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Genetics

Linkage analysis and comparative mapping of canine progressive rod–cone degeneration (*prcd*) establishes potential locus homology with retinitis pigmentosa (RP17) in humans

GREGORY M. ACLAND*†, KUNAL RAY*, CATHRYN S. MELLERSH‡, WEIKUAN GU*, AMELIA A. LANGSTON‡, JASPER RINE§, ELAINE A. OSTRANDER‡, AND GUSTAVO D. AGUIRRE*

*James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca NY 14853; ‡Fred Hutchinson Cancer Research Center, Seattle, WA 98104; and §Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

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ABSTRACT **Progressive rod–cone degeneration (*prcd*) is the most widespread hereditary retinal disease leading to blindness in dogs and phenotypically is the canine counterpart of retinitis pigmentosa (RP) in humans. In previous efforts to identify the genetic locus for *prcd*, the canine homologs for many of the genes causally associated with RP in humans, such as *RHO*, *PDE6B*, and *RDS*/peripherin, have been excluded. In parallel with a recent undertaking to establish a framework map of the canine genome, multiple *prcd*-informative pedigrees have been typed with a panel of more than 100 anchor loci and microsatellite-based markers. Identification of a linkage group flanking *prcd* ([*TK1*, *GALK1*, *prcd*]–[*MYL4*, *C09.173*, *C09.2263*]–*RARA*–*C09.250*–*C09.474*–*NF1*) localizes *prcd* close to the centromeric end of canine chromosome 9 (CFA9), and excludes *RARA* as a candidate gene. The conserved synteny of this region of CFA9 and distal human chromosome 17q establishes the potential locus homology of *prcd* in the dog with RP17, a human retinitis pigmentosa locus for which no gene has yet been identified. Assignment of the *prcd* disease locus to an identified canine autosome represents a powerful application of the developing canine linkage map in medical genetics. The usefulness of this approach is further demonstrated by identification of the correspondence of the *prcd* interval to homologous human and mouse chromosomal regions. The rapid progress that is now occurring in the field of canine genetics will expedite the identification of the genes underlying many of the inherited traits and diseases that make the dog a unique asset for the study of mammalian traits.**

The domesticated dog, *Canis familiaris*, exhibits a striking range of phenotypes that are clearly hereditary. These range from relatively simple traits, such as variation in coat color (1) and numerous single gene disorders (2–4), through a range of well described clinical disorders and other phenotypes exhibiting complex inheritance (3–10). In addition, there is the diverse repertoire of morphological and/or behavioral characteristics that define and distinguish the numerous specific breeds of dog. In many cases these phenotypes are both of interest in and of themselves and have such compelling similarity to recognizable human traits that they promise unique insights into the responsible genetic and metabolic mechanisms. However, despite the enormous range of genetic traits to investigate, progress in mapping the corresponding loci has been hampered, until recently, by the paucity of information about the canine genome. This problem has been compounded by the difficulties in karyotyping the dog genome, which has a large number (38 pairs of autosomes, X, Y) of small, similar, and mostly acrocentric chromosomes (11–14). With the exception of a few X-linked disease phenotypes (see refs. 15 and 16 for examples), so far no heritable trait has been mapped to an identified canine chromosome.

Although the canine genome map is still very sparse (17–19) compared with that of well mapped species, recent efforts to develop a framework linkage map of the canine genome (17, 19), including both gene-specific (type I, or anchor locus) and anonymous (type II) microsatellite markers, enable genomic screening by linkage analysis to be undertaken in informative canine pedigrees. Simultaneously, recent improvements in high resolution canine cytogenetics and chromosome mapping by fluorescent *in situ* hybridization (FISH) have further advanced mapping efforts in the dog by permitting assignment of anchor loci and linkage groups to defined chromosomes (12, 13, 20). Thus, it is now feasible to undertake a comprehensive search by linkage analysis for the loci controlling heritable canine traits, and assign such loci to defined canine linkage groups and chromosomal regions. Furthermore, it is possible, by recognition of interspecies conservations of synteny, to determine the corresponding regions of the human and mouse genomes to which such traits map.

