

RAPID COMMUNICATION

# The Gene Encoding the Mouse Homologue of the Human Osteoclast-Specific 116-kDa V-ATPase Subunit Bears a Deletion in Osteosclerotic (*oc/oc*) Mutants

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Osteosclerosis (oc) is an autosomal recessive lethal mutation that impairs bone resorption by osteoclasts, and induces a general increase of bone density in affected mice. Genetic mapping of the oc mutation was used as a backbone in a positional cloning approach in the pericentromeric region of mouse chromosome 19. Perfect cosegregation of the osteopetrotic phenotype with polymorphic markers enabled the construction of a sequence-ready bacterial artificial chromosome (BAC) contig of this region. Genomic sequencing of a 200-kb area revealed the presence of the mouse homologue to the human gene encoding the osteoclast-specific 116-kDa subunit of the vacuolar proton pump. This gene was located recently on human 11q13, a genomic region conserved with proximal mouse chromosome 19. Sequencing of the 5' end of the gene in oc/oc mice showed a 1.6-kb deletion, including the translation start site, which impairs genuine transcription of this subunit. The inactivation of this osteoclast-specific vacuolar proton ATPase subunit could be responsible for the lack of this enzyme in the apical membranes of osteoclast cells in oc/oc mice, thereby preventing the resorption function of these cells, which leads to the osteopetrotic phenotype. (Bone 26:207-213; 2000) © 2000 by Elsevier Science Inc. All rights reserved.

**Key Words:** Osteopetrosis; oc mutant; V-ATPase; Physical mapping; Mouse chromosome 19.

# Introduction

Osteopetrosis, also known as marble bone disease, is a metabolic bone disorder that is characterized by a general increase of bone density resulting from a defect in osteoclast presence or function. Several genetically inherited forms of the disease have been described in humans. There are four spontaneous osteopetrotic mutations in mice that have been reported: *op/op* (osteopetrosis), *mi/mi* (microphtalmia), *gl/gl* (grey-lethal), and *oc/oc* (osteosclerosis). The osteoclast deficiency in *op/op* mice is due to a mutation in the coding region of the macrophage colony-stimu-

lating factor gene (M-CSF1),<sup>22</sup> and a defect in a gene coding for a helix-loop-helix transcription factor is responsible for the microphtalmia phenotype.<sup>13</sup> Currently, the genes associated with *oc* or *gl* mutations have not been identified.

Osteosclerosis (*oc*) is a mouse osteopetrotic mutation inherited as an autosomal lethal recessive trait that arose spontaneously in 1966 at the Jackson Laboratory in the C57BL/6J-*bf* strain,<sup>5</sup> and has been backcrossed over 20 times to the hybrid C57BL/6JIe × C3HeB/FeJLe-*a/a*  $F_1$ , in order to increase the survival time of the affected *oc/oc* animals. The *oc/oc* homozygote mice usually die around 3 weeks of age, and the mutation has been maintained in *oc/+* heterozygotes, which do not display any particular phenotype. Affected animals (*oc/oc*) exhibit the characteristic radiologic and histologic features of osteopetrosis, including a generalized increase in skeletal density and absence of marrow cavities easily detected by X-ray radiography (**Figure 1**).

Oc was originally mapped to mouse chromosome 19 (MMU19) with fairly loose precision by Lane.<sup>14</sup> In 1985, Marks et al.<sup>17</sup> gave a better characterization of the phenotype and anchored the mutation in the pericentromeric region of MMU19. However, in this initial genetic mapping effort, the closest phenotypic marker brachymorphic (bm) was located 30 cM telomeric of *oc*, and no close markers were shown to segregate with the osteopetrotic phenotype. We have undertaken a comparative mapping study of a syntenic area of the genome that has been conserved through evolution between human 11q13 and the pericentromeric region of mouse chromosome 19.4,8,21 Many genes involved in inherited human pathologies and localized on 11q13 have an homologous gene present on proximal MMU19 region and vice versa.<sup>20</sup> This gene conservation between mice and humans has been very helpful in designing new probes to refine the physical map. Moreover, it has been a source of candidate genes for positional cloning in both species. In our attempt to identify candidate genes for oc, we were able to exclude the Fos-related antigen 1 gene (fra-I), based on its segregation pattern and an allelism test,<sup>19</sup> as well as a putative transporter gene (Roct1),<sup>3</sup> based on physical mapping results (data not shown).

