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The canine copper toxicosis locus is not syntenic with *ATP7B* or *ATX1* and maps to a region showing homology to human 2p21

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Canine copper toxicosis (CT) is an autosomal recessive disorder resulting in accumulation of copper at toxic levels in the liver owing to deficient excretion via the bile (Hardy et al. 1975). This disorder is prevalent in certain breeds, most notably the American and British Bedlington Terrier, where disease allele frequencies as high as 0.5 are present, resulting in phenotype frequencies of 25% affected and 50% carriers (Herrtage et al. 1987). Affected dogs develop excessive amounts of copper in their liver and, if untreated, will die of liver disease between 3 and 7 years of age. The gene responsible for canine CT is unknown, but candidates include *ATP7B*, the gene responsible for Wilson disease in humans (Bull et al. 1993; Tanzi et al. 1993), and the *ATX1* (*ATOX1* or *HAH1*) gene, which codes for a copper chaperone that delivers copper to ATP7B within liver cells (Klomp et al. 1997; Hung et al. 1998).

Wilson disease in humans is similar to canine CT in that it is also an autosomal recessive disorder where copper accumulates in the liver owing to deficient copper excretion in the biliary system (Brewer and Yuzbasiyan-Gurkan 1992; Bull and Cox 1994). The protein product of *ATP7B* is a P-type ATPase which is expressed in the liver, kidney, and brain and functions to transport copper in the secretory pathway. Patients with Wilson disease accumulate excess copper primarily in their liver, and over time copper levels in the brain also increase, leading to a movement-type neurological disorder. Thus, the clinical phenotype is similar to canine CT, but differences exist. Neurological manifestations are not seen in canine CT, and affected Wilson disease patients have low levels of ceruloplasmin in their serum, while affected Bedlington terriers have normal levels of serum ceruloplasmin. In addition, the subcellular localization of copper accumulation in the liver differs between affected Wilson disease patients and affected Bedlington terriers. Wilson disease patients accumulate copper in their periportal hepatocytes, while affected Bedlington terriers accumulate copper in the center of the lobules (Owen and Ludwig 1982).

HAH1 (ATOX1) (Klomp et al. 1997), the human ortholog of yeast Atx1p, is a cytoplasmic protein that functions as a copper chaperone and is thought to shuttle copper from the cell membrane to both ATP7B and ATP7A (Pufahl et al. 1997) localized in the *trans* Golgi complex (Dierick et al. 1997; Payne et al. 1998). While not as strong a candidate as the *ATP7B* gene, it is possible that a mutation in *ATX1* could result in liver cirrhosis via interfering with the normal function of ATP7B without affecting the activity of ATP7A. No mammalian disorders have yet been attributed to a mutation in the *ATX1* gene.

Yuzbasiyan-Gurkan et al. (1997) performed linkage analysis with several Bedlington terrier pedigrees of the American Kennel Club to identify DNA microsatellite marker *C04107* as being tightly linked to the CT locus with a LOD score of 5.96 at recombination fraction of zero. This polymorphic marker has been successfully applied in molecular diagnostic tests for CT in Bedlington terriers (Holmes et al. 1998; Ubbink et al. 1998). In an earlier study (Yuzbasiyan-Gurkan et al. 1993), the CT locus was found to be unlinked to the esterase D (*ESD*) and retinoblastoma (*Rb1*) loci, both of which show strong linkage to Wilson disease in humans. This suggested that the CT and *ATP7B* loci were different and unlinked in the dog, but data on linkage of the canine *ATP7B, Rb1,* and *ESD* loci is lacking and could differ from that seen in the human genome.

In the present study, fluorescent in situ hybridization (FISH) was performed to determine whether candidate genes *ATP7B* or *ATX1* mapped to the same or to different chromosomal locations from *C04107.* If either *ATP7B* or *ATX1* mapped to the same chromosomal locus as *C04107,* it would suggest that CT may be a result of a mutation in that gene. If they mapped to different chromosomes, this would strongly support the hypothesis that another gene involved in mammalian copper transport or homeostasis is responsible for canine CT.

