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# Genomic Organization of the Dog Dystroglycan Gene *DAG1* Locus on Chromosome 20q15.1–q15.2

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Dystroglycan is a laminin binding protein, which provides a structural link between the subsarcolemmal cytoskeleton and the extracellular matrix. It is also involved in the organization of basement membranes. So far the genomic organization of the dystroglycan gene *DAG1* has not been completely investigated. Here we report the cloning and sequencing of 162 kb of dog genomic DNA containing the complete ~71-kb canine *DAG1* gene, which consists of three exons, with the translation start codon located in exon 2. Its 2679-nucleotide ORF encodes a polypeptide of 892 amino acids, which is highly similar to human, rabbit, and bovine orthologs. To further characterize the dog *DAG1* gene we determined the transcription start site and several naturally occurring polymorphisms, which partially result in amino acid substitutions of the dystroglycan protein. The dog *DAG1* gene was assigned to chromosome 20q15.1–q15.2 by FISH analysis. The analysis of the entire reported sequence revealed that the genes for aminomethyltransferase (*AMT*), bassoon (*BSN*), *TCTA* (T-cell leukemia translocation-associated) gene, and an as yet uncharacterized protein are located very close to the *DAG1* gene. Therefore, this study defines a novel syntenic region among dog chromosome 20q15, human chromosome 3p21, and murine chromosome 9F.

[The sequence data described in this paper have been submitted to the EMBL nucleotide database under accession no. AJ012166.]

Dystroglycan is an important part of the skeletal muscle dystrophin-associated glycoprotein complex (DGC) (Durbeej et al. 1998; Hemler 1999). The DGC is thought to be essential for mediating mechanical forces between the actin cytoskeleton and the extracellular matrix of a contracting muscle. In the DGC dystroglycan forms a membrane-spanning link between the intracellular dystrophin and the extracellular laminin  $\alpha$ 2 chain. Besides its structural function in skeletal muscle, dystroglycan has also signaling functions and is found in many other tissues, especially the central and peripheral nervous systems, the retina, and epithelial cells. Absence of dystroglycan in gene-targeted mice is lethal during embryonic development because Reichert's membrane, an early basement membrane, is not properly formed (Williamson et al. 1997). Subsequent studies confirmed that dystroglycan is essential for the correct formation of basement membranes (Henry and Campbell 1998). The protein received additional attention as it serves as a receptor for viral and bacterial pathogens (Cao et al. 1998; Rambukkana et al. 1998).

Dystroglycan consists of two subunits, termed  $\alpha$ -

and  $\beta$ -dystroglycan. The two subunits are encoded by a single gene and arise through post-translational cleavage of a precursor polypeptide. The predicted molecular mass of the  $\alpha$ -subunit is 72 kD; however due to tissue-specific glycosylation, isolated  $\alpha$ -dystroglycan has a molecular mass between 120 and 156 kD.  $\alpha$ -Dystroglycan is secreted and binds to laminin.  $\beta$ -Dystroglycan has a molecular mass of 43 kD and contains a single membrane-spanning domain. Its intracellular part binds to the carboxyl terminus of dystrophin, whereas its extracellular domain interacts with  $\alpha$ -dystroglycan.

The dystroglycan gene *DAG1* has not yet been characterized completely. Highly homologous cDNA sequences have been reported from man (Ibraghimov-Beskrovnaya et al. 1993), mouse (Brancaccio et al. 1994; Gorecki et al. 1994; Yotsomoto et al. 1996), rabbit (Ibraghimov-Beskrovnaya et al. 1992), and cattle. The human *DAG1* gene also has been partially cloned; it was found that the coding portion of the gene is located on two exons, which reside on chromosome 3p21 (Ibraghimov-Beskrovnaya et al. 1993).

To elucidate the complete structure of the *DAG1* gene, we cloned and sequenced the dog *DAG1* gene with extensive flanking regions. The analysis of the reported sequence revealed that several other genes are in close vicinity to the *DAG1* gene in the dog genome.