Progressive rod–cone degeneration (*prcd*) is a canine retinal degeneration inherited as an autosomal recessive trait (21). It is one of several diseases recognized clinically and collectively as progressive retinal atrophy (PRA), the canine phenotypic equivalent of retinitis pigmentosa (RP) in humans. Because of its clinical similarity to RP, *prcd* has been widely studied as a model of the human disease (21–24). The *prcd* phenotype is that of a degenerative disorder, in which rods and then cones degenerate both structurally and functionally after apparently normal postnatal development. For this reason it is classified as a late onset disorder, indicating that the clinical disease is not apparent until early adolescence or early adulthood (25).

Classical genetic studies established that several allelic forms of *prcd* occur in different canine populations (25). Candidate gene studies have excluded several of the known RP loci, such as the β-subunit gene for retinal cGMP phosphodiesterase (*PDE6B*), opsin, transducin α1, and RDS/peripherin (26–28). Until now, however, there has been no information regarding the map location of *prcd*. As a result, little is known about potential candidates or to which, if any, human RP locus *prcd* might correspond.

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Abbreviations: RP, retinitis pigmentosa; ERG, electroretinogram; STS, sequence tagged site; lod, logarithm of odds; cM, centimorgan.

†To whom reprint requests should be addressed at: James A. Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, 47 Hungerford Hill Road, Ithaca NY 14853-6401. e-mail: gma2@cornell.edu.

As part of an investigation into *prcd*, three-generation *prcd*-informative pedigrees were developed. These pedigrees were constructed purposefully to be as genetically polymorphic as possible, yet remain fully informative for *prcd,* and to achieve the largest informative sibships. This construction made them simultaneously attractive for canine genome mapping studies, and thus they were used concurrently for both purposes. Genetic mapping studies have identified a linkage group flanking both sides of the *prcd* locus, with placement of *prcd* close to the centromeric end of canine chromosome 9 (CFA9). Because this region of CFA9 corresponds to distal human chromosome 17q (29), conservation of synteny strongly suggests that *prcd* in the dog is the locus homolog of RP17, a human RP locus for which no gene has yet been identified (30, 31).

METHODS

Animals. The *prcd* strain of dogs is maintained as part of an National Eye Institute-sponsored project (EY06855, "Models of Hereditary Retinal Degeneration") at the Retinal Disease Studies Facility (RDSF) in Kennett Square, Pennsylvania. This strain of dogs derives from the original research colony of purebred miniature poodles in which the phenotype and inheritance of *prcd* were originally characterized (25, 32). To generate informative pedigrees for this study, *prcd*-affected dogs were bred to homozygous normal unrelated miniature poodles, beagles, and beagle-crossbred dogs, and their heterozygous offspring were then backcrossed to *prcd*-affected dogs to yield litters segregating for the *prcd* phenotype. As depicted in Figs. 1 and 2, the nine related three-generation canine families analyzed yielded 70 *prcd*-informative progeny; this represents a subset of the extended pedigree of the breeding colony. DNA isolated from blood and tissue samples from dogs in these families were tested in the present study.

Diagnosis of Phenotype. Ascertainment of *prcd* phenotype relied on a combination of ophthalmoscopic, electrophysiological, and retinal morphological examinations using previously published diagnostic criteria for the disease (25, 32). In dogs maintained to adulthood, initial diagnostic assignment was based on electroretinography at a minimum age of 1 year, when characteristic electroretinographic abnormalities are present in *prcd*-affected dogs from this colony (25). This initial diagnostic assignment was relied upon only for selection of potential breeding animals; in all dogs typed for informative pedigrees, *prcd* phenotype was confirmed by indirect ophthalmoscopy (for dogs over 4 years of age), retinal morphologic examination, or both. For all dogs not maintained to adulthood, diagnosis was based on retinal morphologic examination at a minimum age of 14 weeks.