The positional cloning approach we followed consisted of four phases: defining the smallest candidate region based on proximal and distal meiotic recombination events, constructing a high-density physical map, sequencing the region of interest, and performing genomic database comparisons. In this report, we

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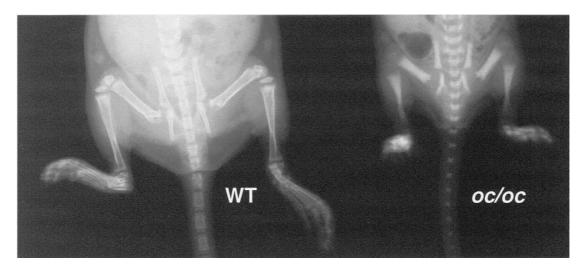


Figure 1. Radiographic images of a 14-day-old normal mouse (wt) and its osteosclerotic littermate (*oc/oc*). A general increase of bone density and disappearance of bone marrow space can be observed in the mutant.

present evidence showing a 1.6-kb genomic deletion in mouse osteosclerotic mutants (*oc/oc*) removing the translation start site in the gene homologous to the human OC 116-kDa osteoclast-specific vacuolar proton pump subunit.

### **Materials and Methods**

#### Mice

Two pairs of (C57BL/6J × C3HheB/FeJ) F1 oc/+ mice were initially obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in our central animal facility in accordance with the general guidelines edicted by the Direction des Services Vétérinaires. Heterozygotes animals (oc/+) were identified 3 weeks after birth by DNA genotyping (tail clipping) with a polymorphic marker (*D19Mit68*) cosegrating with the mutation. Animals suspected to be homozygous at the *oc* locus (smaller size than littermate, circling behavior, absence of or delayed incisors eruption) were systematically submitted to radiographic analysis for final phenotyping. *Mus spretus* mice (SEG imbred strain) were obtained from the Unité de Génétique des Mammifères (Institut Pasteur, Paris).

# Yeast Artificial Chromosome (YACs) and Bacterial Artificial Chromosome (BAC) Identification

We initially identified YACs from the candidate region by screening four different mouse libraries (Princeton Library, MIT/ Whitehead Institute Library, St Mary's Hospital Library, and Imperial Cancer Research Fund (ICRF) Library). The screenings were performed with the help of either the Princeton or Généthon screening facility. BACs were then identified with polymerase chain reaction (PCR)-based screening of pooled libraries (Research Genetics, Huntsville, AL) with various STS from the interval. STS were obtained, mainly from BAC end-sequencing, but also by taking advantage of the conservation between HSA11q13 and the pericentomeric region of MMU19.

#### BAC DNA Purification and BAC End-Sequencing

BAC DNAs were prepared using a Nucleobond PC kit from Macherey-Nagel (Hoerdt, France), according to the manufacturer's instructions. BAC end-sequencing reactions were performed with 5  $\mu$ g of template, using the Thermo Sequenase kit (Amersham, Les Ulis, France), and IRD700- or IRD800-labeled SP6 or T7 primers (MWG Biotech, Ebersberg, Germany). Sequencing reaction products were analyzed on a LI-COR Long ReadIR 4200 DNA sequencer (LI-COR Inc., Lincoln, NE)

#### Polymerase Chain Reaction

Polymerase chain reaction was performed using primers and annealing temperatures reported in **Table 1**. The 25-µL reaction volume contained 25 pmol of each primer, 3.13 pmol dNTPs, 1.5 mmol/L MgCl<sub>2</sub>, and 0.5 U of platinium Taq DNA polymerase (Life Technologies, Cergy Pontoise, France). After 2 min at 95°C, PCR was carried out for 35 cycles with the following steps: 94°C for 30 sec, annealing temperature for 30 sec, and 72°C for 30 sec. A final extension step at 72°C for 10 min concluded each reaction. Amplification was performed in a PTC-100 thermal cycler (MJ Research, Waltham, MA). Polymerase chain reaction products were analyzed by gel electrophoresis in 2% agarose gels.

#### MMUOC116 Positioning on the Physical Map

We used two primer pairs (D19 Car333 and D19Car319) for the positioning of MMUOC116, as illustrated in **Figure 3**. Each pair corresponded, respectively, to the 5' and to the 3' end of the mouse gene, and upstream and downstream primers were as follows: D19Car333, 5'-TAGCTTGAAGCAGATTGTACG-3' and 5'-CTCAACTTCGGCTTAGGATC-3'; D19Car319, 5'-CAGCTCTTTATTCCTGTCCC-3' and 5'-CTTCATGCACCA-AGCAATCC-3'.

# FISH

BAC DNA was labeled using nick-translation with biotin-14dATP (Life Technologies). The labeled probe (1  $\mu$ g/slide) was coprecipitated with 30  $\mu$ g of mouse Cot-1 DNA (Life Technologies), denatured for 5 min at 70°C in hybridization mixture (50% formamide, 2× SSC, 10% Dextran sulfate), and reannealed for 30–60 min at 37°C. The probe was then hybridized on denatured pretreated metaphase chromosomes from mouse SV22-CD cell line<sup>2</sup> overnight in a moist chamber at 37°C. The following steps were performed as described previously.<sup>8</sup>

