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The Sleep Disorder Canine Narcolepsy Is Caused by a Mutation in the *Hypocretin* (*Orexin*) *Receptor 2* Gene

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Summary

Narcolepsy is a disabling sleep disorder affecting humans and animals. It is characterized by daytime sleepiness, cataplexy, and striking transitions from wakefulness into rapid eye movement (REM) sleep. In this study, we used positional cloning to identify an autosomal recessive mutation responsible for this sleep disorder in a well-established canine model. We have determined that canine narcolepsy is caused by disruption of the *hypocretin* (*orexin*) *receptor 2* gene (*Hcrtr2*). This result identifies hypocretins as major sleep-modulating neurotransmitters and opens novel potential therapeutic approaches for narcoleptic patients.

Introduction

Sleep is a vital behavior of unknown function that consumes one-third of any given human life (Dement, 1994). Electrophysiological studies have shown that sleep is a heterogenous state most classically separated into rapid eye movement (REM) sleep and non-REM sleep (Dement, 1994). REM sleep is characterized by vivid dreaming, muscle atonia, desynchronized electroencephalogram (EEG) activity, and REMs. Non-REM sleep is characterized by synchronized EEG activity, partial muscle relaxation, and less frequent dreaming mentation (Dement, 1994). REM and non-REM sleep are indispensable to survival; both REM and total sleep deprivation are lethal in mammals (Rechtschaffen et al., 1983; Kushida et al., 1989). Independent of this organization in REM and non-REM sleep, the propensity to sleep or stay awake is regulated by homeostatic (sleep debt–dependent) and circadian (clock-dependent) processes (Borbély, 1994). Circadian processes are believed to be primarily generated at the genetic level within the suprachiasmatic nucleus of the hypothalamus (Klein et al., 1991; Moore and Silver, 1998). Mutations in selected genes of the basic-helix-loop-helix (bHLH)-PAS domain protein family, such as *Clock*, *per*, and *cycle* (*BMAL1*) or *timeless*, have been shown to selectively alter circadian rhythmicity in *Drosophila* and/or mammals (Huang et al., 1993; Sehgal et al., 1994; King et al., 1997; Shearman et al., 1997; Sun et al., 1997). Protein–protein interactions within the PAS domain and transcription–translation feedback loops have been established to be primary factors in the generation of circadian rhythmicity at the cellular level (Huang et al., 1993; Sehgal et al., 1994; King et al., 1997; Shearman et al., 1997; Sun et al., 1997).

Whereas major progress has been made in our understanding of the generation of circadian rhythmicity both at the molecular and neuroanatomical levels, sleep generation is still poorly understood at the molecular level. One pathway to this understanding is the study of the sleep disorder narcolepsy. Narcolepsy is a disabling neurological disorder that affects more than 1 in 2,000 Americans (Mignot, 1998). The disorder is characterized by daytime sleepiness, sleep fragmentation, and symptoms of abnormal REM sleep, such as cataplexy, sleep paralysis, and hypnagogic hallucinations (Aldrich, 1993; Nishino and Mignot, 1997; Aldrich, 1998). It is the only known neurological disorder that specifically affects the generation and organization of sleep. Narcolepsy has also been reported to occur in animals and has been most intensively studied in canines (Foutz et al., 1979; Baker and Dement, 1985; Nishino and Mignot, 1997; Cederberg et al., 1998). A large number of physiological and pharmacological studies performed over a 20 year period have demonstrated a close similarity between human and canine narcolepsy (Baker and Dement, 1985; Nishino and Mignot, 1997). Strikingly, humans and canines with narcolepsy exhibit cataplexy, sudden episodes of muscle weakness akin to REM sleep–associated atonia that are triggered primarily by positive emotions (Foutz et al., 1979; Baker and Dement, 1985; Nishino and Mignot, 1997).

Although familial cases of narcolepsy have been reported, most human occurrences are sporadic, and the disorder is generally believed to be multigenic and environmentally influenced (Honda and Matsuki, 1990; Mignot, 1998). One predisposing genetic factor is a specific *HLA-DQ* allele, *HLA-DQB1*0602* (Matsuki et al., 1992; Mignot et al., 1994a; Mignot, 1998). Because of the tight *HLA* association, the disorder in humans has been suggested to be autoimmune in nature; however, all attempts to verify this hypothesis have failed (Mignot et al., 1995). In Doberman pinschers, the disorder is transmitted as a single autosomal recessive trait with full penetrance, *canarc-1* (Foutz et al., 1979; Baker and Dement, 1985). To unravel the cause of this unique sleep disorder, we undertook the positional cloning of the canine narcolepsy gene. This project led us to identify a mutation in the gene encoding the receptor for a novel neuropeptide, the hypocretin (orexin) receptor 2 (*Hcrtr2*), as the cause of canine narcolepsy.

Results and Discussion

Linkage Analysis and Region of Initial Linkage in Canine Narcolepsy

Autosomal recessive transmission with full penetrance for canine narcolepsy was first established in Labrador

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retrievers and Doberman pinschers (Foutz et al., 1979). A large number of backcrosses were generated in the late 1980s in preparation for a linkage study using randomly distributed markers and a candidate gene approach (Cederberg et al., 1998). Using this approach, genetic linkage between *canarc-1* and the canine major histocompatibility complex was excluded (Mignot et al., 1991). A tightly linked marker was identified using a human μ switch immunoglobulin variable heavy chain probe (Mignot et al., 1991). This initial μ switch–like linkage marker was cloned using a HaeIII size-selected library (Mignot et al., 1994b). Sequencing of the fragment only revealed a GC-rich repetitive sequence with high homology to the human μ switch locus. Further cloning and sequencing studies using a SauIIIA1 partially digested canine genomic phage library failed to identify a neighboring immunoglobulin gene constant region (Mignot et al., 1994b). This result suggested that the μ switch–like sequence was a cross-reacting repeat sequence of unknown significance rather than a genuine immunoglobulin switch segment.

Chromosome walking using phage and cosmid libraries was difficult because of the small sizes of inserts in available libraries. We therefore decided to build a large insert bacterial artificial chromosome (BAC) canine genomic library for this purpose (Li et al., 1999). The large insert canine genomic BAC library was built using EcoRI partially digested DNA fragments from a Doberman pinscher. An animal born in one of our backcross litters and heterozygous for *canarc-1* was selected to build the library. Having both the control and narcolepsy haplotypes in separate BAC clones would allow us to identify all possible disease-associated polymorphisms, and thus the mutation. Approximately 166,000 clones were gridded on nine high-density hybridization filters. Insert analysis of randomly selected clones indicated a mean insert size of 155 kb and predicted 8.1-fold coverage of the canine genome (Li et al., 1999). A 1.8 Megabase (Mb) contig (77 BAC clones) was built in the region in an attempt to flank the *canarc-1* gene. BAC clones containing our μ switch–like marker were isolated, and chromosome walking was initiated from the ends. Microsatellite markers were developed in the contig, and 11 polymorphic markers were typed in all informative animals. $(GAAA)_n$ repeats (rather than most typically used $(CA)_n$ repeats) were found to be the most informative repeat markers in canines as previously reported by Dr. Ostrander's group (Ostrander et al., 1995; Francisco et al., 1996). All informative animals, whether Dobermans or Labradors, were concordant for all the $(CA)_n$ and $(GAAA)_n$ repeat markers developed in this contig. The absence of any recombination events in this interval made it impossible to determine the location of *canarc-1* in relation to our contig.

Homology Mapping Experiments between Human Chromosome 6 and Canine Chromosome 12

BAC end sequence data obtained through chromosome walks was analyzed with BLAST against appropriate GenBank databases. We identified a BAC end sequence with high homology to *Myo6*, a gene located on the long arm of human chromosome 6 (6q12). A protocol for sequential G banding and canine chromosomal fluorescence in situ hybridization (FISH) was established (Li et al., 1999). Both *DLA* (dog leukocyte antigen), the canine equivalent of *HLA* (6p21), and BAC clones from our contig were found to be on canine chromosome CFA12 but at a very large genomic distance (>30 Mb) (Figure 1). This caused us to suspect a large region of conserved synteny between human chromosome 6 and canine chromosome 12. This large region of conserved synteny has been reported by other investigators (dog chromosome 12 is also called U10 based on radiation hybrid data) (Wakefield and Graves, 1996; Priat et al., 1998; Neff et al., 1999; Ryder et al., 1999).