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In addition to the genomic sequence of the *DAG1* gene, we determined its chromosomal localization, its transcriptional start site, and variations within the *DAG1* coding sequence in different individuals.

## RESULTS AND DISCUSSION

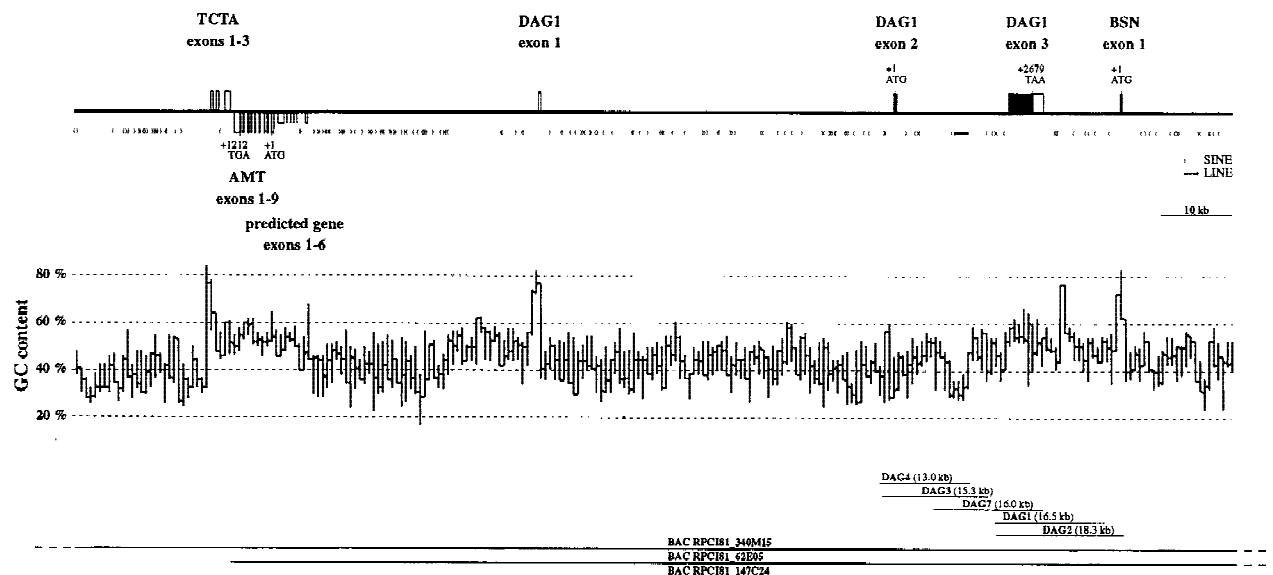
### Cloning of the Dog *DAG1* Gene

To isolate the *DAG1* gene from dog we screened a recombinant phage library with radioactively labeled cDNA probes derived from the two previously reported exons of the *DAG1* gene. This resulted in the isolation of five overlapping clones spanning ~35 kb of genomic DNA. Sequence analysis of this 35-kb contig revealed the presence of two *DAG1* exons with the entire ORF of the gene. However, comparison with the human cDNA sequence revealed that the exon farthest upstream on this contig lacked the 5' end of the cDNA and started with a splice acceptor sequence. To clone the presumably missing authentic first exon of the *DAG1* gene we obtained a human cDNA clone containing the missing 5' end and screened a dog BAC library. This led to the isolation of three overlapping BAC clones spanning ~200 kb of dog genomic sequence. A 162,073-bp fragment from the longest of these BAC clones, designated BAC RPCI81\_340M15 was completely sequenced. This sequence contained the complete dog *DAG1* gene, as well as several flanking genes on both sides of the *DAG1* gene. The positions of the isolated clones and genes are illustrated in Figure 1.