A canine BAC library constructed from Doberman Pinscher DNA (Roswell Park Cancer Institute, RPCI, Buffalo, N.Y.) was screened with random primed (Rediprime™ II DNA Labeling System, Amersham Life Sciences, Arlington Heights, Ill.) ^{32}P-labeled probes prepared from PCR products specific for the *C04107, ATP7B,* and *ATX1* loci. PCR primers (forward-5′ CCGGATCCTTTAGATGGGAC 3′; reverse-5′ CAGGTACCCAAGTCATTTGTCTATC 3′) designed from sequence upstream of the cytosine-adenine (CA) repeat of microsatellite marker *C04107* were used with dog spleen total genomic DNA as template in PCR reactions to generate the CT-specific probe. An *ATP7B*-specific probe was generated from a PCR reaction using primers (forward-5′ GACAAAACTGGCACCATACGCACG 3′; reverse-5′ GTTCTGGAGCTCCTGGACCTTGGCCAG 3′) designed from canine exons 14 and 18 and a canine cDNA subclone, which contains *ATP7B* transmembrane domains 6–8, as template. *HAH1* (*ATX1*) specific primers (forward-5′ CAGTCATGCCGAAGCACGAG 3′; reverse-5′ CTGAGGGTCTCCGCAGGAAC 3′) were used with human cDNA as template in a PCR reaction to generate a probe which was used in cross-species hybridization of the canine BAC filters. All PCR products used as probes were checked by sequencing with an Applied Biosystems model 373A automated sequencer. Positive BAC clones were purchased from RPCI and verified as having the correct loci by PCR and Southern blot analysis as well as sequencing. Canine BAC clones 27N21 and 225B1 contain the CA microsatellite *C04107* as well as the upstream sequence used to generate the CT-specific probe. Minimally, exons 17 and 18 of the *ATP7B* gene are contained within BAC clone 243F13, while BAC clone 84B18 contains the *ATX1* gene.

To map the chromosomal location of these loci, BAC clones

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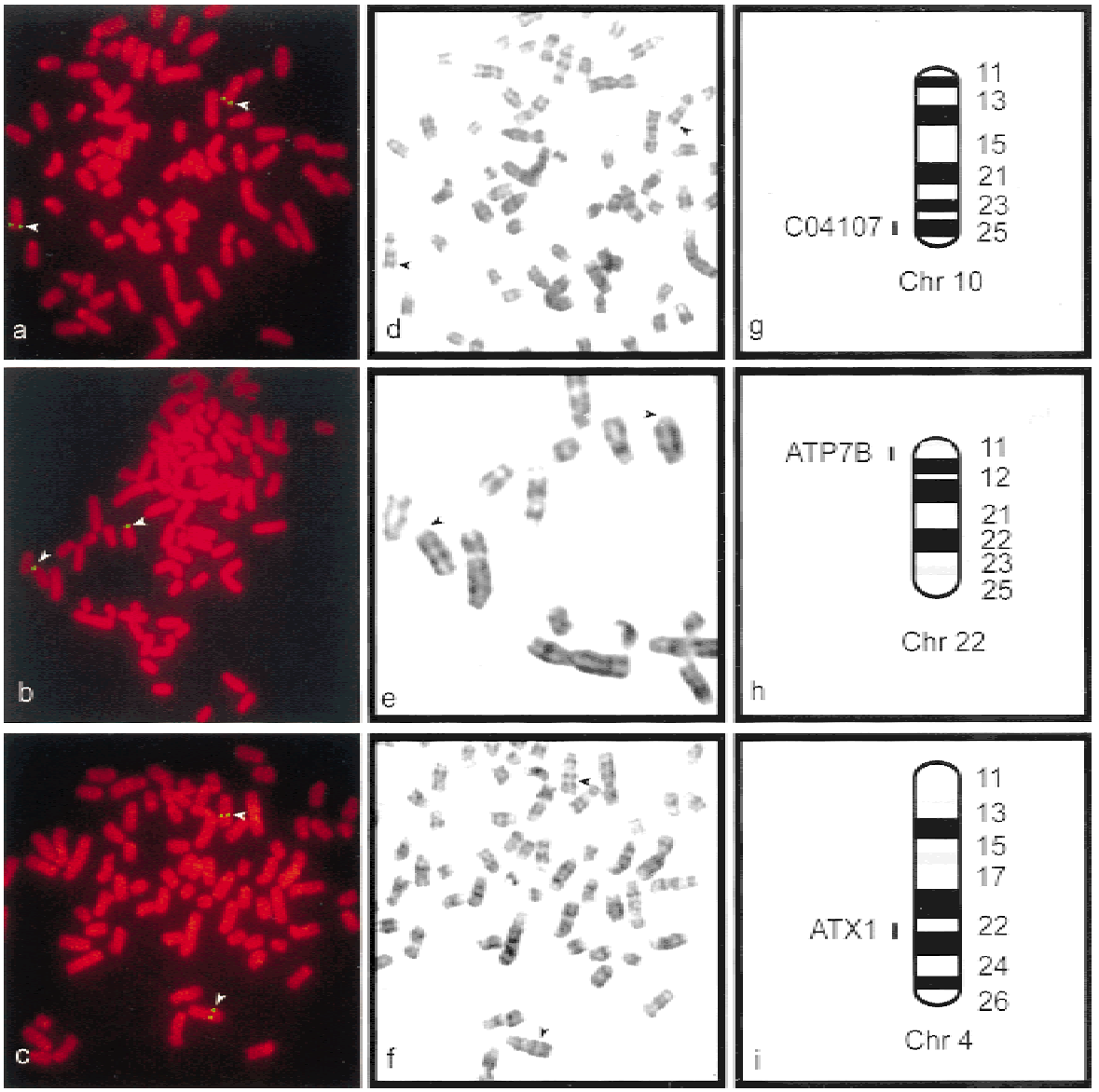