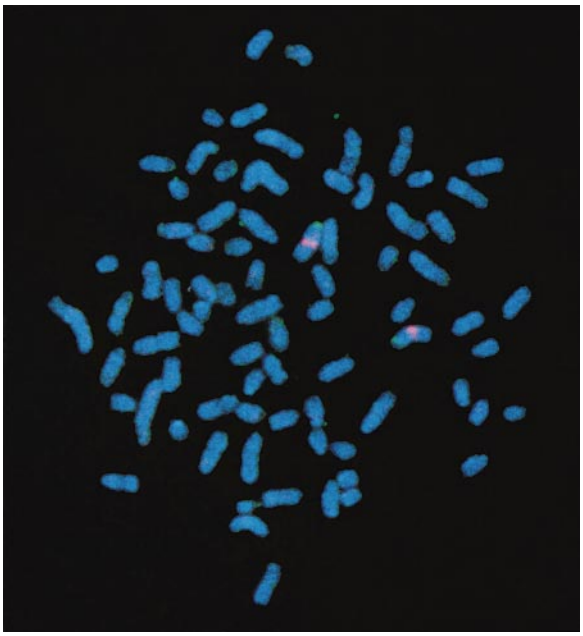

Figure 1. Two-Color Chromosomal FISH Showing Chromosomal Colocalization of *DLA* and *Myo6* on *CFA12*

Note that dog autosomes are all acrocentric. DNA from the *DLA* locus was labeled with biotin and detected with avidin FITC (green); DNA from a canine BAC clone containing the μ switch–like marker and the *Myo6* gene was labeled with digoxigenin and detected with anti-digRhodamine (red) as described in Li et al. (1999). Note that although the published localization of *DLA* is the telomere of CFA12 (Dutra et al., 1996), our result demonstrates a localization of *DLA* to the centromere of CFA12.

We used homology mapping experiments to facilitate identification of our susceptibility region. Human expressed sequence tag clones (ESTs) known to map a few centimorgans distal and proximal to *Myo6* were obtained and used as hybridization probes on the canine BAC library filters. Positive clones were analyzed using two-color FISH on dog metaphase spreads to screen for clones mapping to this portion of CFA12. This novel strategy successfully identified approximately 150 canine BAC clones that were shown to contain the canine equivalents of their corresponding human ESTs through hybridization and sequence analysis of plasmid subclones (data not shown). Minilibraries from these clones were generated to develop dinucleotide and tetranucleotide polymorphic markers, which were typed in our canine crosses and unrelated narcoleptic dog founders. This process was successfully repeated using all available single-copy ESTs mapping within the region in humans until the canine narcolepsy critical region was

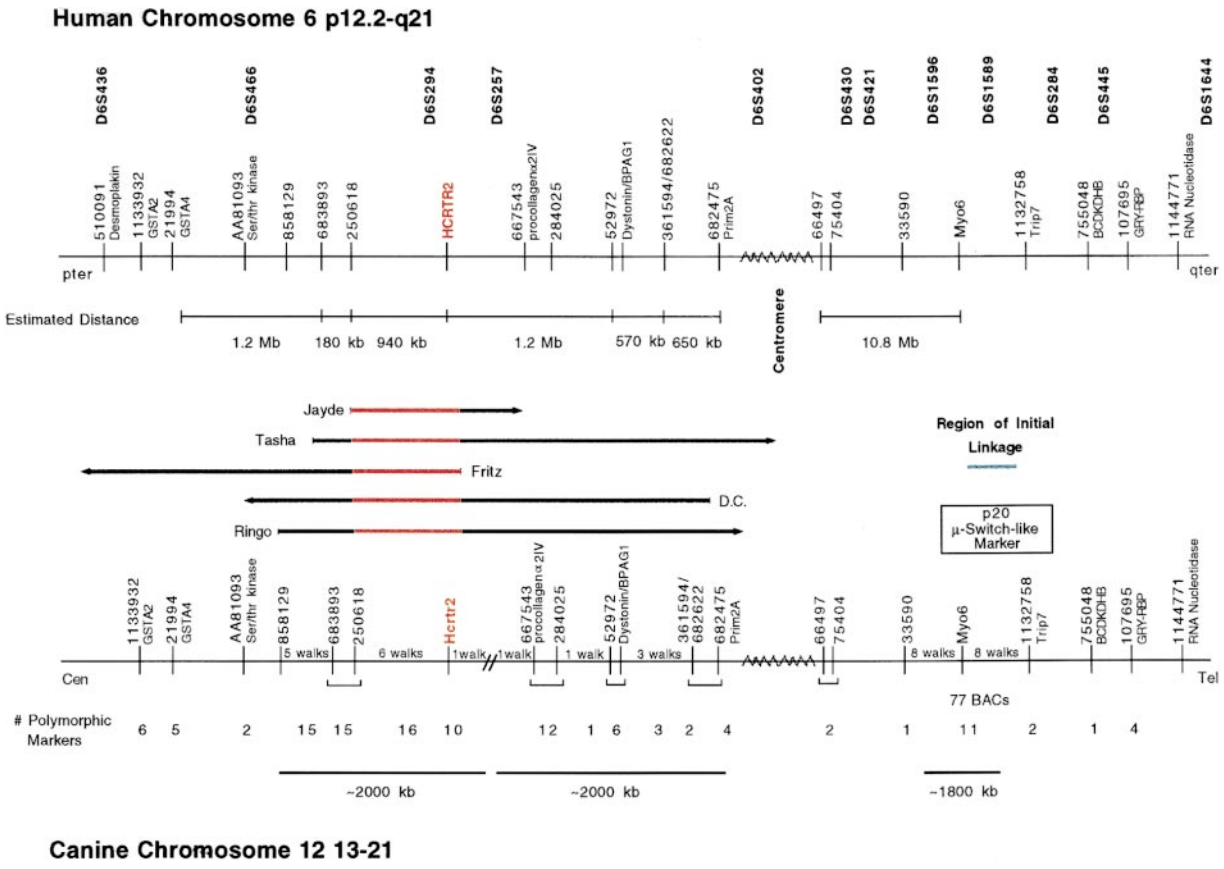

Figure 2. Overview of the Region Containing the Canine Narcolepsy Gene

Human and canine chromosomal regions of conserved synteny are displayed. Human ESTs are displayed on the human map in the top panel. Physical distances in human were estimated by mapping the corresponding clones on the Stanford G3 radiation hybrid panel and using a roughly estimated correspondence of 26 kb/cR. These EST clones were used as probes to isolate 154 cross-hybridizing BAC clones that were verified by FISH to be within the canine region of conserved synteny. Chromosome walking was also performed from these locations, thus resulting in the physical cloning of almost the entire genomic segment. More than 100 polymorphic microsatellite markers were isolated from these clones and tested in narcoleptic and control canines. A total of 77 informative backcross Dobermans, 23 informative backcross Labradors, and 5 informative Dachshund animals were tested. Twenty-five animals belonging to a New Jersey breeder pedigree (7 affected) were also genotyped. Key recombinant animals are listed in the center of the Figure. The canine narcolepsy critical region is labeled in red. The total LOD score in this subsegment is 32.1 at 0% recombination (n = 105 animals) (Ott, 1991).

flanked (the more precise map position of several ESTs was first estimated using the Stanford G3 radiation hybrid panel in several cases). Chromosome walking by filter hybridization was also performed until the region was almost entirely physically cloned (Figure 2).

