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Breakpoint mapping positions the callipyge gene within a 450-kilobase chromosome segment containing the *DLK1* and *GTL2* genes

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We have previously mapped the ovine callipyge (*CLPG*) gene, causing a muscular hypertrophy with parent-of-origin-dependent expression referred to as polar overdominance, to a 4.6-cM chromosome interval on distal OAR18q flanked by microsatellites *IDVGA30* and *OY3* (Cockett et al. 1996; Shay et al. 2000). BAC contigs spanning this interval were constructed by using bovine (Shay et al. 2000) and subsequently ovine (Segers et al. 2000) reagents. We herein report the isolation of eight novel microsatellite markers from these contigs, yielding a marker density of one microsatellite per 68 kilobases and the use of these novel markers to position the *CLPG* gene by breakpoint analysis within a ≈ 450-kilobase chromosome segment.

Five BAC clones jointly spanning most of the *IDVGA30–OY3* interval were selected from the ovine BAC contig (BACs: 724D11, 239G7, 218E10, 497C1, 265F11). BAC DNA was digested to completion with three four-cutters yielding blunt-ended restriction fragments (*Alu*I, *Hae*III, and *Rsa*I) used either separately or combined. Restriction fragments containing microsatellites were detected by standard Southern blotting and hybridization with a $(CA)_{10}$ probe. Fragments measuring between 250 and 500 base pairs were subcloned and their inserts sequenced. Using this procedure, we identified eight distinct microsatellite sequences. One of these, obtained from BAC 724D11, proved to be identical to *IDVGA30;* the seven others were novel microsatellites. Primers were designed to amplify the corresponding microsatellites from genomic DNA. All seven primer sets yielded specific amplification products. Six of the seven novel microsatellites proved to be polymorphic in the callipyge pedigree and were retained for further analysis (*MULGE1-6*). The corresponding primer pairs are given in Table 1. Two markers that were isolated from bovine BACs with the same procedure (*BULGE33* and *BULGE37*) and that yielded polymorphic amplification products in sheep were added to the set of six ovine microsatellites (Table 1). With the previously available *IDVGA30*, *BMS1561*, and *OY3* markers, this yielded 11 microsatellites covering a 755-kb chromosome segment or one polymorphic microsatellite per 68 kb, on average. The precise position of these 11 microsatellites within the ovine BAC contig was determined by STS content mapping after genotyping all available ovine BACs. Figure 1A reports their corresponding map positions. Estimated distance between adjacent markers in this interval ranges from 0 to 170 kb.

To refine the map position of the *CLPG* gene within the *IDVGA30–OY3* interval, we identified chromosomes in our pedigree material that (i) recombined within that marker interval, (ii) were transmitted by a heterozygous CLPG/+ parent, and (iii) whose genotype at the *CLPG* locus could be inferred either from the phenotype of the corresponding individual or its progeny. As defined by Shay et al. (2000), assuming inheritance of the callipyge phenotype according to the polar overdominance model, three types of chromosomes provide unambiguous information about the location of the *CLPG* locus on the basis of the offspring's phenotype: (i) recombinant paternal chromosomes from offspring of (CLPG/+) ram × (+/+) ewe matings, (ii) recombinant paternal chromosomes from offspring of (CLPG/+) ram × (CLPG/+) ewe matings having inherited a non recombinant + chromosome from their dam, and (iii) recombinant maternal chromosomes from offspring of (CLPG/+) ram × (CLPG/+) ewe matings having inherited a nonrecombinant CLPG chromosome from their sire. Twenty-three such chromosomes could be identified in our pedigree material. Two additional recombinant chromosomes whose genotype at the *CLPG* locus could be inferred from its behavior in subsequent generations were identified, yielding a recombinant panel of 25 informative chromosomes. As illustrated in Fig. 2, these two "progeny-tested" chromosomes were recombinant paternal chromosomes from offspring of (CLPG/+) ram × (CLPG/+) ewe matings, having inherited a nonrecombinant CLPG chromosome from their dam.

The corresponding 25 individuals and their parents were genotyped for the newly developed microsatellites. To establish the marker linkage phase of the informative parent (which, for the corresponding 25 individuals, was always the ram), we genotyped two additional non-recombinant sibs that had inherited the alternative homologs (CLPG and +) from their sire. Analysis of the corresponding paternal chromosomes confirmed the complete linkage disequilibrium previously observed between the *CLPG* allele and the microsatellite markers within the *OY5–IDVGA30* interval (Shay et al. 2000): for all newly developed microsatellites, a unique allele proved to be systematically associated with all CLPG chromosomes in the parental generation (data not shown).