Electroretinograms (ERGs) were recorded from halothane-anesthetized dogs, to stimuli and under conditions designed to enable separate evaluation of rod-mediated and cone-mediated response components. The ERG stimuli were controlled for intensity, color, and rate of presentation of 10-μsec light flashes from a Grass strobe white light source, delivered to the cornea by means of a fiber optic light guide (25, 33). For morphologic assessment of disease, eyes were fixed in mixed aldehyde solutions and embedded in an epoxy resin (33). One-micrometer sections of *prcd*-affected retinas demonstrate characteristic and diagnostically reliable morphologic changes by 14 weeks of age (34).

DNA Extraction. Genomic DNA was extracted either from aliquots of fresh, or frozen and thawed, citrated whole blood samples or from frozen samples of splenic tissue, as described previously (26, 35).

Microsatellite Typing. Microsatellites were amplified as described previously, omitting the Hot Start protocol (29, 36–38). PCR products were separated by electrophoresis on denaturing (4–6%) polyacrylamide gels (PAGE) at 55°C and were visualized by autoradiography or by fluorescence on an Applied Biosystems 377 fluorescence DNA sequencer. Genotypes determined by autoradiography were scored independently by two individuals and entered into a consensus database; disputed genotypes were omitted. All microsatellite primers have been reported previously (19, 29, 36–39).

***MYL4* Polymorphism.** The human atrial myosin alkali light chain 1 gene (*MYL4*, GenBank accession nos. M24121 and J03954) maps to human chromosomal region 17q21–qter (ref. 40; see also the Genome Data Base at The John Hopkins University, http://www.gdb.org). Because of our initial evidence of homology between this human chromosomal segment and the *prcd*-linked canine chromosomal region (see below), we cloned and sequenced a 1.5-kb fragment of the canine homologue of *MYL4*. Genomic DNA was amplified by using primers MYL4–2, 5′-CATTGTTTGACCGGACCCCG-ACTGG-3′, and MYL4–4, 5′-CCTTGTTGCGGGAAAT-GTGCTGC-3′ and 2.5 mM $MgCl_2$ for 40 cycles of 94°C for 30 sec, 65°C for 1 min, and 72°C for 2 min. Two restriction fragment length polymorphisms (RFLPs) have been identified in this gene fragment. Digestion with *Bst*NI yields six nonpolymorphic and one polymorphic (140-bp) fragments. When the polymorphic site is present (allele 2) the 140-bp fragment is digested to 120- and 20-bp fragments. Absence of the polymorphic site constitutes allele 1. Digestion of the 1.5 kb-amplified fragment with *Bsr*I generates two nonpolymorphic and one polymorphic (340-bp) fragments. Presence of the *Bsr*I polymorphic site (allele 4) allows cleavage of the 340-bp fragment into 250- and 90-bp fragments. Absence of the polymorphic site constitutes allele 3. Digestion products were analyzed on 6% PAGE.

Linkage Analysis. Using the LINKAGE package of programs in refs. 41 and 42, we undertook two-point linkage analysis (43) between *prcd* and each marker and between each pair of markers. The disease trait was coded as an autosomal recessive trait with full penetrance and no phenocopy (41–43). Because we had prior knowledge of the actual *prcd* genotypes for all parental and grandparental dogs (based on previous breeding studies), *prcd* was also coded as an allele numbers locus. Three-point analyses were also run on selected subsets of loci.

Subsequently, the data were displayed on a pedigree/haplotype-analysis spreadsheet, using gene order best supported by our linkage analysis to identify where recombination events had occurred.

Physical Mapping on Canine–Rodent Hybrid Cell Lines. As described elsewhere (17), a panel of canine–rodent microcell hybrid cells were constructed by fusion of canine fibroblast donors with immortalized rodent recipient cells. Hybrid cell lines were characterized by fluorescence *in situ* hybridization (FISH) using dog genomic DNA probes. Three of the markers utilized in the present study (*C09.250*, *C09.474*, and *C09.173*), as well as two additional canine- and gene-specific sequence tagged site (STS) amplimers, were mapped onto this panel of hybrid cells to determine evidence for physical association of these markers. Primer pairs for each microsatellite or STS amplimer were used for PCR using dog, mouse, and hamster genomic DNA as templates. Cell lines from which the *prcd*-linked marker primers amplified an appropriately sized product were recorded as positive.