Backcross breeding was continued in parallel with the physical cloning effort. A Doberman litter born in our colony yielded our first narcolepsy/μ switch–like marker recombinant animal, which mapped the region proximal to the *Prim2A* locus ("DC," see Figure 2). This finding, together with the observation of a crossover immediately distal to *EST 858129* ("Ringo," Figure 2), reduced the narcolepsy susceptibility interval to an estimated 4 Mb region (*EST 858129* to *Prim2A* in Figure 2) in a total of 100 informative backcross animals. Two pedigrees identified in outside breeder colonies were used to further reduce the segment. The first pedigree (from New York) is a familial narcolepsy Dachshund litter with three affected and two unaffected animals. Linkage with the *canarc-1* locus was considered likely in this litter, considering previously established linkage of this region in other breeds. A maximum LOD score of 2.0 at 0% recombination ($p = 0.01$) was obtained in this litter for the region immediately proximal to and including the *Hcrtr2* locus (all animals concordant). This putative *canarc-1*-positive Dachshund pedigree includes a recombinant asymptomatic animal "Fritz" (Figure 2). The second pedigree (from New Jersey) is a very large Doberman breeder pedigree with seven affected animals. One of the affected animals was donated to the colony and shown to be *canarc-1* positive by breeding. In this pedigree, all narcoleptic animals are identical by descent in a region flanked proximally by *EST 250618* (Jayde and Tasha, Figure 2). These findings allowed us to narrow down the canine narcolepsy susceptibility region to a subsegment of approximately 800 kb flanked by *EST 250618* and *Hcrtr2*.

The distance between the initial linkage marker and the critical region corresponds to a 10 cM distance on the human map (Figure 2) within an extensive region of conserved synteny. However, the canine genetic distance estimated from our breeding studies indicates

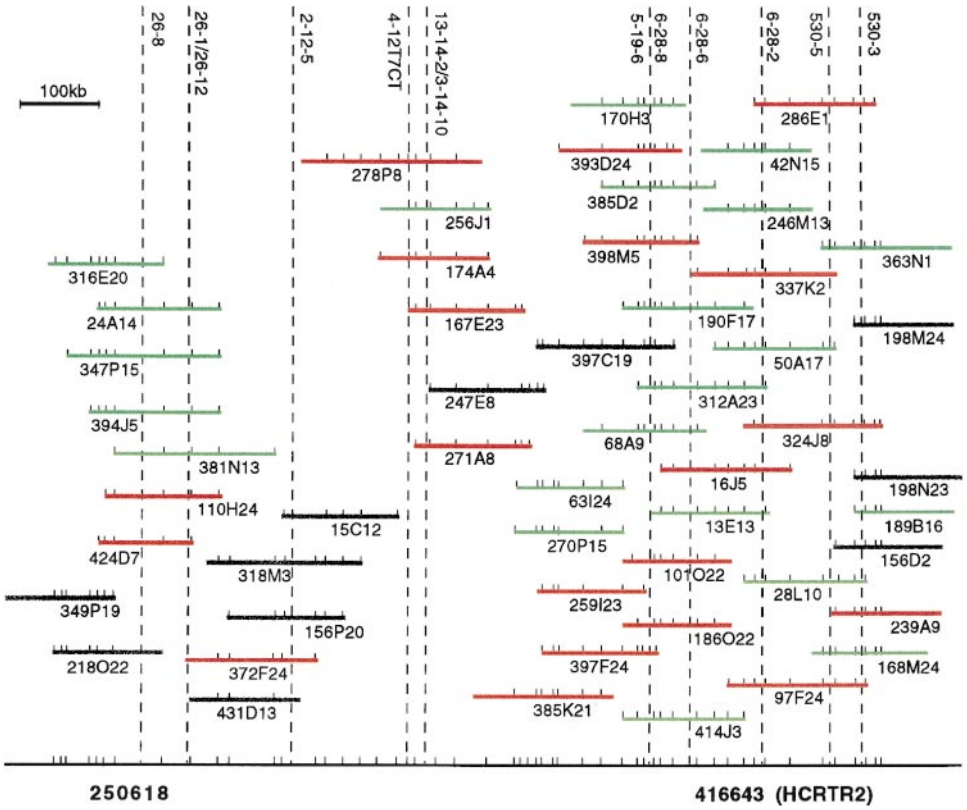

Figure 3. BAC Clone Contig Covering the 800 kb Segment Known to Contain *canarc-1*

The BAC clone sizes are drawn to scale. Selected polymorphic microsatellite markers are indicated by dotted lines. STSs for which locations were not strictly constrained were spaced at roughly equidistant intervals between constrained markers. The canine narcolepsy gene critical region is flanked by marker *26-12* (immediately distal to *EST 250618*) and marker *530-5* (immediately distal to *EST 416643*). All BAC clones were genotyped with available informative markers to determine *canarc-1* associated status. Narcolepsy/control segments are labeled in red and green, respectively. Unclassified clones are shown in gray.

that the distance is 1 cM (only one recombinant animal, "DC," over 100 backcross animals). We suspect the region syntenic to the human chromosome centromere may have repressed recombination for an unknown reason. A map of the region as currently characterized is depicted in Figure 2. The *EST 858129* to *Prim2A* segment is approximately 4 Mb in humans (Figure 2) as estimated through radiation hybrid data (3 cM on the human map). Interphase and metaphase FISH data in canines suggest the region is approximately of the same physical size in canines (data not shown). A small gap (estimated at 400 kb, based on the human radiation hybrid data, canine clone contig size, and canine FISH data) remains in the contig, between *Hcrtr2* and the *procollagen* α*2 IV* gene (Figure 2). The precise location of the canine narcolepsy gene is between *EST 250618* and a region immediately distal to *Hcrtr2* between markers *26-12* and *530-5* (Figure 3). The estimated overall LOD score in the critical region is 32.1 at 0% recombination (n = 105 animals) (Ott, 1991). Twenty-five dogs born in the New Jersey breeder colony are not included in the calculation due to inbreeding loops, missing animals, and the difficulty in establishing precise family relationships in some cases.

Identification of an RFLP in the Vicinity of the *Hcrtr2* Gene

Only one previously identified gene, *Hcrtr2*, was known to reside within the critical region. This gene encodes a G protein–coupled receptor with high affinity for the hypocretin neuropeptides. The hypocretins (orexins) have primarily been implicated in the regulation of feeding (De Lecea et al., 1998), but the broad neuroanatomical projections for hypocretin neurons (Peyron et al., 1998; Nambu et al., 1999; van den Pol, 1999) and corresponding receptor distribution (Trivedi et al., 1998) for this system suggest other possible functions (Peyron et al., 1998). To explore the possibility of an involvement of *Hcrtr2* in the etiology of canine narcolepsy, BAC clones containing either the *canarc-1* or the wild-type-associated haplotypes were identified using previously identified polymorphic markers (see Figure 3). Narcolepsy and control allele–associated BAC clones were digested with four enzymes (HindIII, BglII, TaqI, and MspI), Southern blotted, and probed with a human *HCRTR2* EST probe. A clear restriction fragment length polymorphism (RFLP) pattern was observed with three of the four enzymes (BglII, TaqI, and MspI), suggesting a possible genomic alteration in the vicinity of or within the canine *Hcrtr2* gene (Figure 4).

