forward (5′-ACG CGC TGC TGG AGA AGA AG-3′) primer was used in a 30-μl tercycle reaction with 4 mM $MgCl_2$, 12 μl BigDye Terminator v3.0 Ready Reaction Mix (Applied Biosystems) and 800 ng BAC DNA. The tercycle consisted of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 10 s at 56 °C, and 4 min at 60 °C. Tercycle products were purified using multiscreen 96-well filtration plates (Millipore), and sequenced in 15 μl distilled water with an ABI 3100 Genetic Analyzer (Applied Biosystems).

Three primer pairs (3 and 4, 5 and 6, and 7 and 8; see Table 1) were designed based on the preliminary canine coding sequence. These pairs amplified overlapping products in PCR reactions containing 1 ng BAC 18L8 DNA. The first PCR product was produced in a 25-μl reaction volume with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM primers 3 and 4, and 1.25 U Platinum® *Taq* DNA polymerase. The second PCR product was produced in a 50-μl reaction volume with 4 mM $MgCl_2$, 0.34 mM dNTPs, 0.2 μM primers 5 and 6, and 0.5 U *Taq* DNA polymerase. The third PCR product was produced in a 20-μl reaction volume with 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μM primers 7 and 8, and 0.7 U AmpliTaq Gold® DNA polymerase. All PCR products were purified with a QIAquick PCR purification kit and their DNA sequence was analysed with the automatic sequencer. Tercycle reactions were performed in a 10-μl volume and contained 10 ng PCR product, 3.2 pmol primer, and 1 μl BigDye Terminator v3.0 Ready Reaction Mix in 1× sequence buffer (80 mM Tris, 2 mM $MgCl_2$, pH 9.0). Tercycle products were purified using multiscreen 96-well filtration plates (Millipore) and diluted several times before sequencing. In this way, every base in the preliminary DNA sequence was confirmed. The 5′ and 3′ ends of the preliminary sequence were confirmed by repeated direct BAC DNA sequencing. Note that there is a mismatch in primer 5: the 10th base is a T, where the corresponding position in the exon shows a C, but this apparently did not affect the specificity of the primer.

Homologies with human, murine and porcine coding sequences were calculated with MegAlign (DNA Star Software, clustal W method). These DNA sequences have the following GenBank accession numbers: M89478 (human), M85151 (mouse) and AF188626 (pig). Predicted positions of the transmembrane regions and key amino acid residues in the protein were derived from the SWISSPROT website (http://us.expasy.org/sprot/; Swissprot accession number P28222), the GPCRDB information system for G protein-coupled receptors (http://www.gpcr.org), and from several publications referred to in the relevant sections.

2.5. FISH mapping

Metaphase chromosomes were prepared from concanavalin A stimulated peripheral blood lymphocytes from karyotypically normal dogs. Fluorescence in situ hybridisation was performed on GTG-banded metaphase spreads as described by Zijlstra et al. (1997). Prior to FISH, well-banded metaphases were captured using a Leica DMRA microscope equipped with the GENUS Image Analysis software of Applied Imaging. After capturing, slides were washed twice with 4× SSC/0.05% Tween 20, dehydrated and air-dried. Total DNA of BAC clone 18L8 was used as probe and labelled with biotin-16-dUTP by nick-translation. Labelled BAC DNA was precipitated with a 50-fold excess of salmon ssDNA (Sigma) and a 100-fold excess of fragmented total dog DNA as competitor. The precipitate was resuspended in hybridisation solution containing 50% formamide/2× SSC/10% dextransulfate to a final probe concentration of 5 ng/μl. Specific sites of hybridisation were detected using avidin-FITC, and signals were amplified twice using additional layers of biotinylated goat anti-avidin and avidin-FITC. Chromosomes were counterstained with propidium iodide. Previously captured metaphases were reexamined after FISH, recaptured and analysed using the GENUS software. Chromosomes showing specific hybridisation signals were identified on the

basis of their banding patterns in accordance with the recommendations of the Committee for the Standardized Karyotype of the Dog (*Canis familiaris)* (Switonski et al., 1996).