As previously described (Shay et al. 2000), examination of the genotypes of the recombinant chromosomes allows one to distinguish two types of chromosome segments: (i) the "excluded" segments, which cannot contain the *CLPG* locus (because the corresponding marker genotypes are not compatible with the phenotype of the individual), and (ii) the "included" segments, which can contain the *CLPG* locus (either because the marker genotypes are in agreement with the phenotype or because they are not informative). Figure 1B shows the recombinant chromosomes sorted according to the segment from which the *CLPG* gene could be excluded. Figure 1C combines this information by showing the number of recombinant chromosomes that exclude a specific chromosome interval. It can be seen from this figure that the exclusion rate is lowest in the *MULGE4–OY3* interval, therefore pointing towards this ≈ 450-kb interval as the most likely position of the *CLPG* gene.

Note that the breakpoints corresponding to the two "progeny-tested" chromosomes flank the *MULGE5–OY3* interval nested within the previously defined *MULGE4–OY3* interval. Because the inference of the CLPG genotype of these progeny-tested chromosomes is based on the phenotype of multiple (9) descendants rather than that of a single offspring, the corresponding breakpoints can

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Table 1. Primer pairs for microsatellite amplification.

Marker	UP-Primer (5′-3′)	DN-primer (5′-3′)
MULGE1	GGGTGCTCCCTAGTCTCGAACATTC	CTTCAGAAGCAGGACCGCTTGG
MULGE2	AAGACAAATACCAGGCATGTGACC	ACATGCCCTGGTATTTGTCACAG
MULGE3	CTTAAAGGCAGAGTGGTGAGCAC	GGAACAGTGAGGAGTCTGTGTGAC
MULGE4	GCAACCCTTCTGATGTCATGAACC	AAAAGCACAACTCCCCTCAAATCC
MULGE5	CATCACACTCACCTCATTTGTTTG	GAGTAGCAAATTTACCCTCCAGTC
MULGE6	AGCCTTCCAGATTCAATAAAGC	GTGTGAAGAGGAAAACATAAGACG
BULGE33	CCAAGGGCCTCGGCGGTCGTGA	ATTCCCTCTCCTCTCGCCTCCCA
BULGE37	GTGAGGATGTTACAGAATGATGAG	CTCATCATTCTGTAACATCCTCAC