Canine Narcolepsy Is Caused by a Mutation in the *Hcrtr2* Gene

Degenerate consensus primers were designed based on the 5′ and 3′ sequences of the published human and rat *Hcrtr2* cDNAs. cDNAs were prepared from the brains

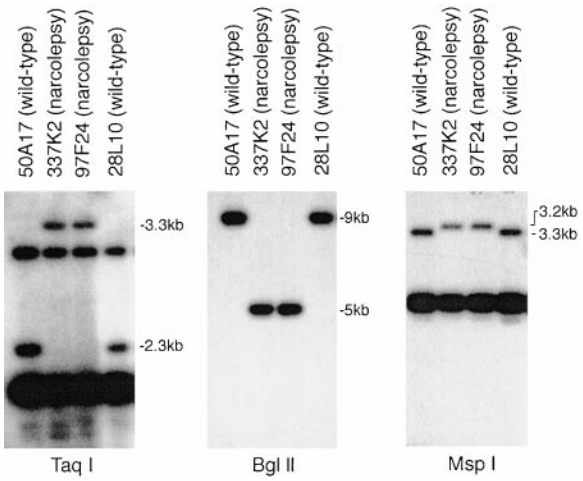

Figure 4. Autoradiograms Showing Alternate RFLP Alleles Associated with the Control versus Narcolepsy-Associated BAC Clones When Hybridized with an *HCRTR2* Probe

Overlapping BAC clones (50A17, 337K2, 97F24, and 28L10) containing the canine homolog of the *Hcrtr2* gene were digested with restriction endonucleases TaqI, BglII, MspI, and HindIII, electrophoresed, transferred to nylon membrane, and hybridized with the IMAGE clone *416643* (*HCRTR2*) probe. Haplotypes of BAC clones were previously identified by polymorphic markers. HindIII digest showed no restriction length polymorphism (data not shown).

of four control and four narcoleptic Dobermans born in our colony. Amplified products from the cDNA of narcoleptic dogs significantly differed in size from the products of the controls (1.5 versus 1.6 kb). This finding suggested a deletion in the transcripts of narcoleptic animals (Figure 5A). Sequence analysis of the RT–PCR product in narcoleptic and control animals indicated a 116 bp deletion, a result also confirmed by nested PCR experiments on cDNA templates (data not shown). PCR primers scattered throughout the entire coding sequence were used to directly sequence the corresponding BAC clones representing both control and narcoleptic haplotypes. This allowed us to determine exon–intron boundary sequences of the locus in control and mutant alleles. The 116 bp deletion in the transcript corresponds to the fourth exon (Figure 6). Genomic sequencing of the intron–exon boundary immediately preceding this intron indicated that a 226 bp canine short interspersed nucleotide element (SINE) (Minnick et al., 1992; Coltman and Wright, 1994) was inserted 35 bp upstream of the 5′ splice site of the fourth encoded exon (Figure 7). This insertion falls within the 5′ flanking intronic region needed for pre-mRNA lariat formation and proper splicing. The efficiency of pre-mRNA splicing is strongly affected by alterations of the site within the intron that binds to the U2 small nuclear ribonucleoprotein. This region of complementarity includes the branchpoint sequence (BPS) at the site of lariat formation (Reed and Maniatis, 1985, 1988). In mammals the BPS is a poorly conserved element that conforms to a very loose consensus sequence (PyXPyTPuAPy) in which the adenine residue is of primary importance. The BPS is typically located between 18 and 40 nucleotides upstream of the 3′ splice junction, but this position may also vary considerably. Despite the loose constraints on the consensus sequence and relative position of the BPS, alterations in the sequence may nearly abolish splicing (Reed and Maniatis, 1985, 1988). The SINE insertion may thus have moved the functioning branchpoint sequence beyond the acceptable range for efficient splicing (Figure 7). PCR primers were designed in the immediate flanking area, and PCR analysis was performed in control and *canarc-1*-positive narcoleptic dogs of three breeds (Dobermans, Labradors, and Dachshunds). We identified the same SINE insertion in all 17 narcoleptic Dobermans tested, including 6 dogs not known to be related by descent by at least four generations but likely to be identical by descent as a result of a founder effect. The SINE insertion was not found in the 36 control dogs tested, including 14 Dobermans, 13 Labradors, and 9 Dachshunds (Figure 5B). Based on this result and the associated cDNA analysis, we conclude that the SINE insertion mutation is the cause of narcolepsy in Dobermans. Similar retrotransposon insertion mutations have been reported to cause human disease (see Kazazian,

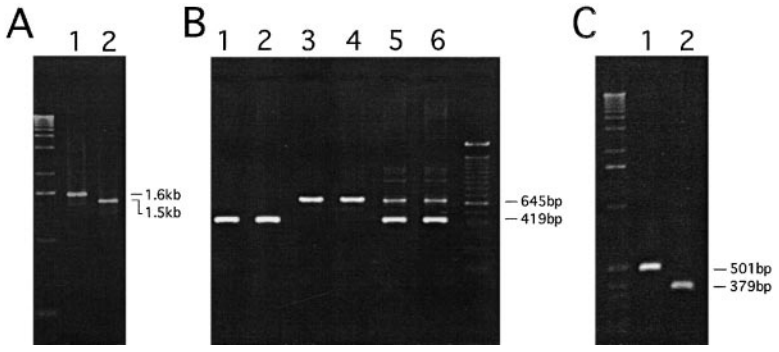

Figure 5. PCR Amplification Studies of the *Hcrtr2* Locus in Narcoleptic and Control Dogs

(A) Amplification of *Hcrtr2* cDNA from control and narcoleptic Doberman Pinschers. PCR primers were designed in the 5′ and 3′ untranslated regions of the *Hcrtr2* gene (exon 1 and exon 7). The amplification product from the control dog (lane 1) is the expected 1.6 kb size, whereas the product from the narcoleptic dog (lane 2) is 1.5 kb. Forward PCR primer, 5-2 (5′GCTGCAGCCTCCAGGGCCG GGTCCCTAGTTC 3′); reverse primer, 3-2 (5′ATCCCTGTCATATGAATGAATGTTCTAC CAGTTTT 3′).

(B) Amplification of narcoleptic and wild-type Doberman Pinscher genomic DNA with PCR primers flanking the SINE insertion. PCR primers designed to flank the SINE insertion site produce a 419 bp amplification product from DNA of wild-type dogs and a 645 bp product from narcoleptic Doberman Pinscher DNA. Products of both sizes are amplified from the DNA of Dobermans known to be carriers of narcolepsy, and they also display prominent heteroduplex bands. Lanes 1 and 2, wild-type Dobermans (Alex and Paris); lanes 3 and 4, narcoleptic Dobermans (Tasha and Cleopatra); lanes 5 and 6, heterozygous carrier Dobermans (Grumpy and Bob). Forward primer w554-65seqF (5′GGGAGGAACAGAAGGAGAGAATTT3′) located in intronic sequence upstream of the insertion. Reverse primer R4/7-6R(110) (5′ATAGTTGTTAATGTGTACTTTAAGGC3′) located in intronic sequence downstream of exon 4.

(C) Amplification of narcoleptic and wild-type Labrador retriever *Hcrtr2* cDNAs. Forward primer F6 5′AGCGCTGT(G/C)GC(G/T)GCTGA(A/G)ATAAAGCAGATC3′ and reverse primer 3-2 were used. The amplification product from the control dog (lane 1) is the expected 501 bp size, whereas the product from the narcoleptic dog (lane 2) is 379 bp.