### Analysis of the Genomic Structure of the Dog *DAG1* Gene

Exon/intron boundaries of the canine *DAG1* gene were determined by comparison of the dog genomic DNA sequence with the human cDNA sequence. In addition, a dog cDNA fragment containing the junction between the first two exons was generated by RT-PCR, sequenced, and used for comparison with the genomic sequence. From these analyses it became clear that the dog *DAG1* gene consists of three exons separated by two introns. The gene spans ~71 kb, of which 48.8 kb is contained in the first intron. All splice donor/splice acceptor sites conform to the GT/AG rule. The exon/intron junctions in the protein coding region of the *DAG1* gene are well conserved between human and dog. However, at the end of the first exon the dog cDNA lacks ~120 bp compared to the human cDNA sequence. The additional human cDNA sequence shows high homology to the beginning of the first intron of the dog genomic sequence. This is probably due to the use of a different splice donor site in humans than in dogs. The above-mentioned RT-PCR did not show any additional bands representing alternatively spliced transcripts, which could also have explained the discrepancy between human and canine cDNA sequence. The DNA sequences at the exon/intron boundaries are summarized in Table 1.

The transcription start site of the dog *DAG1* gene was determined by primer extension analysis with two different primers located in the first and second exon,



**Figure 1** Genomic organization of the dog *DAG1* locus. Exons of the genes in the analyzed region are shown as boxes. Boxes above the solid line represent exons in sense orientation; boxes below the solid line indicate exons in antisense orientation. ORFs, which are translated into proteins of known function, are indicated by numbering and the respective exons are shown as solid boxes. Untranslated exons, or exons where the translation product is not characterized, are shown as open boxes. The positions and orientations of SINE and LINE repetitive elements are indicated by arrows. (Middle) GC content. A 300-bp window size was used for the calculation of the GC content. (Bottom) The position of the isolated phage and BAC clones.

**Table 1.** Exon–Intron Junctions of the Dog *DAG1* Gene

No.	Exon (bp)	Splice donor	Intron (bp)	Splice acceptor
				– 228 ...gcgtcgacgGACTG
1	121	– 108 GCCCGgtacgtgctc...	48842	– 107 ...tccaaaccaGCTCA
2	383	276 GTC AATgtgagactgg...	16535	277 ...tctcttttaG GTG TCA Val Lys Val Ser
3	>4455	4731 AAAATAAACAATTCCA...		

Transcribed sequences are shown in uppercase letters, and nontranscribed regions in lowercase letters. Untranslated regions are shown in italics. The conserved GT/AG exon/intron junctions are shown in boldface type. For the first exon the transcription start site is shown instead of a splice acceptor; for the last exon the first of two closely associated polyadenylation signals is shown in bold italics instead of a splice donor. Amino acid residues are indicated with respect to each boundary. Numbers refer to the corresponding positions in the *DAG1* cDNA starting with +1 at the adenosine of the translation initiation codon.

respectively (Fig. 2). The longest extension product of both primers corresponded to the same G residue 228 nucleotides upstream from the start codon of the dog cDNA sequence. The region immediately downstream

of the transcription start site is well conserved between human and dog. The reported human cDNA sequence starts only two nucleotides downstream of the dog transcription start site, therefore supporting the results of the primer extension analyses.

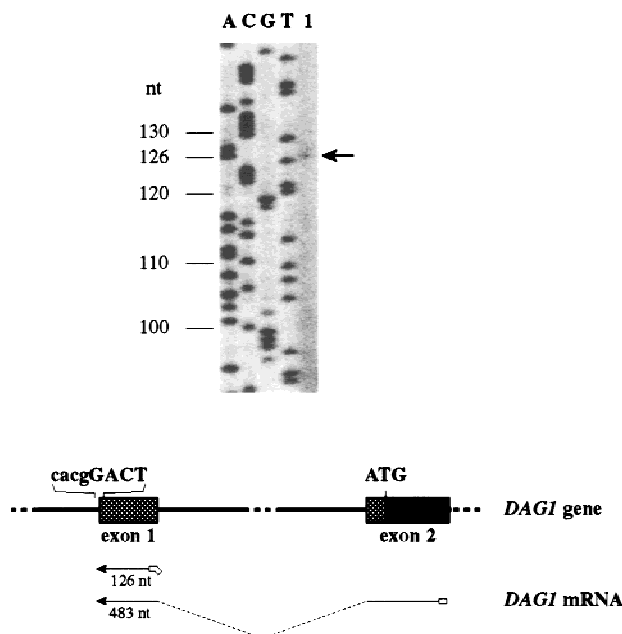
The promoter of the dog *DAG1* gene is GC-rich with a GC-content of 78.7% in the 300 nucleotides preceding the transcription start site (see also Fig. 1). Like other GC-rich promoters it does not contain a TATA box or a CAAT box.