RESULTS

The set of pedigrees used in this study is illustrated in Figs. 1 and 2. Initially, a panel of approximately 100 anonymous canine-specific microsatellites (19, 36–39) was typed on a subset of the *prcd*-informative pedigrees. Linkage was detected between marker *C09.173* (GenBank L15680 and L15681) and the *prcd* disease locus with 4 recombinants from 27 scored meioses [recombination fraction = 0.148, logarithm of odds (lod) score = 3.21]. This marker, *C09.173*, had previously been

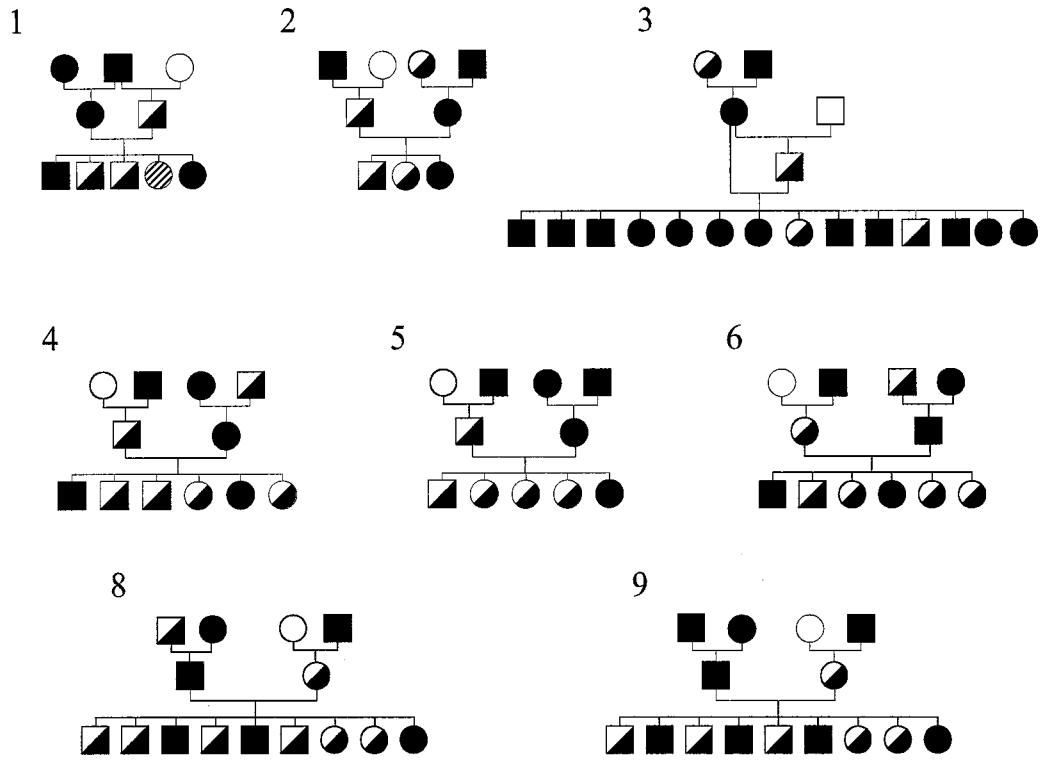

FIG. 1. Pedigrees of *prcd*-informative three-generation families 1–6, 8, and 9, used in linkage studies. Circles represent females, squares represent males, open symbols indicate homozygous normal at *prcd* locus, solid symbols indicate *prcd*-affected, and half-filled symbols indicate *prcd* heterozygous. The hatched symbol in family 1 indicates a sibling for whom *prcd* phenotype could not be reliably ascertained. Although shown as separate pedigrees, these families together form part of a much larger *prcd* colony.

mapped to two of three canine–rodent hybrid cells—MDE6, MDE15, and MDL9 (17)—which also contained the canine homolog of the human breast cancer 1 susceptibility gene (*BRCA1*, GenBank U50709; refs. 17 and 44). These data enabled identification of two further anonymous microsatellites (*C09.250*, GenBank L15688 and L15689; and *C09.474*, GenBank L24352 and L24353) which mapped to the same canine–rodent hybrid cells, and a fourth (*C09.2263*) that was linked to *C09.173* in different pedigrees. These four microsatellites were then tested on a further set of *prcd*-informative pedigrees, as well as on additional unrelated pedigrees to determine the linkage distances and map order of the four microsatellites (19). Analysis of these data placed *prcd* close to *C09.173* and *C09.2263*, and on the opposite side of these two markers from both *C09.250* and *C09.474*, as summarized in Table 1 and illustrated in Fig. 2*A*.