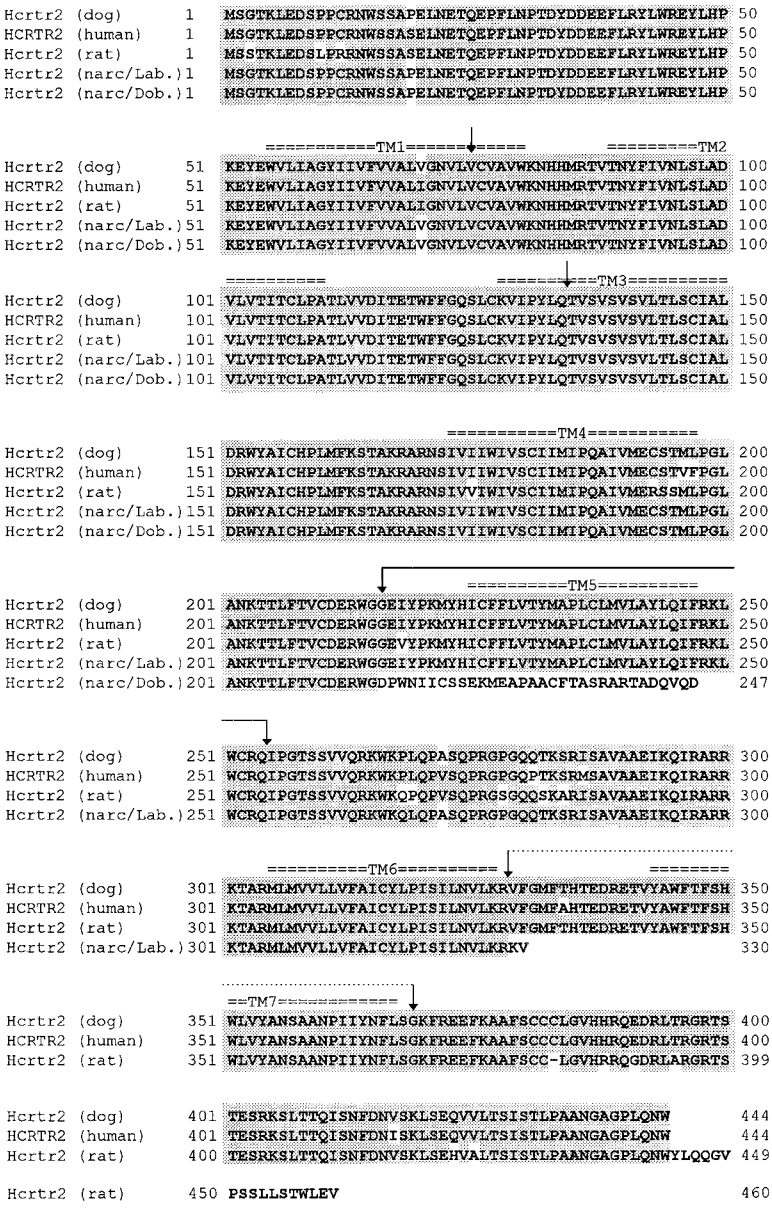

Figure 6. Deduced Amino Acid Sequences of *Hcrtr2* in Wild-Type Dog, Human, Rat, and Narcoleptic Dogs

Amino acid residues that are not identical in at least two sequences are boxed. Putative transmembrane (TM) domains are marked above the aligned sequences. Arrows indicate exon/intron boundaries in the gene structure of the dog. Sequence analysis of RT–PCR products revealed that exon 4 (continuous line between arrowheads) and exon 6 (dotted line between arrowheads) are missing in RNA transcripts of narcoleptic Doberman pinschers (narc/Dob.) and Labrador retrievers (narc/Lab.), respectively. In both cases, the deleted exon results in a frameshift and the creation of a premature stop codon. The full-length nucleotide sequences of the canine *Hcrtr2* cDNA can be retrieved from GenBank under accession number AF164626.

1999 and the human gene mutation database http://www.uwcm.ac.uk/awcm/mg/hgmd0.html).

The SINE insertion was not observed in *canarc-1*-positive animals from other breeds (three Labrador retrievers and one Dachshund; data not shown). This led us to suspect that other mutations in the *Hcrtr2* gene might be involved in these cases. RT–PCR analysis was performed using cDNAs prepared from the brains of two control and two narcoleptic Labrador retrievers born in our colony. Dachshund cDNA samples were not studied because no brain samples were available. A shorter PCR product was observed in narcoleptic versus control Labrador retrievers (Figure 5C). Sequencing indicated a deletion of exon 6 (122 bp) in the narcolepsy-associated cDNA. Analysis of the intron–exon boundaries and sequencing of exon 6 revealed a G to A transition in the 5′ splice junction consensus sequence (position +5, exon 6–intron 6) in genomic DNA of narcoleptic Labrador retrievers (Figure 7). This G to A transition was not observed in the corresponding sequences of 24 control dogs (11 Labradors, 10 Dobermans, and 3 Dachshunds) and 11 non-Labrador narcoleptic dogs (10 Dobermans and 1 Dachshund). The consensus position for the +5 nucleotide is G (84%), and an A in this position is rarely observed (Shapiro and Senapathy, 1987; Krawczak et al., 1992). A G to A transition reduces the likelihood functional score for the 8 nucleotide splicing consensus sequence from 88.4% to 74.8% (Shapiro and Senapathy, 1987). Mutations in this position have been shown to produce 100% exon skipping (Krawczak et al., 1992; McGrory and Cole, 1999; Teraoka et al., 1999).

The *Hcrtr2* transcripts produced in narcoleptic animals are grossly abnormal mRNA molecules. In Doberman pinschers, the mRNA potentially encodes a protein with 38 amino acids deleted within the fifth transmembrane domain followed by a frameshift and a premature stop codon at position 932 in the encoded RNA. The C terminus of the protein encoded by narcoleptic Labradors is also truncated and does not include a seventh transmembrane domain. These changes most likely disrupt

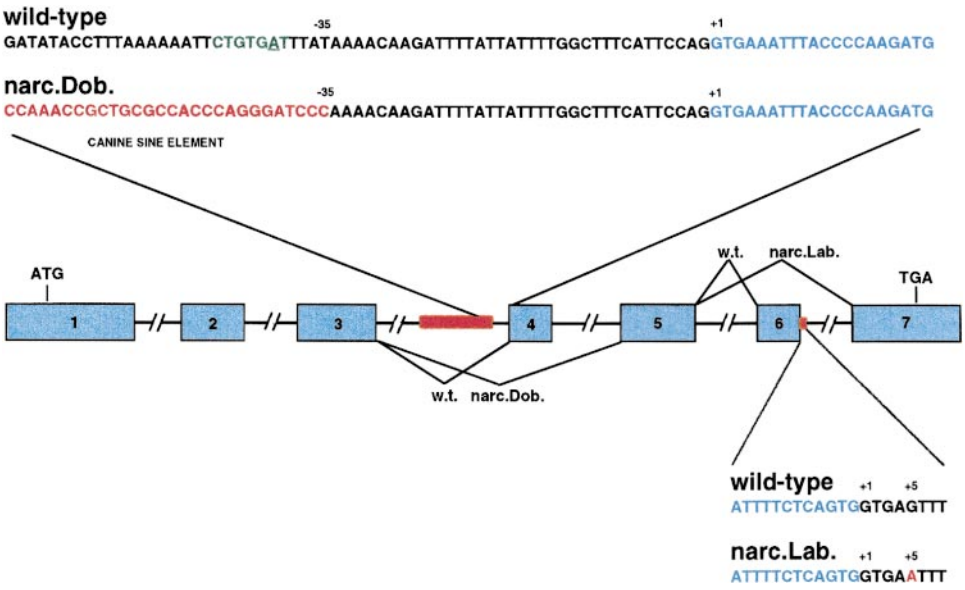

Figure 7. Genomic Organization of the Canine *Hcrtr2* Locus, which Is Encoded by Seven Exons

In transcripts from narcoleptic Doberman pinschers, exon 3 is spliced directly to exon 5, omitting exon 4 (narc. Dob. versus wild type). The genomic DNA of narcoleptic Dobermans contains an 226 bp insertion corresponding to a common canine SINE repeat element (shown in red) located 35 bp upstream of exon 4. The insertion of the SINE displaces a putative lariat branchpoint sequence (BPS, shown in green) located at position −40 through −46 upstream of the 3′ splice site in control animals. The critical A is underlined. No candidate BPS sequences are present in this vicinity in the narcolepsy-associated intron. In transcripts from narcoleptic Labrador retrievers, exon 5 is spliced directly to exon 7, omitting exon 6 (narc. Lab. versus wild type). Genomic DNA analysis revealed a G to A transition in the 5′ splice site consensus sequence (shown in red).

proper membrane localization and/or cause loss of function of this strongly evolutionarily conserved protein. These mutations are consistent with the observed autosomal recessive transmission of the disorder in these breeds.