2.6. Radiation hybrid mapping

We used the RHDF5000 canine whole genome radiation hybrid (RH) panel (Vignaux et al., 1999) for radiation hybrid mapping of the 18L8 clone. Primer pair 7 and 8 (Table 1) was used to establish retention of *htr1B* in the 126 hybrids. The primers were tested on genomic canine, hamster and 2:1 hamster/canine DNA in order to confirm their specificity for dog DNA. Conditions of the PCR reactions were as described in Section 2.4, except for the total reaction volume (10 μl instead of 20 μl). Fifty nanogram hybrid DNA was used in each reaction.

The program "126to118" (http://www-recomgen.univ-rennes1.fr/Dogs/126to118.html) was used to convert the results to a 118 vector to be computed on the 3270-marker version of the RH map. The typing data were then incorporated into this map (Guyon et al., 2003), using the two-point analysis of the Multimap package (Matise et al., 1994). RH maps for all chromosomes and additional information can be found at http://www-recomgen.univ-rennes1.fr/doggy.html.

2.7. Microsatellite marker isolation and analysis

In order to isolate a microsatellite marker from BAC 18L8, 1 μg of BAC18L8 DNA was subcloned as described in Section 2.4. Instead of hybridising zeocin-resistant colonies with probe htr1B-997, we randomly picked 96 colonies and grew them for 6 h in 100-μl LB medium with 50 μg/ml zeocin. The cultures were diluted 10 times, and a PCR was performed directly using M13 forward (5′ -GTA AAA CGA CGG CCA GT-3′) and reverse (5′ -CAG GAA ACA GCT ATG AC-3′) primers. Sequencing was performed as described in Section 2.4, with M13 forward primer. We blasted all DNA sequences on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) and examined them for the presence of microsatellite markers. A $(GA)_{13}$ repeat was identified.

In order to establish whether the marker is polymorphic, primers 9 and 10 (Table 1) were designed around the repeat and tested in the BAC, two Beagles, two Dobermanns and six Golden Retrievers. The 5′ end of primer 9 was labelled with 6-FAM fluorescent dye (Eurogentec). PCR reactions were performed in a 20-μl volume containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 μM of both primers, 0.7 U AmpliTaq Gold® DNA polymerase in 1 × AmpliTaq Gold buffer. The following PCR program was used: 5 min initial denaturation at 95 °C, followed by 34 cycles of 30 s 94 °C, 30 s 57 °C, and 30 s 72 °C, followed by a final extension step of 2 min at 72 °C. Labelled PCR products were diluted to a concentration of approximately 0.5 ng/μl and 2 μl of this dilution was mixed with 9.85 μl formamide and 0.15 μl TAMRA-GS500 size standard (Applied Biosystems) for analysis on the ABI 3100 Genetic Analyzer (Applied Biosystems). The results for genomic DNA were compared to the results for BAC DNA in order to determine the accurate size of the fragments.

2.8. Sequence analysis of htr1B in two Beagles, two Dobermanns and six Golden Retrievers

We analysed base pairs 7–1271 of *htr1B* in genomic DNA of two Beagles, two Dobermanns and six Golden Retrievers by DNA sequencing of PCR products, using primer pairs 3 and 4, 5 and 6, and 7 and 8. Twenty-five nanogram of genomic DNA was used in each reaction. The PCR reactions and sequencing were performed as described in Section 2.4. The 752- and 680-bp PCR products were sequenced with alternative forward primers (5′-ACA TCC TCT ACA CCG TGT ACT C-3′ and 5′-GGT CAC CTC CGT TAA CTC G-3′, respectively) in some dogs because primers 5 and 7 did not give satisfying results.

3. Results

3.1. BAC library screening and Southern blot

BAC library screening with probe *htr1B*-997 resulted in six positive BAC clones: 1I5, 18L8, 86N14, 91D4, 163B17 and 164D24. In a Southern blot analysis with the same probe, all BAC DNA and genomic dog DNA showed a single *Eco*RI fragment of approximately 8 kb. Southern blots of genomic dog DNA digested with *Bgl*II, *Bam*HI and *Pst*I also showed only one band (of approximately 6.6, 8, and 2 kb respectively). These results confirm the presence of *htr1B* on the BAC clones and imply that *htr1B* is likely to be a single-copy gene in the canine genome. We continued our experiment with clone 18L8.