#### Analysis of the Dog *DAG1* cDNA Sequence

The dog *DAG1* cDNA sequence is 4.5 kb long, with an ORF of 1679 nucleotides (including the stop codon) and a 5' UTR of 228 nucleotides. At the 3' end two canonical polyadenylation signals, AATAAA, are found within 350 bp similar to the human *DAG1* gene. Depending on which polyadenylation signal is used this would result in 3' UTRs of 2.1 and 2.4 kb, respectively. Upstream from the main ORF is a very short ORF encoding for only seven amino acids. A similar upstream ORF is also present in the human *DAG1* cDNA sequence. According to the criteria of Kozak (1996), such an upstream ORF is compatible with translation of a second ORF from the same transcript, although reduced protein yields are to be expected. The translation product starting at the second ATG is 892 amino acids long and exhibits 94%, 92%, and 91% identity to the corresponding human, rabbit, and bovine dystroglycan precursor proteins. The dog protein sequence lacks a stretch of three amino acids shortly after the signal peptide, which is present in all of the other species but otherwise contains no insertions or deletions.

#### Analysis of Polymorphisms Within the *DAG1* Coding Sequence

To search for different *DAG1* alleles within the dog



**Figure 2** Determination of the transcription start site of the *DAG1* gene. (Top) A detail of the sequencing gel with the DNA marker sequence (lanes A, C, G, T) and the product of the primer extension reaction of primer exon1\_Ext (lane 7) is shown. Sizes are indicated on the left side of the gel. The size of the extension product is actually 5 nucleotides longer than the size of the first exon, as the primer contained a *RsaI* tag at its 5' end for cloning purposes. (Bottom) The diagram outlines the experimental strategy. Primers exon1\_Ext and exon2\_Ext (open boxes) were annealed to total RNA and elongated as described in Methods. The thin dotted line indicates the spliced first intron. Important DNA sequence elements (transcription start site, ATG start codon) are shown. Crosshatched boxes within exons represent the 5' UTR.

population we isolated genomic DNA from four different dogs and amplified the three *DAG1* exons with their flanking sequences. To amplify the complete 4.5-kb third exon, five overlapping PCR products from this exon were generated. The PCR-amplified exons from the four dogs were directly sequenced and the sequences compared to each other. A total of eight polymorphic sites within the dog *DAG1* cDNA sequence were identified and are summarized in Table 2. Three of these eight polymorphisms led to amino acid exchanges. All of these amino acid exchanges are clustered in a relatively short region near the amino terminus of the protein. To verify that the protein-changing mutations are not caused by PCR artifacts, the amplification and sequencing of these sites was repeated with identical results.

### Analysis of the Genomic Organization of *DAG1* and Flanking Genes

Comparisons of the entire 162,073 bp of the reported sequence with the nucleotide database using the BLAST program revealed the presence of four other putative genes flanking the *DAG1* gene (Fig. 1). Upstream from *DAG1* on the same DNA strand as *DAG1*, the dog homolog of the human T-cell leukemia translocation-associated (*TCTA*) gene is located. This gene was first identified at the site of a t(1;3) translocation commonly occurring in T-cell acute lymphoblastic leukemia (Aplan et al. 1995). In humans it contains a short ORF that encodes a protein of 103 amino acids without homology to any other known protein. In the canine *TCTA* gene the corresponding ORF cannot be translated because an upstream ATG in a different reading frame is present, which is followed by a stop codon after only a few codons. Therefore, the dog *TCTA* seems to be a pseudogene.