A Role for Hypocretins in the Pathophysiology of Narcolepsy and the Regulation of REM Sleep

Hypocretins were first identified by De Lecea et al. (1998) using a direct tag PCR subtraction technique aimed at the isolation of hypothalamic-specific transcripts. These authors isolated the *preprohypocretin* mRNA and showed that the corresponding precursor protein is likely to be processed into two related peptides, hypocretin-1 and -2. Hypocretins are localized in the synaptic vesicle and possess neuroexcitatory effects (De Lecea et al., 1998). The existence of hypocretins was independently confirmed by Sakurai et al. (1998) using biochemical purification and ligand binding to cell lines expressing selected orphan G protein–coupled receptors. Two orphan receptors were found to bind hypocretin-1 (also called orexin-A) and hypocretin-2 (orexin-B) with different affinity profiles (Sakurai et al., 1998). The first of these receptors, now called hypocretin receptor 1 (Hcrtr1), was shown to selectively bind hypocretin-1, whereas Hcrtr2 binds both hypocretin-1 and -2 with a similar affinity.

Initially, the finding that *preprohypocretin* RNA molecules and hypocretin-immunoreactive cell bodies were discretely localized to a subregion of the dorsolateral hypothalamus and a hypothesized colocalization of hypocretins with melanin-concentrating hormone (MCH), a potent orexigeneic peptide, suggested a possible role of this system in the control of feeding (De Lecea et al., 1998). In support of this hypothesis was the observation that centrally administered hypocretin-1 and -2 stimulate appetite in rodents (Sakurai et al., 1998) and that *preprohypocretin* mRNA is upregulated by fasting (Sakurai et al., 1998). More recent experiments suggest a more complex picture. First, the suggested initial colocalization with MCH was not substantiated by further studies (Broberger et al., 1998). Second, there is controversy regarding the magnitude of the effect of hypocretins on food consumption in rodents (Lubkin and Stricker-Krongrad, 1998; Edwards et al., 1999; Ida et al., 1999; Moriguchi et al., 1999; Sweet et al., 1999). For example, while hypocretins stimulate short-term food intake, these peptides do not alter 24 hr total food consumption (Ida et al., 1999). Some authors have also suggested that hypocretins exert a shift in the diurnal pattern of food intake. The effect on energy metabolism seems to be more pronounced than that on feeding behavior (Lubkin and Stricker-Krongrad, 1998) and differs with the circadian time of administration (Ida et al., 1999). These two observations may have to be reconsidered in the light of potential wake-promoting effects of hypocretins. Recent studies suggest complex interactions between hypocretins, MCH-containing neurons, neuropeptide Y, agouti gene–related protein systems, and leptins in the control of feeding and energy balance (Broberger et al., 1998; Beck and Richy, 1999; Horvath et al., 1999; Kalra et al., 1999; Marsh et al., 1999; Moriguchi et al., 1999; Yamamoto et al., 1999).

Further neuroanatomical work on hypocretins and their receptors suggests a broader role than the regulation of energy balance and feeding. Immunocytochemical

studies have shown that while the preprohypocretin-positive neurons are discretely localized in the perifornical nucleus and in the dorsal and lateral hypothalamic areas, their projections are widely distributed throughout the brain (Peyron et al., 1998; Date et al., 1999; Mondal et al., 1999; Nambu et al., 1999; van den Pol, 1999). Consistent with the potential role of hypocretins in the regulation of feeding, projection sites include intrahypothalamic sites such as the arcuate nucleus and paraventricular nucleus. Other major projection sites, however, include the cerebral cortex, the spinal cord (dorsal horn), medial nuclei groups of the thalamus, the olfactory bulb, basal forebrain structures such as the diagonal band of Brocca and the septum, limbic structures such as the amygdala and the medial part of the accumbens nucleus, and brainstem areas including periaqueductal gray, reticular formation, pedunculopontine nucleus, parabrachial nucleus, locus coeruleus, raphe nuclei, subtantia nigra pars compacta, and ventral tegmental area (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999; van den Pol, 1999). The neuroanatomical distribution of *Hcrtr1* and *Hcrtr2* mRNAs was studied in rats by Trivedi et al. (1998). Of special interest is the finding that the *Hcrtr1* transcript is mostly localized in the ventromedian hypothalamic nucleus, hippocampal formation, dorsal raphe, and locus coeruleus. In contrast *Hcrtr2* mRNA molecules are more abundant in the paraventricular nuclei and in the nucleus accumbens (Trivedi et al., 1998). Experiments using radioligand binding and immunocytochemical techniques are needed to further establish the respective pattern of expression of these receptors in relation to hypocretin projection sites.

How could a defect in the *Hcrtr2* gene produce narcolepsy? There is no direct published evidence suggesting significant sleep/wake effects for hypocretins. Pharmacological, neurochemical, and physiological studies implicate monoaminergic and cholinergic neurotransmissions as the main modulators in narcolepsy (Mignot et al., 1993b; Nishino and Mignot, 1997). The human sleep disorder is treated using amphetamine-like stimulants for the control of daytime sleepiness and antidepressant drugs for the control of abnormal REM sleep manifestations (e.g., cataplexy). Pharmacological analysis using the canine model has shown that inhibition of dopamine uptake and/or stimulation of dopamine release mediates the wake-promoting effects of amphetamine-like stimulants (Nishino and Mignot, 1997). Other experiments have shown that inhibition of noradrenergic uptake mediates the anticataplectic effects of antidepressive therapy (Mignot et al., 1993b). The effect on cataplexy parallels the well-established REM suppressant effect of adrenergic uptake inhibitors. Stimulation of cholinergic transmission using acetylcholine esterase inhibitors or direct M2 agonists also stimulates cataplexy (Nishino and Mignot, 1997). These results suggest that the pharmacological control of cataplexy, a symptom resembling REM sleep atonia, is very similar to the control of REM sleep and involves a reciprocal interaction between pontine cholinergic REM-on cells and aminergic locus coeruleus (LC) REM-off cells and their projection sites (Mignot et al., 1993b; Nishino and Mignot, 1997).

In order to determine the neuroanatomical basis for the sleep abnormalities observed in narcolepsy, several complementary approaches have been taken. In both human and canine subjects with narcolepsy, brain neurotransmitter levels and receptors have been measured (Miller et al., 1990; Aldrich, 1993). In narcoleptic animals, the most consistent abnormalities were observed in the amygdala where significant increases in dopamine and metabolite levels were reported in two independent studies (Miller et al., 1990). These results were interpreted as suggesting decreased dopamine turnover and accumulation of dopamine in presynaptic terminals. Another important finding was the observation of increased muscarinic M2 receptors in the pontine reticular formation (Baker and Dement, 1985; Kilduff et al., 1986), a region where cholinergic stimulation triggers REM sleep in normal animals. The functional significance of these abnormalities has been addressed in experiments where active pharmacological agents are locally injected or perfused in specific brain areas of freely moving narcoleptic animals. These experiments have shown that widespread hypersensitivity to cholinergic stimulation likely explains abnormal REM sleep tendencies in narcolepsy (Nishino and Mignot, 1997). Local injection or perfusion of cholinergic agonists in the pontine reticular formation or the basal forebrain area triggers REM sleep and/or REM sleep atonia in narcoleptic and control canines (Nishino and Mignot, 1997). In narcoleptic animals, however, much lower doses can trigger muscle atonia, thus suggesting hypersensitivity to cholinergic stimulation. We have also found that dopaminergic autoreceptor stimulation (D2/D3) in the ventral tegmental area (VTA) induces cataplexy and sleepiness in narcoleptic but not in control canines (Reid et al., 1996). As this dopaminergic system and its projection to the nucleus accumbens and other limbic structures are involved in the perception of pleasurable emotions, this could explain the triggering of cataplexy by positive emotions (Reid et al., 1996; Nishino and Mignot, 1997). Narcolepsy may thus result from abnormal interactions between REM-on cholinergic pathways and mesocorticolimbic dopaminergic systems (Nishino and Mignot, 1997).