3.2. Sequence analysis of htr1B

The entire coding sequence of *htr1B* on BAC clone 18L8 was determined, including 102 bp at the 5′-flanking side and 184 bp at the 3′-flanking side (Fig. 1). Canine *htr1B* consists of one exon with an open reading frame (ORF) of 1167 bp. The nucleotide sequence in this ORF is highly similar to human, murine, and porcine *htr1B* sequences (91%, 89% and 91% identity, respectively). We discovered a 66-bp segment with 85% sequence homology to the human 5′ region in the canine 5′ flanking flanking region, but no homology was found with the murine 5′ sequence (canine nucleotides −68 up to −3, where the A in the ATG start codon is designated number 1). The 3′ flanking sequence contained a segment homo-

CAAGCGGGACACCGGACTGTGGTATCTCCGCGGGCCTTCCGCCCTTTCCGTCGTTTGCTCCATGCCCCAGGGCTGCGCTC
CGGGGCCCCGGCGAGGAGAGAC

ATG	GAA	GCA	GCC	GGC	GCT	CCG	TGC	GCC	CCG	CCG	CCG	CCC	GCG	GGC	TCC	CAG	ACC	54
M	E	A	A	G	A	P	C	A	P	P	P	P	A	G	S	Q	T	18
GGG	GCT	CCT	CCA	GCC	AAC	CTG	TCT	TCG	GCG	CCG	CAC	AAC	TGC	AGC	GCC	GAG	GGC	108
G	A	P	P	A	N	L	S	S	A	P	H	N	C	S	A	E	G	36
TAC	ATC	TAC	CAG	GAC	TCC	GTC	GCG	CTG	CCC	TGG	AAA	GTG	CTC	CTG	GTC	**A**TT	CTG	162
Y	I	Y	Q	D	S	V	A	L	P	W	K	V	L	L	V	I(L)	L	54
CTG	GCA	CTC	ATC	ACC	CTG	GCC	ACC	ACG	CTC	TCC	AAC	GCC	TTT	GTG	ATC	GCC	ACG	216
L	A	L	I	T	L	A	T	T	L	S	N	A	F	V	I	A	T	72
GTG	TAC	CGG	ACC	CGG	AAG	CTG	CAC	ACG	CC**G**	GCC	AAC	TAC	CTG	ATC	GCC	TCC	CTG	270
V	Y	R	T	R	K	L	H	T	P	A	N	Y	L	I	A	S	L	90
GCC	GTC	ACC	GAC	CTG	CTC	GTC	TCC	ATC	CTG	GTG	ATG	CCC	ATC	AGC	ACC	ATG	TAC	324
A	V	T	D	L	L	V	S	I	L	V	M	P	I	S	T	M	Y	108
ACG	GTC	ACC	GGC	CGC	TGG	ACG	CTG	GGC	CAG	GTG	GTC	TGC	GAC	TTG	TGG	CTG	TCG	378
T	V	T	G	R	W	T	L	G	Q	V	V	C	D	L	W	L	S	126
TCG	GAC	ATC	ACC	TGT	TGC	ACG	GCT	TCC	ATC	CTG	CAC	CTC	TGC	GTC	ATC	GCC	CTG	432
S	D	I	T	C	C	T	A	S	I	L	H	L	C	V	I	A	L	144
GAC	CGC	TAC	TGG	GCC	ATC	ACG	GAC	GCC	GTG	GAG	TAC	TCC	GCC	AAA	AGG	ACT	CCC	486
D	R	Y	W	A	I	T	D	A	V	E	Y	S	A	K	R	T	P	162
AAG	AGG	GCC	GCG	GTC	ATG	ATC	GCG	CTC	GTG	TGG	GTC	TTC	TCC	ATC	TCC	ATC	TCG	540
K	R	A	A	V	M	I	A	L	V	W	V	F	S	I	S	I	S	180
CTG	CCG	CCC	TTC	TTC	TGG	CGC	CAG	GCC	AAA	GCC	GAG	GAG	GAG	GTG	TCG	GAC	TGC	594
L	P	P	F	F	W	R	Q	A	K	A	E	E	E	V	S	D	C	198
GTG	GTG	AAC	ACC	GAC	CAC	ATC	CTC	TAC	ACC	GTG	TAC	TCC	ACG	GTG	GGC	GCT	TTC	648
V	V	N	T	D	H	I	L	Y	T	V	Y	S	T	V	G	A	F	216
TAC	TTC	CCC	AC**G**	CTG	CTC	CTC	ATC	GCC	CTC	TAC	GGC	CGC	ATC	TAC	GTG	GAA	GCC	702
Y	F	P	T	L	L	L	I	A	L	Y	G	R	I	Y	V	E	A	234
CGC	TCC	CGG	ATT	TTG	AAA	CAG	ACG	CCC	AAC	AGG	ACC	GGC	AAG	CGC	CTG	ACC	CGA	756
R	S	R	I	L	K	Q	T	P	N	R	T	G	K	R	L	T	R	252
GCC	CAG	CTG	ATA	ACC	GAC	TCC	CCC	GGC	TCC	ACG	TCC	TCG	GTC	ACC	TCC	GTT	AAC	810
A	Q	L	I	T	D	S	P	G	S	T	S	S	V	T	S	V	N	270
TCG	CGG	GCT	CCC	GAC	GTG	CCC	AGC	GAA	TCC	GGG	TCC	CCG	GTG	TAC	GTG	AAC	CAA	864
S	R	A	P	D	V	P	S	E	S	G	S	P	V	Y	V	N	Q	288
GTC	AAA	GTG	CGG	GTC	TCC	GAC	GCG	CTG	CTG	GAG	AAG	AAG	AAA	CTC	ATG	GCC	GCT	918
V	K	V	R	V	S	D	A	L	L	E	K	K	K	L	M	A	A	306
AGG	GAG	CGC	AAA	GCC	ACC	AAG	ACC	CTG	GGA	ATC	ATC	**C**TG	GGA	GCC	TTT	ATC	GTG	972
R	E	R	K	A	T	K	T	L	G	I	I	L	G	A	F	I	V	324
TGC	TGG	CTG	CCC	TTC	TTC	ATC	ATC	TCC	CTG	GTG	ATG	CCT	ATT	TGC	AAG	GAC	GCC	1026
C	W	L	P	F	F	I	I	S	L	V	M	P	I	C	K	D	A	342
TGC	TGG	TTC	CAC	CTG	GCC	ATC	TTC	GAC	TTC	TTC	ACG	TGG	CTG	GGC	TAT	CTC	AAC	1080
C	W	F	H	L	A	I	F	D	F	F	T	W	L	G	Y	L	N	360
TCC	CTT	ATC	AAC	CCC	ATC	ATC	TAT	ACC	ATG	TCC	AAT	GAG	GAC	TTC	AAA	CAA	GCG	1134
S	L	I	N	P	I	I	Y	T	M	S	N	E	D	F	K	Q	A	378
TTC	CAT	AAA	CT**C**	ATA	CGC	TTT	AAG	TGC	GCA	GGT	TGA							1170
F	H	K	L	I	R	F	K	C	A	G								389