Recognition of the physical association of these four markers, and thus *prcd,* to a canine chromosomal region including *BRCA1* suggested that *prcd* was located on a canine chromosomal segment homologous to the region of human 17q where human *BRCA1* maps. We therefore developed a set of PCR primers to amplify a canine-specific fragment of the γ-subunit gene for retinal cGMP phosphodiesterase (*PDE6G*; GenBank U49359), which we had previously cloned and sequenced (45). The human homolog of this gene is distal to *BRCA1* on chromosome 17q (46). PCR amplification with these *PDE6G*-STS primers yielded positive results for cell lines MDE6 and MDE15 to which the loci *BRCA1*, *C09.250*, *C09.474*, and *C09.173* had previously been mapped (17).

Thus, the canine–rodent cell lines MDE6, MDE15, and MDL9 (17) each contain all or a fragment of a canine chromosome with strong homology to human chromosome 17q24–25, and the *prcd* locus is physically located in this region. Because we have not yet identified any informative polymorphisms within the small (2.8-kb) *PDE6G* gene for these pedigrees, the linkage distance between *prcd* and *PDE6G* could not be determined.

Because of the positive result with *PDE6G*, we then genotyped the *prcd*-informative pedigrees for two restriction fragment length polymorphisms identified in a 1.5-kb amplified fragment of the canine homolog of *MYL4*. This locus also demonstrated linkage to *prcd* yielding a recombination fraction (θ) of 0.033 (lod score = 14.24).

Concurrently and independently, a set of gene-specific microsatellites were described that map to the centromeric end of CFA9 and correspond to loci on distal human chromosome arm 17q (29). We therefore typed four of these microsatellites, identified as thymidine kinase 1 (*TK1*), galactokinase 1 (*GALK1*), retinoic acid receptor α (*RARA*), and neurofibromin (*NF1*) on *prcd*-informative pedigrees. Two of these markers, *TK1* and *GALK1*, cosegregated with *prcd* (θ = 0.000; lod scores = 10.84 and 4.21, respectively). *RARA* mapped to the interval bounded by markers *C09.250* and *C09.474* at one end and *C09.173*, *C09.2263,* and *MYL4* at the other, yielding a recombination fraction of 0.091 with *prcd* (lod score = 7.42), which excludes this locus as a candidate for *prcd*. *NF1* mapped further from *prcd* than any previous marker (θ = 0.032), but with only 25 informative individuals the lod score (0.72) associated with this linkage result was not significant. *NF1* did, however, show significant linkage to either *RARA* (θ = 0.172; lod score = 3.27) or *C09.250* (θ = 0.000; lod score = 4.82) both of which were significantly linked to *prcd,* thus confirming that *NF1* is part of the conserved syntenic region surrounding *prcd*. The results of the linkage analysis are summarized in Table 1, and illustrated in Figs. 2 and 3. No obligate recombinants were observed in *prcd*-informative families for the interval defined by markers *C09.173*, *C09.2263*, and *MYL4*. Three-point analysis yielded no differences among all possible orders for these three markers together, or for any two with *prcd*.