The discovery that a mutation in the *Hcrtr2* locus produces canine narcolepsy suggests that hypocretins and Hcrtr2 are major neuromodulators of sleep in interaction with aminergic and cholinergic systems. This effect might be especially important during early development, since canine narcolepsy typically develops between 4 weeks and 6 months of age and severity increases until animals are approximately 1 year old (Mignot et al., 1993a; Riehl et al., 1998). We have also found that *canarc-1* heterozygote animals may exhibit brief episodes of cataplexy when pharmacologically stimulated with a combination of cholinergic agonists and drugs depressing monoaminergic activity but only during early development (Mignot et al., 1993a). Projection sites and reported *Hcrtr2* localization are in agreement with a concerted effect of hypocretins, monoamines, and acetylcholine on sleep–wake regulation. Hypocretin neurons are known to project to monoaminergic brainstem cell groups, such as the LC or the VTA, and to the cholinoceptive pontine reticular formation and associated cholinergic cell groups (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999). Other major projection sites, such as the cortex, the basal forebrain area, the nucleus accumbens, and the amygdala (Peyron et al., 1998; Date et al., 1999; Nambu et al., 1999), are also consistent with

interactions at the level of target projections for these neurotransmitters. Functional experiments aimed at studying the effects of hypocretins on sleep after systemic and central (e.g., intracerebroventricular injection and/or local perfusion in selected brain areas) administration are now critically needed. We expect that the central and peripheral administration of hypocretins will be potently wake promoting and suppress REM sleep via a stimulation of Hcrtr2 in control but not in narcoleptic animals.

The phenotypes of human and canine narcolepsy and associated neurochemical abnormalities are strikingly similar (Baker and Dement, 1985; Nishino and Mignot, 1997). Abnormalities in the hypocretin neurotransmission system are thus likely to be also involved in human cases. Mutations in the *HCRTR2* gene or other hypocretin family genes may be involved in some familial cases of human narcolepsy, some of which are non-HLA associated (Mignot, 1998). The observation that rare cases of symptomatic secondary narcolepsies are most typically associated with lesions surrounding the third ventricle is also consistent with a destruction of hypocretin-containing cell groups (Aldrich and Naylor, 1989). As most cases of human narcolepsy are nonfamilial and strongly *HLA* associated (Mignot, 1998), an autoimmune process directed against the Hcrtr2 or the very localized paraventricular hypothalmic nucleus, or more complex neuroimmune interactions may also be involved in the pathophysiology of human narcolepsy.

Finally, hypocretins or analogs may offer new therapeutic avenues in narcolepsy and other sleep disorders. Hypocretin-1 has been shown to enter the brain after peripheral administration (Kastin and Akerstrom, 1999). A reduction of Hcrtr2-mediated neurotransmission might be supplemented in some cases by increasing ligand availability.

Experimental Procedures

Canine Subjects and Genetic Linkage Analysis

Backcross narcoleptic Dobermans and Labradors were produced in our breeding colony at the Center for Narcolepsy as described in Cederberg et al. (1998). The procedure to determine phenotypic status for these dogs is described in Mignot et al. (1993a). All experimental procedures were done in accordance with the NIH guidelines for laboratory animal care. Two familial cases of canine narcolepsy were reported to our attention for therapeutic advice by a veterinarian and a breeder, respectively (see text). Diagnosis for these cases was verified by phone interview and breeding into the colony whenever possible (one of two cases). Linkage analysis was performed as described in Mignot et al. (1991), with kind assistance of Neil Risch (Stanford, CA).

Radiation Hybrid Mapping of EST Candidate Loci and Human EST Clone Selection

At the time our project started, EST mapping information obtained from various online sources, such as Genemap '96 (http://www.ncbi.nlm.nih.gov/SCIENCE96/), Whitehead Institute (http://carbon.wi.mit.edu:8000/cgi-bin/contig/phys_map), and Sanger Center (http://www.sanger.ac.uk/HGP/Rhmap/maps.html), was often contradictory or of a low resolution, so that the precise location of these genes was not reliably known. Radiation hybrid (RH) mapping is a simple and reliable method for mapping genes (Cox et al., 1990), and whole genome RH panels have been developed to quickly localize genes to their unique chromosomal location. We made use of the 83 hybrid Stanford G3 radiation hybrid panel to map a number of candidate anchor ESTs in order to verify that the EST was likely to lie within our relatively large region of interest (*GSTA4-PRIM2A*) and to attempt to characterize the relative order of our selected EST loci. One microliter of each hybrid DNA, plus positive and negative control parental cell DNA, was transferred for PCR in a 96-well PCR plate. PCR primers published in dbSTS (http://www.ncbi.nlm.nih.gov/dbSTS/index.html) or the previously mentioned online sites were obtained and used in a 10 μl PCR reaction to amplify these genes. Amplification was performed as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 52°C–62°C (primer dependent) for 30 s, 72°C for 30 s, and a final extension of 72°C for 10 min. The reaction products were run on a 2% agarose gel and scored for the presence of a human specific PCR product of the expected size. A positive result was denoted with the number one (1), and negative result a number two (2), and an ambiguous result was given an R. The data vector was submitted to the Stanford Human Genome Center (SHGC) radiation hybrid server (http://www-shgc.stanford.edu/RH/rhserverformnew.html) in order to perform a two-point analysis with the genetic markers contained in their database. The most tightly linked RH marker and the estimated distance from that marker were returned by the RH server.

Screening of the Canine BAC Library with Human EST Probes

Human IMAGE consortium clones mapping to the pericentromeric region of human chromosome 6 were identified through scrutiny of available data from maps constructed by the Whitehead Institute for Genomic Research (http://carbon.wi.mit.edu:8000/cgi-bin/contig/phys_map), GeneMap 96 (http://wwwncbi.nlm.nih.gov/SCIENCE96/), GeneMap 98 (http://www.ncbi.nlm.nih.gov/genemap98), the Sanger Centre (http://www.sanger.ac.uk/HGP/Rhmap/maps.shtml), the Stanford Human Genome Center (http://www-shgc.stanford.edu/RH/rhserverformnew.html), and through Unigene (http://www.ncbi.nlm.nih.gov/UniGene/Home.html). Selected clones were obtained from Research Genetics (Huntsville, AL) and verified through sequence analysis of extracted DNA. IMAGE clone inserts were excised and band-purified on agarose gels (Qiaquick spin columns, Qiagen) for use as hybridization probes. Probes were evaluated by hybridization of strips of Southern-blotted canine genomic DNA. Those not producing high background signal or obvious nonspecific hybridization signals were used to screen the Canine Genomic BAC library. Hybridizations and washes were performed in standard BAC library buffers as described in Li et al. (1999) but were carried out at 51°C–53°C to reduce stringency. Positive clones were selected from the library, streaked onto LB plates supplemented with chloramphenicol, and DNA was extracted from 5 ml minicultures of single clones. BAC DNA was digested with EcoRI and SacI, electrophoresed in agarose gels, and Southern blotted onto nylon filters. Filters were hybridized with the appropriate EST probes to identify true positive clones. Positive clones were grouped into bins based on patterns produced by ethidium bromide staining and hybridization results. Clones from each bin were further characterized through two-color chromosomal FISH using a previously characterized CFA12 BAC (as described in Li et al., 1999) clone as a positive control to verify that the clones were in the narcolepsy region. In most cases, plasmid minilibrary clones were also hybridized with the EST probes, and resulting subclones were sequenced in order to identify homologous canine exon sequences.