CTTGTCAGTTGCAATGGGGTAGCCTGAGCGACCTTTGGGGACCATGCTGGGTCTGGTTCCACAGGTAGGTCGACTCTTCT
TTCACTGTTACTGGGTCAGGTTGAGGCTCTGTCTTCTGGCCAGTGGATCCTGAGAAGCCAGGACAGCCCTGAGGGCGCTC
CGCTCCCAGAGGAGACCTGTTCCA

Fig. 1. Nucleotide sequence and deduced amino acid sequence of canine *htr1B*. The sequence has been deposited in the GenBank database under accession number AY323909. A preliminary sequence was obtained by means of sequencing subcloned BAC 18L8 with T7 and SP6 primers and direct BAC DNA sequencing. This sequence was confirmed by sequencing BAC DNA-based PCR products. Nucleotides printed in bold are polymorphic and the alternative amino acid is printed between parentheses in case of an amino acid change.

logous to both human and murine sequences (canine nucleotides 1179–1267 were 89% identical to the human sequence, and 1171–1266 were 86% identical to the murine sequence). The evolutionary conservation of these two segments suggests that they are involved in the function of the gene. The 5-HT_{1B} protein consists of 389 amino acids in the dog, 390 amino acids in man and the pig, and 386 in the mouse. Canine amino acid identity is 95% with the human, 93% with the murine, and 94% with the porcine protein. The conservation in the transmembrane domains is high (Fig. 2), although it does not compare with the homology in the serotonin receptor 1A gene (*htr1A*), where these domains are 100% identical in dogs and humans (Van den Berg et al., 2003).