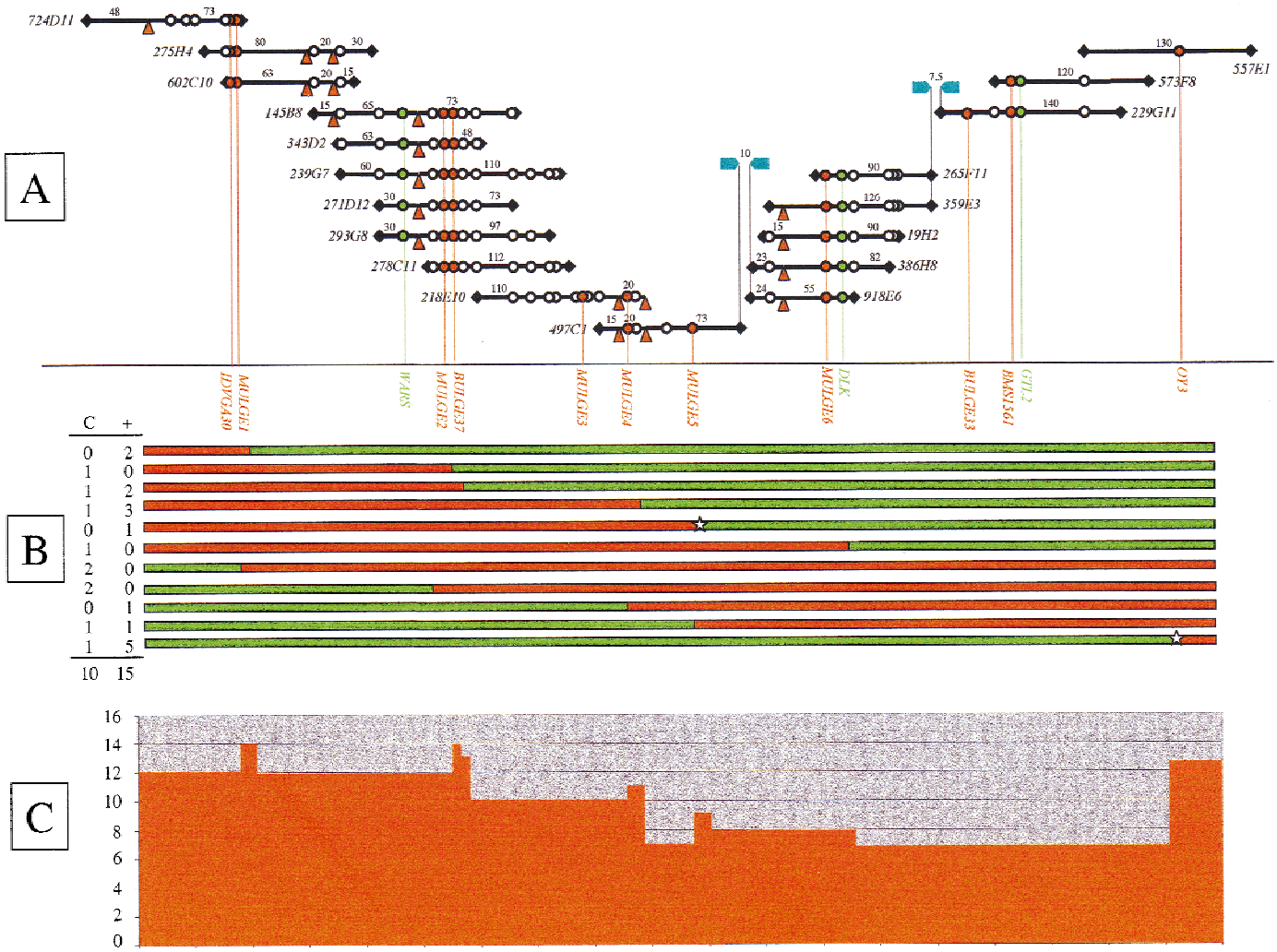

Fig. 1. (A) Ovine BAC contig spanning the *CLPG* gene. White dots correspond to the positions of sequence tagged sites (STS) used for contig construction and described in detail in Segers et al. (2000). Green dots correspond to the positions of three genes (*WARS, DLK1,* and *GTL2*); red dots correspond to the positions of the three previously available microsatellites (*IDVGA30, BMS1561,* and *OY3*), and the eight newly developed markers (*MULGE1–6; BULGE33, BULGE37*). The blue arrows flank gaps in the contig bridged by long-range PCR (Segers et al. 2000). The red triangles correspond to *Not*I sites, and the numbers above the BACs report the size of the corresponding *Not*I restriction fragments or long-range PCR products in kilobases. **(B)** Recombinant chromosomes in the *IDVGA30–OY3* interval sorted according to the chromosome segment from which the *CLPG* gene could be excluded and shown in red. The number of CLPG and "+" chromosomes with a given breakpoint are reported on the left. Positions of progeny-tested breakpoints are marked by asterisks. **(C)** Distribution along the BAC contig of the number of recombinant chromosomes excluding the corresponding marker interval. These numbers are obtained by summing, for each interval, the number of red chromosomes in (B).

be regarded as highly reliable. This strongly suggests, therefore, that the *CLPG* locus is located within this *MULGE5–OY3* interval.

Assuming that the location of the *CLPG* gene within the *MULGE5–OY3* interval is indeed correct, a number of observations remain puzzling. Indeed, it is noteworthy that this entire interval is excluded by a minimum of 7 out of the 25 informative chromosomes (= 28%). This value has to be compared with an exclusion rate less than 1% (3/364) when considering only individuals that had inherited a nonrecombinant chromosome (interval *GMBT16–TGLA122;* Shay et al. 2000). In other words, this observation seems to indicate that discrepancies between phenotype and genotype would be higher when individuals inherit a recombinant versus a non-recombinant chromosome. This could reflect the fact that the CLPG chromosomes differ from the "+" chromo-