Canine Fluorescence In Situ Hybridization

BAC clones were analyzed by FISH on canine metaphase spreads to confirm location on CFA12. Briefly, BAC clones were labeled with digoxygenin or biotin conjugated nucleotides using nick translation kits (Boehringer Mannheim and GIBCO–BRL). Following nick translation, 100–500 ng of labeled DNA was twice precipitated together with 10 μg of sheared total dog genomic DNA and 1 μg of salmon sperm DNA. After resuspension with 10 μl of formamide hybridization buffer, DNAs were denatured for 10 min at 70°C, directly transferred to 37°C, and allowed to preanneal for at least 15 min. Canine metaphase chromosome spreads were prepared from peripheral lymphocytes according to standard methods (Barch, 1997). Prior to hybridization, chromosome slides were treated with RNase and subjected to dehydration in an ethanol series (70%, 80%, 90%, 100%) for 5 min in each concentration, and allowed to air dry. The chromosome spreads were next denatured in 70% formamide, 2×

SSC at 65°C for 5 min, quenched in iced 70% ethanol, and again dehydrated in an ethanol series. After air drying, slides were hybridized to labeled BACs at 37°C overnight. Some BAC clones were analyzed by sequential G-banding-FISH to allow specific chromosomal assignments. GTW-banded slides were photographed and destained by three 1 min washes in 3:1 methanol/acetic acid. Slides were then dried and treated in 2× SSC at 37°C for 30 min and then dehydrated in an ethanol series. Thirty microliters of probe mix was added and sealed under a 24 × 50 mm coverslip. Chromosomal and target DNAs were denatured together by incubating on a slide warmer at 65°C for 30 s, and then transferred to 37°C overnight for hybridization. Following hybridization, slides were washed at 45°C for 20 min in 50% formamide/2× SSC, two times for 10 min each in 1× SSC, and two times for 10 min each in 0.5× SSC. Slides were then blocked for 15 min at 37°C with 4× SSC/3% BSA, and signals were detected with Rhodamine-coupled sheep anti-digoxigenin FAB fragments (Boehringer Mannheim) or avidin-fluorescein DCS (Vector Labs). Following detection, slides were washed three times in 4× SSC/0.1% Triton X100 for 5 min each and mounted/counterstained with Vectashield containing Dapi and/or propidium iodide (Vector Labs) and viewed on a Nikon Axioskop microscope with epifluorescence.

Chromosome Walking Using Canine BAC End Probes

The development of a high-density BAC contig map was primarily based on chromosome walking and PCR assay results. The BAC clones were obtained through library screening by hybridization and verified through PCR of derived sequence tag site (STS) markers. For the purposes of contig extension, the outlying STS-PCR products from each side of the contig were selected for hybridization of the high-density gridded filters of the library as described in Li et al. (1999). STS markers were designed to each end of each BAC clone. BAC end sequences were first analyzed with BLAST to identify common dog repetitive elements. PCR primers for STS markers were designed in regions of unique sequence using the Primer3 program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Amplification was performed as follows: 95°C for 5 min, 25 cycles of 94°C for 1 min, 55°C–60°C (primer dependent) for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were analyzed on 1.5% agarose gels followed by staining in ethidium bromide solution.

Polymorphic Marker Isolation and Genetic Typing in Canine Clones and Genomic BAC Clones

Microsatellite markers were isolated using minilibraries constructed from selected genomic BAC clones. Briefly, BAC clones were triple digested with DraI, SspI, and EcoRV (Amersham), and the resulting digests were ligated to pBluescript, transformed, and plated on LB/Agar plates covered with a Duralose-UV (Stratagene) membrane. Following overnight growth in a 37°C incubator, replica filters were made using a second duralose membrane, applying pressure, and marking by puncture. Replica filters were transferred to LB/Agar plates, allowed to grow, and then colonies were lysed in situ by alkaline lysis as follows. Membranes were placed on Whatman paper wet with 10% SDS for 5 min, and then transferred to denaturing and neutralizing solutions for 5 min each. DNA was then cross-linked using UV light and washed in 2× SSPE/1% SDS. Next, the membranes were hybridized with [γ-^{32}P]dATP radiolabeled (CA)15, (GAAA)8, (GAAT)8, and/or (GATA)8 oligonucleotides and washed in 0.1× SSPE/0.1% SDS or 1× SSPE/0.1% SDS (65°C or 55°C, respectively, for dinucleotide versus tetranucleotide probes). Plasmid DNAs were extracted from all positive colonies (Qiagen) and sequenced on an ABI 377 DNA sequencer using T3 and T7 primers. The program Primer3 was used to design flanking primers on all sequence traces containing a repeat sequence longer than ten compound repeats. Amplification and detection of the fragment length polymorphism were performed as described in Lin et al. (1997).

STS Typing and Contig Building

The majority of the STS markers were developed by direct sequencing of BAC clone ends with T7 and SP6 using an ABI 377 DNA sequencer and by designing PCR primers. Other STSs were developed as part of our effort to clone dinucleotide and tetranucleotide microsatellite repeat markers in the region. These markers were used to test all BAC clones. BAC clone insert sizes are determined using NotI digestion followed by pulsed field gel electrophoresis in 1% agarose with a CHEF-DRII system (BioRad) as described in Li et al. (1999). STSs for which location was not strictly constrained were spaced at roughly equidistant intervals between constrained markers. To verify clone integrity, fingerprinting was performed on all clones using EcoRV, HindIII, and BglII. Fragment sizes were estimated after ethidium bromide staining using established molecular weight markers and the Biorad 200 imaging system. Contig assembly was performed manually with assistance of the contig ordering package (Whitehead Institute) and Segmap for STS mapping (Green and Green, 1991) and FingerPrint contig (http://www.sanger.ac.uk/Users/cari/fpc.shtml) for fingerprinting (Soderlund et al., 1997).

RT–PCR Amplification and Sequencing of the *Hcrtr2* cDNA in Control and Narcoleptic Canines

Total RNA extraction and mRNA purification from wild-type (four Dobermans, two Labradors) and narcoleptic (four Dobermans, two Labradors) dog brain were performed using the RNeasy Maxi (Qiagen) and Oligotex mRNA Midi Kits (Qiagen), respectively. We generated first-strand cDNA using mRNA (1 μg), AMV reverse transcriptase (SuperScript II RT; 200 U; GIBCO–BRL), and *E. coli* RNaseH (2 U) according to the manufacturer's recommendation. PCR primers and conditions for RT–PCR amplification are described in the Figure 5 legend.

PCR Amplification of the *canarc-1* SINE Insertion

Genomic DNA was amplified with PCR primers flanking the SINE insertion using w554-65seqF 5′(′GGGAGGAACAGAAGGAGAGAATT T3′) and R4/7-6R(110) (5′ATAGTTGTTAATGTGTACTTTAAGGC3′). PCR conditions were 95°C for 2 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

PCR Amplification and Sequencing of Exon 6–Intron 6 Boundary for Identification of the Labrador *canarc-1* Mutation

Genomic DNA was amplified with PCR primers flanking exon 6 and intron 6 using 6INF(162) (5′GACTTCATTTGGCCTTTGATTTAC3′) and 7EXR(1620) (5′TTTTGATACGTTGTCGAAATTGCT3′). PCR conditions were 94°C for 2 min; five cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min. Cycle sequencing on the PCR product was performed using the 6INF(162) primer, and reactions were analyzed on an ABI 377 DNA sequencer.

Bioinformatics

Sequence contig and sequence comparisons were performed using the Sequencher 3.0 program (Gene Codes). cDNA-genomic DNA comparisons were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast). Genemap 1996 (The Human Transcript Map) and Genemap 1998 can be found at http://wwwncbi.nlm.nih.gov/SCIENCE96/ and http://www.ncbi.nlm.nih.gov/genemap98, respectively. The Sanger Center Human Chromosome Radiation Hybrid Maps can be found at http://www.sanger.ac.uk/HGP/Rhmap/maps.html. The Stanford Human Genome Center RHServer submission form can be found at http://www-shgc.stanford.edu/RH/rhserverformnew.html. The Whitehead physical mapping project can be found at http://carbon.wi.mit.edu:8000/cgi-bin/contig/phys_map. The FPC (Software for FingerPrinting Contigs) can be found at http://www.sanger.ac.uk/Users/cari/fpc.shtml. The human gene mutation database (Institute of Medical Genetics, Cardiff) can be found at http://www.uwcm.ac.uk/awcm/mg/hgmd0.html.

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GenBank Accession Number

The full-length nucleotide sequence of the canine *Hcrtr2* cDNA has been submitted to GenBank with the accession number AF164626.