3.3. FISH mapping of BAC 18L8

For the chromosomal localisation of BAC clone 18L8, 27 GTG-banded metaphase spreads were analysed after fluorescence in situ hybridisation (FISH). Nineteen of these metaphases showed specific hybridisation signals on the chromosomes of pair 12, band q1.6–1.7 (Fig. 3). This is in agreement with previous localisation of *htr1B* in the dog using radiation hybrid mapping (Guyon et al., 2003).

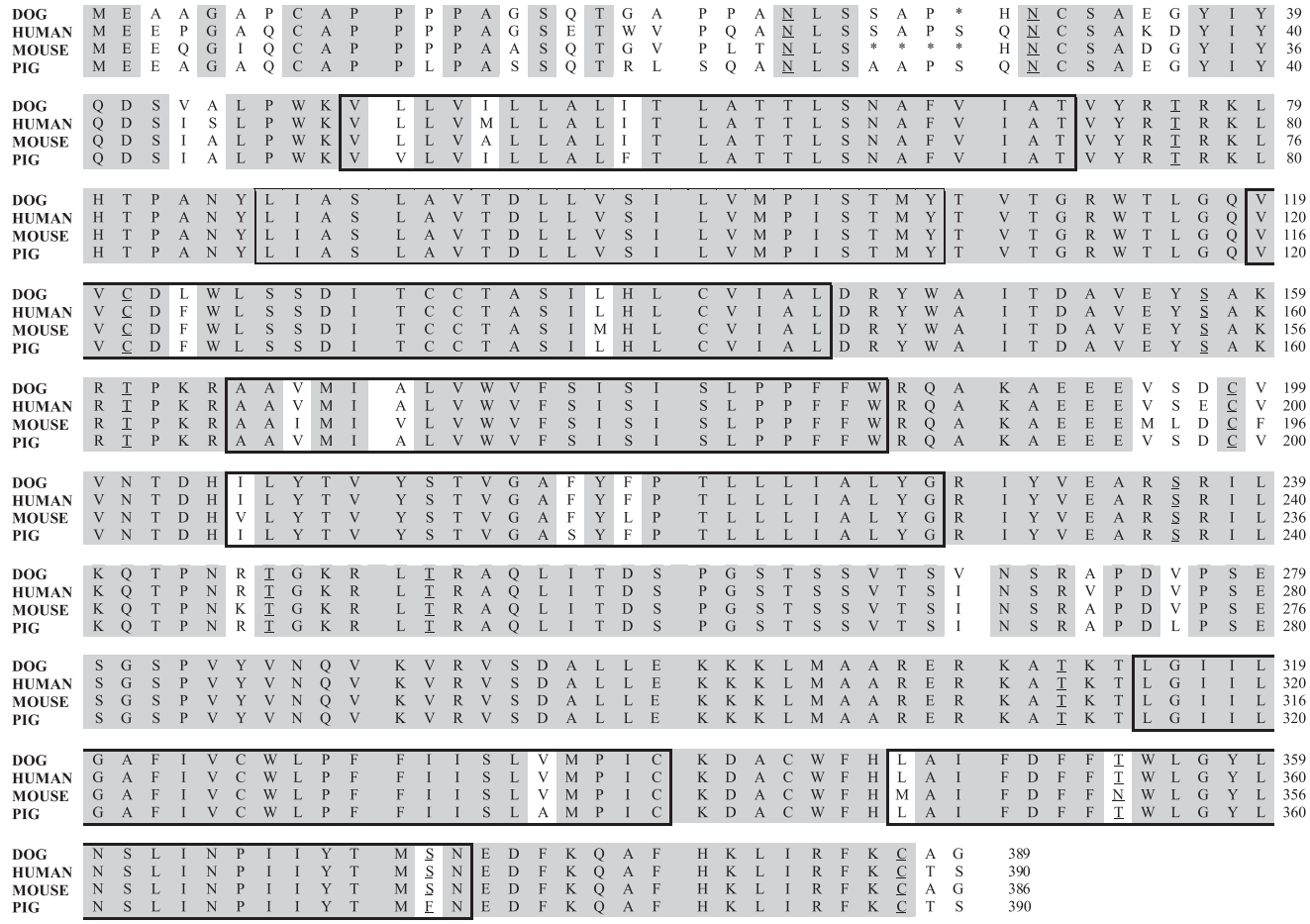

Fig. 2. Alignment of the amino acid sequence of canine serotonin receptor 1B with human (accession number M89478), murine (accession number M85151), and porcine (accession number AF188626) sequences. Amino acids are presented in groups of 10. Residues that are conserved in the four species are shown in grey background and predicted transmembrane regions according to SWISSPROT are boxed. Residues 1–48 form the amino terminus; residues 73–85 form the first intracellular loop, etc. Key residues that are highlighted in Section 4.1 are underlined: 121C and 198C (disulfide bridge), 387C (palmitoylation), 24N and 31N (glycosylation), 76T, 157S, 161T, 236S, 246T, 251T, 312T, and 371S (phosphorylation) and 354T (ligand binding). (All positions of key residues refer to the canine protein).

3.4. Radiation hybrid mapping

htr1B was retained in 23% of the hybrids of the RHDF5000 panel, which is in accordance with its average retention rate of 21%. Computation of the vector on the 3270 RH map (Guyon et al., 2003) revealed that *htr1B* mapped on CFA12 with high LOD scores with the previously mapped *htr1B* (LOD score = 22) and EST 5H8 (LOD score = 20).

3.5. Microsatellite marker isolation and analysis

Ninety-six random subcloned fragments of BAC 18L8 were sequenced in order to find microsatellite markers. The sequences were blasted, and among the results was a 228-bp fragment with 84% identity to human clone RP11-551A13 on chromosome 6 (GenBank accession number AL390316). In addition, a 22-bp fragment with 100% identity to the 3′ UTR of human *htr1B* (GenBank accession number D10995) was found. Another sequence contained a $(GA)_{13}$ repeat. The latter sequence has been deposited in the GenBank database under