Immediately downstream of the *TCTA* gene but on the complementary DNA strand, the gene for aminomethyltransferase *AMT* is located. *AMT*, or glycine cleavage system T-protein, is a mitochondrial enzyme

involved in glycine metabolism (Nanao et al. 1994). The gene consists of nine exons and encodes a protein with 91% identity to the human *AMT*.

Adjacent to the *AMT* gene, the program GeneMark of the EBI online tools predicted another protein coding region. Sequence comparisons revealed homologies to several ESTs but to no known genes. Based on the homology to human ESTs with GenBank/EMBL/DBJ accession numbers H86023 and AA701640, a putative gene with six exons could be localized.

Downstream of the *DAG1* gene, again on the same strand as *DAG1*, the gene for the brain-specific synapse-associated protein bassoon (*BSN*) follows. Bassoon has been identified as a component of the synaptic cytoskeleton (tom Dieck et al. 1998; Winter et al. 1999). The human gene is >100 kb in size with a large first intron. In the reported dog sequence there is only the first exon of *BSN* present, which is to be expected when one assumes that the first intron of the dog *BSN* is similar in size to the first intron of the human gene.

The GC content of the reported sequence correlates extremely well with the presence of genes. Three CpG islands with very high scores were detected using the program CPG of the EBI at the 5' ends of the genes *TCTA*, *DAG1*, and *BSN*. Two more CpG islands with moderately high scores were detected at the 5' end of the novel predicted gene and at the 3' end of the *DAG1* gene. The GC content of the reported sequence is 46%. This relatively low value is due to the presence of the polyadenosine tracts of 153 copies of a dog tRNA-SINE element (Das et al. 1998; Bentolila et al. 1999). In the second *DAG1* intron there is also an incomplete LINE-1 element with a relatively low GC content. The distribution of the GC content and repetitive elements along the sequence are illustrated in Figure 1.

### Chromosomal Localization of the Sequenced Contig

To determine the chromosomal location of the dog *DAG1* gene as well as the other genes in the reported sequence, we used the genomic phage clone DAG2 as

**Table 2.** Polymorphisms in the Dog *DAG1* Sequence

Location of polymorphic site (exon)	Nucleotide position <sup>a</sup>	cDNA position <sup>b</sup>	Nucleotide polymorphism	Amino acid substitution
2	113708	+99	A → G	silent
3	130489	+345	G → C	E <sup>115</sup> → D <sup>115</sup>
3	130760	+616	G → A	D <sup>206</sup> → N <sup>206</sup>
3	130817	+673	G → A	V <sup>225</sup> → M <sup>225</sup>
3	131869	+1725	T → C	silent
3	132229	+2085	G → A	silent
3	132562	+2418	G → C	silent
3 (3' UTR)	134574	+4430	G → T	

<sup>a</sup>Numbering refers to GenBank accession no. AJ012166.

<sup>b</sup>+1 corresponds to the adenosine of the start codon ATG of the *DAG1* cDNA.

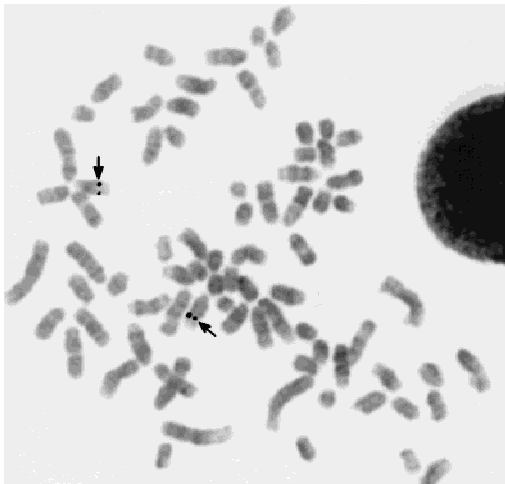
probe in FISH analysis. As shown in Figure 3, a hybridization signal was detected on CFA 20q15.1–q15.2. All of the genes described (*AMT*, *BSN*, *DAG1*, *TCTA*) have been localized in human to HSA 3p21, which is a region within the evolutionarily conserved chromosome segment shared with CFA 20q11–15.2 (Breen et al. 1999a). Therefore, these four genes seem to form a syntenic region between human and dog, and the information about the relative position and orientation of these genes in the dog genome might promote human gene mapping efforts as well. In mice, *Bsn* and *Dag1* have been mapped to chromosome 9F. Assuming synteny between dog and mouse in this region, one would also expect to find the murine *Amt* and *Tcta* genes on chromosome 9F.