Similarly, no obligate recombinants were observed in the F_2 generation of *prcd*-informative families for the interval defined

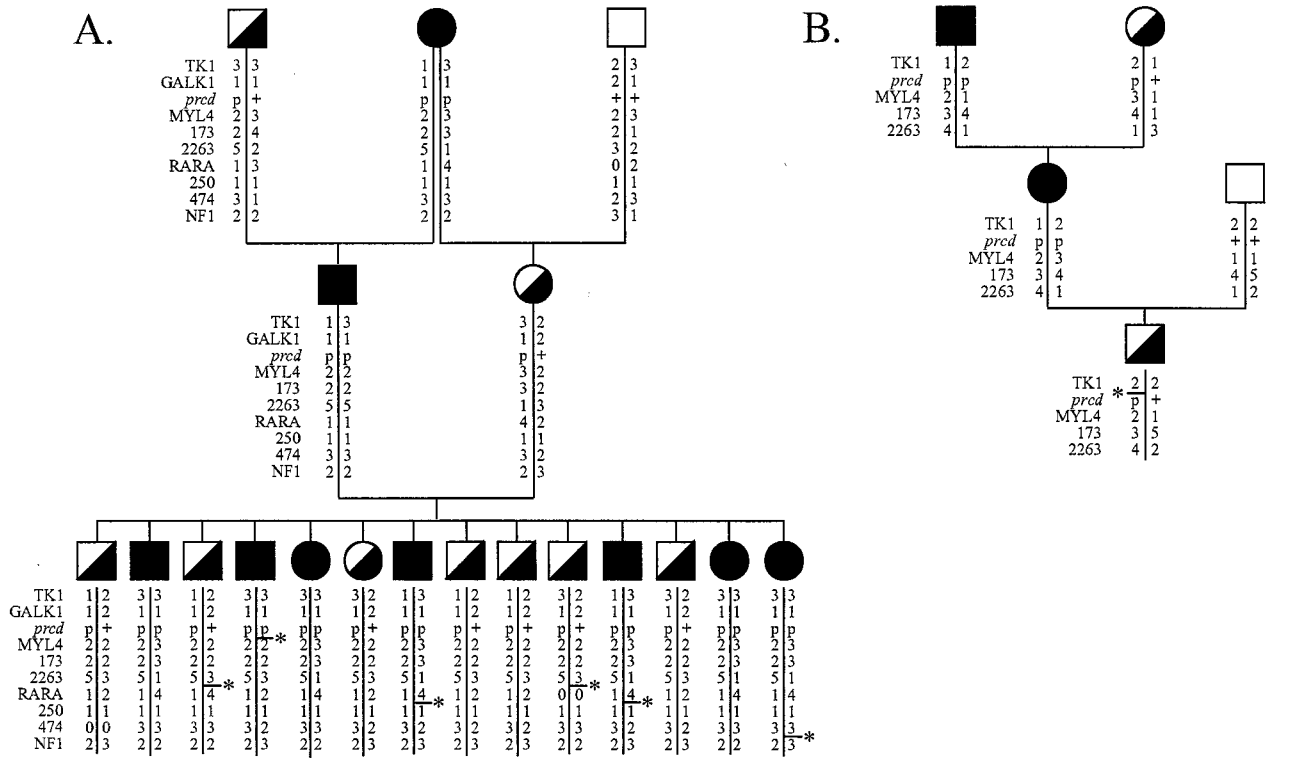

FIG. 2. (*A*) Pedigree of *prcd*-informative family 7, with haplotype data. The order of loci is according to results of two-point linkage analyses in the present study. For the *prcd* locus, p indicates disease allele and + indicates wild-type allele. For *MYL4*, allele numbers are assigned as described in the text. For all other loci, allele numbers were assigned according to ref. 19. Loci 173, 2263, 474, and 250 represent microsatellite markers *C09.173*, *C09.2263*, *C09.474*, and *C09.250*, respectively. Haplotypes demonstrating recombination events are indicated (—*), although indicated position does not always correspond to the only possible site of the recombination. (*B*) Pedigree of sire of F_2 litter in family 3, with haplotypes indicating that this individual received a *TK1–prcd–MYL4* recombinant chromosome from his dam. Although this recombination can equally be interpreted to have taken place between *TK1* and *prcd,* or between *prcd* and *MYL4*, the haplotypes also indicate that two different *TK1* alleles are segregating in phase with *prcd* in this dog's pedigree.