Fig. 1.

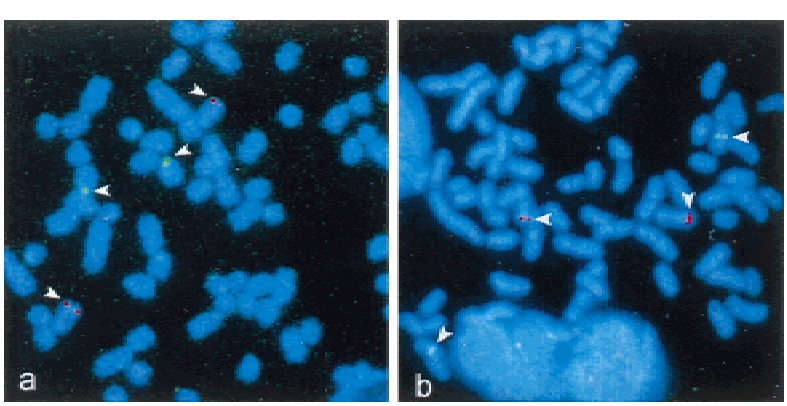

Fig. 2. Two-color FISH results. BAC 225B1 [red, **(a, b)**] hybridizes to chromosomal homologs different from BAC 243F13 [green, (a)] or BAC 84B18 [green, (b)]. BAC clones 243F13 and 84B18 were labeled with biotin as previously discussed. BAC clone 225B1 was labeled by Nick translation with dioxigenin-11-dUTP (DIG-11-dUTP, Boehringer Mannheim, Indianapolis, Ind.). Denatured metaphase spreads were simultaneously hybridized overnight in the presence of the biotin-labeled probe and the digoxigenin-labeled probe and then washed as described in the legend for Fig. 1. To visualize both colors, the slides were simultaneously incubated in the presence of FITC-conjugated avidin DCS for the biotin-labeled probe and sheep anti-digoxigenin rhodamine (Boehringer Mannheim) for the DIG-labeled probe. The slides were incubated with secondary antibodies fluorescein-conjugated anti-avidin IgG and Texas Red-conjugated anti-sheep IgG (Vector Laboratories) and counterstained with DAPI. A triple pass filter was used to visualize the signals.