In conclusion, for the first time, our results provide the complete genomic structure of the *DAG1* gene containing three exons. The reported 162 kb of DNA sequence adds significantly to our knowledge of the dog genome, as there are currently only four other database entries with >10 kb of dog DNA sequence available, of which the 16-kb mitochondrial sequence is the longest. As predicted, the localization of a total of five genes on the sequenced contig defines a syntenic region between dog chromosome 20q15.1–q15.2, human chromosome 3p21, and murine chromosome 9F.

## METHODS

### DNA Library Screening

A dog genomic DNA library constructed in  $\lambda$ FIX II (Stratagene, Heidelberg, Germany) was screened according to standard



**Figure 3** Chromosomal assignment of the dog *DAG1* gene by FISH analysis. The biotinylated genomic phage clone DAG2 containing the 3' end of the dog *DAG1* gene was hybridized to metaphase chromosomes of a normal dog. Double signals are visible on both CFA 20q15.1–q15.2, indicated by arrows. The chromosomes are counterstained with propidium iodide. They were subsequently identified by DAPI staining.

protocols (Ausubel et al. 1995). Two  $^{32}$ P-labeled DNA fragments were used simultaneously as probes: the insert of the IMAGE clone 267199 containing 1.2 kb of exon 3 of the human *DAG1* gene, and a 270-bp human DNA fragment of exon 2 that was PCR-amplified on human genomic DNA. The IMAGE clone was provided by the Resource Center of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics (RZPD, IMAGp998C08586). To isolate the missing 5' end of the dog *DAG1* gene, the dog BAC library RPCI81 (Osoegawa et al. 1998) was also screened. High-density BAC filters were probed according to the RPCI protocols (<http://bacpac.med.buffalo.edu/>) with a  $^{32}$ P-labeled 670-bp *EcoRI*–*EcoRV* fragment of the human cDNA clone HD-4 (Ibrahimov-Beskrovnyaya et al. 1993) corresponding to exons 1 and 2 of the *DAG1* gene.

### DNA Sequence Analysis

Isolated phage or BAC DNA was restricted with appropriate restriction enzymes, and the resulting fragments were subcloned into the polylinker of pGEM-4Z (Promega, Mannheim, Germany). Recombinant plasmid DNA was sequenced with the thermosequencing kit (Amersham–Pharmacia, Freiburg, Germany) and a LICOR 4000L automated sequencer. Within extremely GC-rich regions, some fragments were sequenced manually with the T7 sequencing kit and  $\alpha$ - $^{35}$ S-labeled thio-dATP as label. The plasmid clones were subcloned further with different restriction enzymes. After sequencing a collection of plasmid subclones, existing gaps were closed by a primer walking strategy until both strands were completely sequenced. Sequence data were analyzed with Sequencher 3.1.1 (GeneCodes, Ann Arbor, MI) and MacDNASIS 3.6 (Hitachi, Hess, Oldendorf, Germany). Further analyses were performed with the online tools of the European Bioinformatics Institute (<http://www.ebi.ac.uk/>).