by *TK1*, *GALK1*, and *prcd*. However, haplotype analysis indicates that an F_1 individual, the sire of the informative litter in family 3, received a chromosome that was recombinant in the *TK1-prcd-MYL4* interval from his dam (see Fig. 2*B*). The site of the recombination is interpretable as equally likely to be between *TK1* and *prcd* or between *prcd* and *MYL4*. Formal

Table 1. Recombination fractions and lod scores for markers flanking the *prcd* locus on canine chromosome 9 (CFA9)

		TK1	*GALK1*	*MYL4*	173	2263	*RARA*	250	474	*NF1*
prcd	θ	**0.000**	**0.000**	**0.033**	**0.049**	**0.051**	**0.091**	**0.156**	0.244	0.320
	Z	**10.84**	**4.21**	**14.24**	**13.17**	**12.61**	**7.42**	**3.61**	2.45	0.72
	n	**36**	**14**	**70**	**61**	**61**	**37**	**47**	41	25
GALK1	θ	**0.000**								
	Z	**4.21**								
MYL4	θ	**0.039**	0.071							
	Z	**10.48**	2.35							
173	θ	**0.048**	0.071	**0.044**						
	Z	**12.55**	2.65	**15.94**						
2263	θ	**0.049**	0.071	**0.039**	**0.000**					
	Z	**12.27**	2.65	**16.15**	**25.29**					
RARA	θ	**0.161**	0.154	**0.097**	**0.058**	**0.056**				
	Z	**3.99**	1.49	**8.57**	**13.84**	**14.67**				
250	θ	**0.148**	NI	**0.121**	**0.131**	**0.139**	0.143			
	Z	**3.21**		**4.50**	**5.01**	**4.54**	2.58			
474	θ	0.250	0.357	**0.167**	**0.167**	**0.167**	**0.151**	0.154		
	Z	1.59	0.25	**3.49**	**4.42**	**4.42**	**3.84**	1.49		
NF1	θ	0.368	0.429	0.277	0.266	0.276	**0.172**	**0.00**	0.071	
	Z	0.29	0.06	0.73	1.48	1.31	**3.27**	**4.82**	2.65	

Markers 173, 2263, 474, and 250 indicate canine microsatellite loci *C09.173*, *C09.2263*, *C09.474*, and *C09.250*, respectively. Data are shown in boldface where lod score exceeds 3.0. The order shown is that supported by two-point linkage analyses of data in the present study. θ, Maximum likelihood estimate of recombination fraction between loci; *Z*, lod score; *n*, number of informative meioses typed for two-point linkage analysis; and NI, no pedigrees informative.

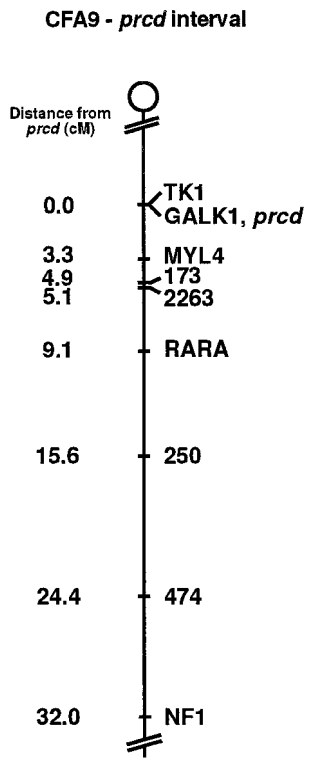

FIG. 3. Linkage map of the *prcd* interval on canine chromosome 9 (CFA9). The location of *prcd* is shown relative to that of nine loci corresponding to type I and II markers. Loci listed on a single line indicate no recombinations observed between these markers in the present study. *TK1* and *GALK1*/*prcd* are on separate lines but at a single location because, although no obligate recombinations were observed in this interval, indications of possible recombinations between *TK1* and *prcd* were detected. Order and distances (in cM, centimorgans) shown are based on two-point linkage analyses of the data in this study.