225B1 (CT), 243F13 (*ATP7B*), and 84B18 (*ATX1*) were labeled with biotin using the BioNICK™ Labeling System (Life Technologies, Gaithersburg, Md.) and then used as probes for hybridization to denatured canine (Golden Retriever) metaphase spreads for single-color FISH as described in the legend for Fig. 1. A minimum of 30 metaphases was scored for each hybridization, and metaphase hybridization efficiency was >90% in all experiments with no other specific hybridization signals observed. Map loca-

Fig. 1. Hybridization of biotin-labeled BAC probes 225B1 **(a),** 243F13 **(b)** and 84B18 **(c)** to canine metaphase chromosomes. A triple pass filter was used for visualizing and photographing panels a–c, while panels d–f are G-banded images of the same metaphase spreads. Ideograms of the mapping locations are depicted in g–i. For chromosome preparations, 0.4 ml of heparinized whole dog blood was cultured for 70–72 h at 37°C and 5% CO_2 in 9 ml RPMI 1640 medium (Life Technologies) containing 15% fetal bovine serum (Life Technologies), 2 mM L-glutamine (Life Technologies), and 100 units/ml of penicillin/streptomycin (Life Technologies). To stimulate lymphocyte growth, 75 μl of pokeweed (Life Technologies) and 75 μl of phytohaemagglutanin ((PHA), Life Technologies) were added to the medium. Cultures were harvested by standard procedures and consisted of 30, 40, and 60 min of KaryoMAX Colcemid® (Life Technologies) treatment (0.07 μg/ml) followed by a 20-min hypotonic treatment (0.075 M KCl) and multiple changes of fixative (3:1 methanol-glacial acetic acid) before preparation of the slides. For simultaneous banding with FISH, 5-bromodeoxyuridine (BrdU)-synchronized lymphocyte cultures were grown as described above, but after 70–72 h 200 μl of BrdU (10 μg/μl, Sigma, St. Louis, Mo.) was added and the cultures were incubated at 37°C and 5% CO_2 for an additional 14–16 h. Cells were washed with complete RPMI medium once and then resuspended with 10 ml complete RPMI medium. A 100 μl volume of 2×10^{-3} M thymidine (Sigma) was added, and the culture was grown for an additional 4 h at 37°C and 5% CO_2. Cells were harvested as described above. Total BAC DNA was biotin labeled (biotin-14-dATP) according to manufacturer's instruction with the BioNICK™ Labeling System (Life Technologies). 300 ng of labeled BAC DNA and 75×---100× excess genomic dog DNA was precipitated with 1/10 volume 3 M sodium acetate and 2× volume 200-proof ethanol. The pellet was resuspended in 10 μl hybridization mix consisting of 2 × SSC, 10% dextran sulfate, 55% formamide, and 1% Tween-20. The DNAs were denatured for 8 min at 75°C and then preannealed at 37°C for 1 h prior to hybridization. Chromosome banding was obtained by DAPI banding of BrdU-substituted chromosomes as described by Lemieux and associates (1992; data not shown) and by standard trypsin-Giemsa banding (G-banding). After G-banding, slides were photographed and destained as described by Meltzer and colleagues (1992). Briefly, the slides were washed in methanol for 15–20 min with one or two changes to remove immersion oil, destained by sequential washes in 70% and 85% ethanol for 1 min each, fixed in Carnoy's fixative for 10 min, 3.7% formaldehyde in PBS for 10 min, and finally two 5-min washes in PBS. After air drying, the slides were denatured for 2 min at 70°C in 70% formamide/2 × SSC (pH = 7.0), and then 2 min each in an ethanol series of 70%, 85%, and 100% on ice. Hybridization was overnight at 37°C. The slides were washed three times at 42°C in 55% formamide/2 × SSC (pH = 7.0) and then three times at 60°C in 1 × SSC (pH 7.0). After blocking the slides for 1 h at 37°C with 4 × SSC/3% BSA, signals were visualized by incubation with two layers of FITC-conjugated avidin-DCS and fluorescein-conjugated anti-avidin IgG (Vector Laboratories, Burlingame, Calif.) and then counterstained with propidium iodide (PI). Chromosomes were analyzed with a Zeiss Axiophot epifluorescence microscope, and images were photographed on Kodak technical pan film and Kodak Royal Gold 1000 film. Photographic images were scanned and processed with Adobe Photoshop software and printed on a dye sublimation printer.