accession number AY325269 and we have named this marker UU18L8. To determine whether the marker was polymorphic, it was analysed in 10 dogs (six Golden Retrievers, two Beagles, and two Dobermanns). Three different alleles were detected with product lengths of 139, 143, and 147 base pairs. The genotypes of the dogs are presented in Table 2.

3.6. Sequence analysis of htr1B in Dobermanns, Beagles and Golden Retrievers

Sequence analysis of base pairs 7–1271 of *htr1B* in 10 different dogs revealed five single nucleotide polymorphisms (SNPs): A157C, G246A, C660G, T955C, and G1146C (Fig. 1 and Table 2; note that the nucleotide with the highest evolutionary conservation is put in front in this notation, e.g. at the equivalent of position 157, human and

porcine *htr1B* contain an A, while murine *htr1B* contains a G). All SNPs displayed variation between the six Golden Retrievers, indicating that they are not breed-specific. Table 2 reveals that there exist at least four extended haplotypes in the Golden Retrievers tested. Golden 3710 is homozygous for all markers, so the haplotype must be 143-C-A-C-T-G ("haplotype 1") in this dog. In the simplest situation Goldens 1264, 1265, 3031, and 3346 would also carry haplotype 1. Golden 1264 would then carry a second haplotype, 143-A-G-C-C-C ("haplotype 2"); Golden 1265 and 3031 would carry a third version, 143-A-G-C-T-G ("haplotype 3"); and Golden 3346 a fourth variant, 139-A-G-G-C-C ("haplotype 4"). Golden 3032 would carry haplotypes 3 and 4.

Table 2
Genotype of 10 dogs for microsatellite marker UU18L8 (isolated from BAC 18L8), and five SNPs in the *htr1B* ORF

	UU18L8	157[a]	246	660	955	1146
Golden 1264[b]	143/143	A/C	A/G	C/C	C/T	G/C
Golden 1265	143/143	A/C	A/G	C/C	T/T	G/G
Golden 3031	143/143	A/C	A/G	C/C	T/T	G/G
Golden 3032	139/143	A/A	G/G	G/C	C/T	G/C
Golden 3346	139/143	A/C	A/G	G/C	C/T	G/C
Golden 3710	143/143	C/C	A/A	C/C	T/T	G/G
Dobermann 5058	139/139	A/A	G/G	C/C	T/T	G/G
Dobermann 5126	139/139	A/A	G/G	C/C	T/T	G/G
Beagle 5260	139/147	A/A	G/G	C/C	T/T	G/G
Beagle 5265	139/143	A/A	G/G	G/C	C/T	G/C

[a] Numbers of the SNPs refer to their position in *htr1B*.
[b] Numbers of the dogs refer to their numbers in our DNA database.