### PCR Amplification and Sequencing of *DAG1* Exons

PCR was performed in 100- $\mu$ l reactions containing 100 ng of genomic dog DNA, 200  $\mu$ M dNTPs, 1  $\mu$ M each primer, and 2.5 units of *Taq* polymerase in the reaction buffer supplied by the manufacturer (Qiagen, Hilden, Germany). After a 5-min initial denaturation at 94°C, 32 cycles of 1 min at 94°C, 1 min at the annealing temperature of the specific primer pair, and 1.5 min at 72°C were performed in a Hybaid Omnigene thermocycler (MWG AG Biotech, Ebersberg, Germany). Primers were designed to amplify the complete coding sequence, all exon/intron junctions, and 5'- and 3' UTRs. Because the last exon could not be amplified in a single PCR, it was divided into four overlapping fragments (a,b,d,e). Fragment b was difficult to sequence; therefore, a smaller subfragment of b, termed c, was also amplified for sequence analysis. Primer sequences and annealing temperatures are given in Table 3. The amplified products were purified by electrophoresis through 1% agarose gels and sequenced directly using IRD800-labeled internal primers.

### Primer Extension Analysis

Total RNA from dog skeletal muscle was prepared with Trizol (Life Technologies, Karlsruhe, Germany). Ten picomoles of IRD800-labeled primer were annealed to 2  $\mu$ g of total RNA and reverse transcribed in a 20- $\mu$ l reaction volume with 200 units of MMLV reverse transcriptase (Life Technologies, Karl-

**Table 3. Primer Sequences**

Primer (exon) <sup>a</sup>	Sequence (5'-3')	Length	T <sub>m</sub> (°C)	Product (bp)
1, F	AGCCTGGGTCGGTTGTACG	19		
1, R	AGCAGGTGTTCCCTCGGGAG	19	60	684
2, F	ATTGTGTCTTTCATGACTTC	19		
2, R	GAATTCCTCCCTCATTAC	18	50	953
3a, F	AGCTGTTGTGATCATTGTGTC	19		
3a, R	GAGTTGTGGTGGTTGAAG	18	54	1122
3b, F	TCCCACGACTACCACCAAG	19		
3b, R	ACCGGTCTCCACAGACAAC	19	55	1507
3c, F	GCCGGCACCTCCAGTTTATCC	21		
3c, R	GTCAATGTTCTTGGGACGGGAGC	22	68	514
3d, F	CCTTTACGGCCCCCATGGAG	20		
3d, R	CGCCACCAGACTTTCCACAG	20	65	1296
3e, F	TCCCAACAGTCCACAGCCC	19		
3e, R	TCCCCTCTTCCCTCTGTCAACA	21	61	1402

<sup>a</sup>(F) Forward; (R) reverse.

sruhe, Germany). After a 50-min incubation at 44°C and 10 min at 75°C, 5 µg of RNase A was added and the reaction was maintained at 37°C for 30 min. The synthesized cDNA was precipitated by addition of 4 µl of 0.1 M spermine and subsequent centrifugation for 40 min at 22,000 rpm and 4°C. The cDNA pellet was dissolved in 2.5 µl of formamide loading dye and denatured at 70 °C for 5 min. One microliter was loaded on an automated sequencer together with a sequencing reaction as size standard. The primer sequences for the experiment were exon1\_Ext, 5'-CGTACCGGGCGCTGAGAG-3'; exon2\_Ext, 5'-GGTCCCTACCCTCTGTAC-3'.

## FISH

A total of 150 ng of the dog *DAG1* gene phage DAG2 was labeled with digoxigenin-11-dUTP and hybridized to dog metaphase preparations as described by Breen et al. (1999b). The chromosomes were counterstained in DAPI prior to being mounted with Vectashield (Vector Laboratories, Burlingame, CA) and sealed with a coverslip. Images were captured using an Axiophot fluorescence microscope (Zeiss, Jena, Germany), equipped with an FITC/Texas Red/DAPI filter set and a cooled KAF1400 CCD camera (Photometrics, Munich, Germany), both driven by SmartCapture software (Vysis Inc., Downers Grove, IL). Hybridization signals were scored from 30 metaphase preparations using image-enhanced DAPI banding and assigned according to the DAPI-banded ideogram of Breen et al. (1999c).

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