tions were determined by three independent cytogeneticists and were based on location on banded chromosomes according to a recent canine standardized karyotype ideogram proposed by Switonski and associates (1996; 225B1 and 84B18) and another by Reimann et al. (1996; 243F13). As shown in Fig. 1a and 1d, BAC clone 225B1 hybridized to both homologs of canine Chr 10 at 10q25. In Figure 1b and 1e, BAC clone 243F13 hybridized to both homologs of canine Chr 22 at 22q11. The probe from BAC clone 84B18 hybridized to 4q22, as shown in Fig. 1c and 1f. These results indicate that the CT locus maps to a chromosome different from that of the *ATP7B* or *ATX1* loci, and thus the loci are not physically linked or syntenic.

To further verify these results that the *C04107* locus is not syntenic with or physically linked to either *ATP7B* or *ATX1*, we performed two-color FISH as described in the legend to Fig. 2. As shown in Fig. 2, the two-color FISH confirms that the CT-specific probe generated from BAC clone 225B1 (red) hybridizes to chromosomal homologs different from that of the *ATP7B* BAC clone 243F13 (green, Fig. 2a) and *ATX1* BAC clone 84B18 (green, Fig. 2b).

In the course of BAC isolation, we identified four BAC clones containing an *ATP7B* pseudogene(s). PCR assays were performed to distinguish clones containing the *ATP7B* gene from clones containing an *ATP7B* pseudogene. Using primers specific for exons 14 and 18, we generated an 800-bp PCR product from the DNA of BAC clones containing an intronless *ATP7B* pseudogene, but not from BACs with *ATP7B.* The PCR product from one BAC clone, 163P18, was sequenced to verify the *ATP7B* pseudogene. This clone mapped to canine Chr 4q17, based on the Switonski and colleagues (1996) standardized karyotype (data not shown).

These results strongly suggest that the gene responsible for canine copper toxicosis is not the *ATP7B* gene responsible for Wilson disease in humans and further rule out the copper chaperone *ATX1.* Because the CT gene has not been cloned, these conclusions are based on the map location of the tightly linked marker *C04107.* While the distance between the two loci is not known, few recombinants have been observed in hundreds of meioses (Yuzbasiyan-Gurkan et al. 1997; Holmes et al. 1998; Ubbink et al. 1998), and thus it is highly likely that they are syntenic and tightly linked.

The canine CT marker maps to canine Chr region 10q25. This region shows homology to human 2p21 (Priat et al. 1998). Although only a few human orthologs have been mapped to this or any region of the canine genome, human 2p21 is a candidate region for human genes involved in copper metabolism. A database search for candidate human genes in the 2p21 region that might function in copper transport or homeostasis did not result in definitive candidates. However, the gene for human vacuolar proton-ATPase subunit M9.2 (Accession No. R07157) maps in or adjacent to 2p21 and should be considered as a possible candidate gene. Vacuolar ATPases are found in membranes bounding the acidic compartments of cells and as proton pumps are responsible for acidification of a variety of intracellular organelles. The M9.2 subunit gene contains a conserved sequence motif (Ludwig et al. 1998), CSVCC, similar to those of metal-binding proteins (MTCXXC). Whether or not this is a functional domain is yet to be determined. In addition, Szczypka and coworkers (1997) have shown that in yeast deficient in vacuolar ATPase, and hence abnormal vacuolar acidification, copper and iron homeostasis is abnormal.

Our results suggest that the gene responsible for CT is probably a novel mammalian gene involved in copper transport or homeostasis in liver cells. Mutations in the homologous human gene(s) could give rise to a disease phenotype similar to canine copper toxicosis. Such conditions might be Indian childhood cirrhosis or Tyrollean childhood cirrhosis (Tanner 1998). These disorders both appear to share environmental and genetic influences, with an autosomal recessive inheritance of a gene involved in the threshold of copper tolerance being most widely suggested. Based on our results, human Chr region 2p21 is a candidate location for a gene involved in these disorders.

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