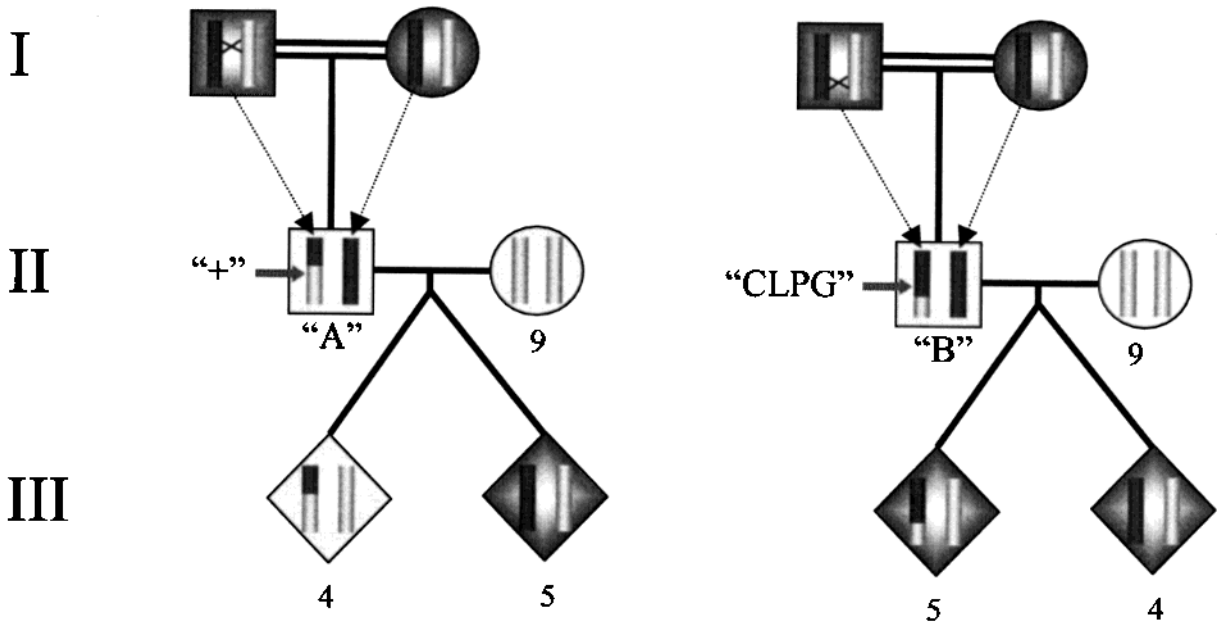

Fig. 2. Schematic representation of the two progeny-tested recombinant chromosomes. Individuals with callipyge phenotype are shown in gray; individuals with normal phenotype in white. CLPG chromosomes are shown in black; "+" chromosomes in white. The two informative offspring result from (CLPG/+) × (CLPG/+) matings. The number of individuals having a given phenotype/genotype combination are indicated. **(A)** Offspring A has inherited a nonrecombinant CLPG chromosome from its dam, while a recombinant chromosome from the sire. Under the polar overdominance model, the genotype (CLPG or +) of the recombinant chromosome cannot be inferred from individual A's phenotype. The fact that, in generation III, half the offspring (those having inherited the grand paternal homolog from their sire) are of normal phenotype indicates that recombinant chromosome of individual A was of "+" genotype. This allows exclusion of the black segment of that chromosome. **(B)** Offspring B has also inherited a nonrecombinant CLPG chromosome from its dam, and a recombinant chromosome from the sire. Under the polar overdominance model, the genotype (CLPG or +) of the recombinant chromosome can, therefore, not be inferred from individual B's phenotype. The fact that, in generation III, all the offspring have the callipyge phenotype irrespective of the paternal homolog inherited indicates that the recombinant chromosome of individual B was of "CLPG" genotype. This allows exclusion of the white segment of that chromosome.

somes at two or more tightly linked mutations. Recombinations between these mutations would then generate "pseudo +" chromosomes. This hypothesis could account for the slight excess of "+" (15) over CLPG (10) chromosomes observed in the recombinant panel (see Fig. 1B). However, this hypothesis also predicts that the location of the *CLPG* gene should then be unambiguous when considering CLPG chromosomes only. This is not the case (see Fig. 1B): the entire *IDVGA30–OY3* interval can be excluded whether considering CLPG or "+" chromosomes separately. The biological significance of this observation is being examined.

It is important to notice that the imprinted *DLK1* and *GTL2* genes (Schmidt et al. 2000; Takada et al. 2000; Charlier et al. unpublished data), previously shown to map within the contigs spanning the IDVGA30–OY3 interval (Shay et al. 2000; Segers et al. 2000), are also mapping to the the *MULGE5–OY3* interval. This strengthens the evidence in favor of the *CLPG* gene(s) being located within a novel evolutionary conserved imprinted domain.

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