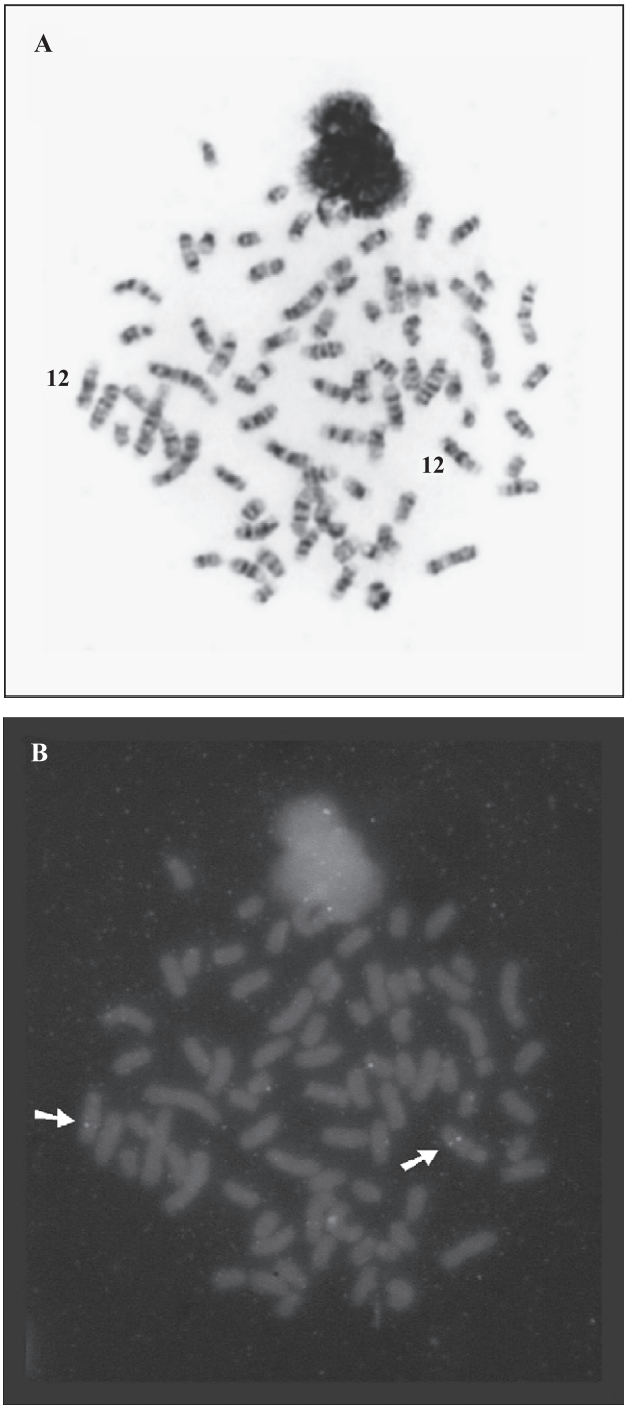

Fig. 3. Chromosomal localization of BAC clone 18L8 by fluorescence in situ hybridisation (A) GTG-banded metaphase spread, where chromosomes of pair 12 are marked; (B) same metaphase after FISH with clone 18L8 as probe. Hybridisation signals on chromosomes of pair 12 are marked with arrows.

Only A157C is expressed at the amino acid level; the other four SNPs are silent polymorphisms. A157C gives rise to an isoleucine/leucine polymorphism of amino acid 53, which is situated in the amino terminus of the first transmembrane segment of the receptor. We do not expect this polymorphism to have functional consequences because Ile and Leu have similar chemical properties.

4. Discussion

4.1. Canine htr1B sequence

We have isolated a canine BAC clone containing the gene encoding serotonin receptor 1B and we have determined the entire coding sequence of this gene. Sgard et al. (1996) previously published a partial sequence of *htr1B* (GenBank accession number S82461), running from position 573 to 1056 in the ORF. At that time, the gene was called *htr1Dβ*, but it is now generally accepted that *htr1Dβ* is in fact a species variant of *htr1B* (Hartig et al., 1996). The Sgard et al. DNA sequence is completely identical to the DNA sequence published in this paper, except at positions 660 and 955 due to polymorphism of the gene.