linkage analysis gave a recombination distance of zero between *TK1* and *prcd* but, if this dog is recombinant between *TK1* and *prcd*, then the order is *TK1-prcd-MYL4*. The observation of two different *TK1* alleles segregating in phase with *prcd* can also be taken to indicate recombination events taking place between *TK1* and *prcd* in previous generations of this pedigree, lending further support to this order. Furthermore, although no recombinations were observed between *GALK1* and *prcd,* relatively few of the typed individuals were informative for the *GALK1* $(CA)_n$ repeat. Thus the supported linkage distance is broader for *GALK1* (1-lod support interval = 15.2 cM) than for *TK1* (1-lod support interval = 9.05 cM). The one dog indicated by haplotype analysis (Fig. 2*B*) as possibly recombinant between TK1 and *prcd* was not informative for *GALK1*. Three-point analysis yielded no differences among all possible orders for *TK1*, *GALK1*, and *prcd*.

Although, from the present data, *C09.474* maps further from *prcd* ($\theta = 0.244$) than does *C09.250* ($\theta = 0.156$), recombinations observed on haplotype analysis would also support placement of *C09.474* between *C09.250* and *prcd*. This order is in agreement with mapping data derived from more extensive pedigrees (19).

DISCUSSION

The linkage and cell line data presented here firmly establish that the canine *prcd* locus maps to the canine chromosome identified as CFA9 (12, 13, 29); previously this chromosome has also been referred to as canine chromosome 23 (14, 47). More specifically, *prcd* maps to the centromeric end of CFA9 in a region exhibiting conservation of synteny and gene order with distal human 17q (29, 47), mouse chromosome 11 (29, 40, 48, 49), and bovine chromosome 19 (50–52). Fig. 3 summarizes our linkage data for the centromere–NF1 region of CFA9, representing the extended *prcd* interval.

Linkage analysis and obligate recombinations locate *prcd* to the interval between *TK1* and *MYL4*. Our data, as well as those presented elsewhere (19, 29), allow us to estimate that this distance is between 3.9 and 5.1 cM. Both *TK1* and *GALK1* yielded recombination fractions of 0.000 with each other and with *prcd*, and three-point analysis of our data does not establish a preferred order for these loci. However, haplotype analysis revealed one dog that demonstrated a possible recombination between *prcd* and *TK1*, placing *TK1* on the opposite side of *prcd* from marker *C09.173*. We also observed in this dog's family two different *TK1* alleles in phase with *prcd*. Thus, although we did not obtain a nonzero recombination distance for the *TK1--prcd* interval, we do see evidence that recombinations have occurred in the extended pedigree. Similarly, no recombinants were observed between *GALK1* and *prcd* in this study, thus placing *GALK1* in the *TK1–MYL4* interval. However, because relatively few individuals were informative for *GALK1*, our data are interpretable to support the placement of *GALK1* centromeric to *TK1*—i.e., on the other side from *prcd,* which would be in better agreement with other studies (19, 29). Resolution of these issues requires additional linkage or physical mapping data.

Bardien *et al.* (30, 31) identified a locus (RP17) for autosomal dominant RP in two South African human kindred in the interval between markers *D17S1607* and *D17S1874* and close to 17q22. They initially (30) suggested three nearby genes as candidates for RP17: *PDE6G,* tissue inhibitor of metalloproteinases-2 (*TIMP2*; ref. 53), and protein kinase C α (*PRKCA*; refs. 54 and 55); but subsequently (31) presented evidence excluding each of these loci. *RARA* has also been suggested as a candidate for RP17 (31), but our data exclude it as a candidate for *prcd*. No genes have yet been implicated causally in RP17. Although RP17 is autosomal dominant and *prcd* is recessive, it is possible, perhaps likely, that these diseases arise from mutations in homologous genes. Dominant and recessive forms of RP have been identified that arise from mutations in the same gene (56, 57). The RP17 candidate region lies within the interval defined by *MYL4* at 17q21–qter (ref. 40 and http://www.gdb.org), and *TK1* at 17q25.2–25.3 (58), which loci also encompass the *prcd* interval on CFA9. As this interval forms part of the largest conserved syntenic group among mammals (40, 47–52), it seems likely that the canine homolog of RP17 lies within the corresponding interval on CFA9, and that *prcd* in the dog may be a true locus homolog of RP17 in humans.

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