In future studies on the association between mutations or polymorphisms in *htr1B* and behavioural disorders in dogs, information about key functions of residues in the amino acid chain is indispensable. The key ligand binding areas of G-protein coupled receptors are located mainly in the top one-third of transmembrane domains 3–7 (Hartig, 1997). In particular, residue 354Thr in the serotonin receptor 1B is

believed to be critical for ligand binding. The second and third intracellular loop appear to be important for coupling to the inhibition of adenylate cyclase (Albert and Tiberi, 2001). In Fig. 2, several specific key amino acid residues can be recognised: residues involved in a disulfide bridge (121Cys and 198Cys, SWISSPROT website http://us.expasy.org/sprot/; Swissprot accession number P28222), and potential sites for palmitoylation (387Cys), N-linked glycosylation (24Asp and 31Asp), phosphorylation by protein kinase C (76Thr, 157Ser, 161Thr, 236Ser and 246Thr), phosphorylation by tyrosine kinase (371Ser), and phosphorylation by protein kinase A (251Thr and 312Thr) (Jin et al., 1992; Hamblin et al., 1992; Maroteaux et al., 1992).

Most of the amino acid residues highlighted above are conserved in dogs, humans, mice and pigs, except for residues 354Thr and 371Ser. The presence of an asparagine residue at the equivalent of position 354 in the mouse is remarkable. Evolutionary conservation of key residues is usually high, because a mutation in such an amino acid is often deleterious and therefore removed from the population by natural selection. (Majewski and Ott, 2003). *htr1B* illustrates an exception from the rule: the Thr354Asn mutation causes a marked difference in the pharmacology of murine and human 5-HT_{1B} receptors, but it is not deleterious (Oksenberg et al., 1992).

4.2. Localisation of canine htr1B

Radiation hybrid mapping of *htr1B* was previously performed with primers at positions 862–881 (forward) and 947–969 (reverse) in the *htr1B* ORF (Guyon et al., 2003). The PCR product we used was longer (680 bp) and our reverse primer was located in the 3′ non-coding flanking area. The high accordance between the two RH mapping results and the FISH mapping confirms the position of *htr1B*. In the human genome, *htr1B* maps to HSA 6q13. Homology between CFA12 and HSA6 has previously been demonstrated by reciprocal chromosome painting studies (Breen et al., 1999).

4.3. Polymorphic markers

Six polymorphic markers, one microsatellite repeat and five SNPs, were identified in this study. The four silent SNPs are located in conserved regions of the gene, whereas the SNP at nucleotide position 157 is located in a variable region of *htr1B*. The frequency of SNPs in the coding region of canine *htr1B* is remarkably high compared to the average of one in every 834 bp of coding region (Brouillette et al., 2000). A high frequency of polymorphisms has also been reported for human *htr1B*: the coding sequence of the human gene contains nine SNPs, at nucleotide positions 129, 276, 371, 655, 705, 772, 861, 1099, and 1120 (Sanders et al., 2002). Future studies will have to reveal the frequency of the different alleles in various dog breeds.

The presence of at least four haplotypes in the small number of Golden Retrievers that we studied suggests a large variation of the *htr1B* sequence within this breed. Dog breeds are considered to be genetically homogeneous (Ostrander et al., 2000). However, some dog breeds are more inbred than others, and the Golden Retriever breed has previously been reported to be relatively heterogeneous (Nielen et al., 2001). The haplotypes will be useful for genetic studies in dog populations for which behavioural data (for example about aggressive behaviour) are available.

4.4. Conclusion

We extended the available data on canine *htr1B* with its coding and flanking DNA sequence, developed primer sets for genomic sequencing of the coding region, and identified five single nucleotide polymorphisms within the ORF. In addition, the localization of *htr1B* on the canine genomic map was confirmed by fluorescent in situ hybridisation (FISH) and radiation hybrid (RH) mapping, and we have isolated a nearby microsatellite marker. This work provides a starting point for mutation scans and association studies on dogs with behavioural problems.

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