Digcar5" $S'$ -GCT GGC TTT AGA CTG ATT TG-3' $S'$ -GCTDigcar0 $S'$ -CTC CTG AAG ATG ACA TT ACA-3' $S'$ -GCTDigcar14 $S'$ -GCT TCT CTG ATG ACA TT AAT G-3' $S'$ -GCTDigcar51 $S'$ -GCT TCT CTG ATG ACA TT AAT G-3' $S'$ -GCTDigcar51 $S'$ -GCT TCT CTG ATG ACA TT AAT G-3' $S'$ -GTGDigcar53 $S'$ -GTC TC ATG ACA GG GGA GG-3' $S'$ -AGADigcar55 $S'$ -TTC ATC ACC TCT AGG GGA GG-3' $S'$ -AGADigcar51 $S'$ -GTG GG GT TCC TTC ACA GG-3' $S'$ -GTGDigcar53 $S'$ -AGA AGG GTT TCC TTC ACA GG-3' $S'$ -GTGDigcar51 $S'$ -AGA GG GTT TTC ACA GG-3' $S'$ -GTGDigcar53 $S'$ -AGA AGG GTT TCC TTC AGA GG-3' $S'$ -GTGDigcar71 $S'$ -AGC CTC CAA GTC CTA GGA ATC-3' $S'$ -GTGDigcar73 $S'$ -AGC CTC CAA GGC TAG GG-3' $S'$ -GCADigcar73 $S'$ -AGC TTC AAG GAT GG CAA GG-3' $S'$ -GCADigcar73 $S'$ -AGC TTC AAG GAT GG GG-3' $S'$ -GCADigcar73 $S'$ -AGC TTC AAG GAT GG GG-3' $S'$ -GCADigcar73 $S'$ -AGC TTC AAG GAT GG GG-3' $S'$ -GCADigcar237 $S'$ -AGC TTC AAG GAT GG GG-3' $S'$ -GCADigcar239 $S'$ -AGC TTC AAG GAT GG GG-3' $S'$ -GCADigcar239 $S'$ -AGC TTG ATG GC TAA GAT C-3' $S'$ -GCADigcar237 $S'$ -AGC TTG ATG GAT GG GG-3' $S'$ -GCADigcar237 $S'$ -AGC TTG ATG GAT GG GG-3' $S'$ -GCADigcar239 $S'$ -AGC TTG ATG GAT GG GG GAS $S'$ -GCADigcar239 $S'$ -AGC TTG ATG GAT GAT GC-3' $S'$ -GCADigcar239 </th <th>CTT</th> <th></th> <th>(do) mont</th> <th>Kerence</th>	CTT		(do) mont	Kerence
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<ul> <li>S'-GAT CTC CTT TGC CGA TTA CA-3'</li> <li>S'-CTG TCT CTG ATG ACA TAT AAT G-3'</li> <li>S'-CTG TCT GCG GCA GTG AGG-3'</li> <li>S'-TTC ATC ACC TGT CAG ACA GG-3'</li> <li>S'-TTA ATG CCA GCA CTT GGG GA-3'</li> <li>S'-TTA ATG CCA GCA CTT GGG GA-3'</li> <li>S'-AGG CTT ACC AGG TTA CAG GC-3'</li> <li>S'-AGC CTC CAG CTC TTC ACA GC-3'</li> <li>S'-AGC CTC CAA CTC CTG GG AA-3'</li> <li>S'-AGC CTC CAA CTC CTG GG AA-3'</li> <li>S'-AGC CTC CAA CTC CTG GG GA-3'</li> <li>S'-AGC CTC CAA CTC CTG GG GA-3'</li> <li>S'-AGC CTC AAC TGC AGG GCA GG-3'</li> <li>S'-AGC TCT ATC CAC TGC AGG GG-3'</li> <li>S'-ACT AAC AGG GG GG AA ATC-3'</li> <li>S'-ACT AAC AGG GG GG GA-3'</li> <li>S'-ACT AAC AGG GG GG GA-3'</li> <li>S'-ACT AAC AGG GG GG GA-3'</li> <li>S'-ACT AAC AGG GG GG GC G-3'</li> <li>S'-ACT GG GG GG ATG ACC AGC C-3'</li> <li>S'-ACT GG GG GA ACT AGG GC G-3'</li> <li>S'-AGC TGG AAG ACT GG GC G-3'</li> <li>S'-AGC TGG CTT GG ATG ACC C-3'</li> <li>S'-GGT CTC TGG AAC ACC C-3'</li> <li>S'-GGT CTC AAT ACT CC-3'</li> <li>S'-GGT CTC AAT GAC ACC C-3'</li> <li>S'-GGT CTC AAT ACT CC-3'</li> <li>S'-GCT GGC CAA ATC AGA CC 3'</li> <li>S'-GCT AAT AGA CC 3'</li> <li>S'-GCT AAT AGA CC ATC C-3'</li> <li>S'-GGT CTC AAT AGT CC-3'</li> <li>S'-GCT AAT AGA CC ATC C-3'</li> <li>S'-GCT AAT AGA CC AGG CC AGG C-3'</li> <li>S'-GCT AAT AGA CC AGG CC AGG C-3'</li> <li>S'-GCT AAT ACA ATC AGA CTC AAT AGT C-3'</li> <li>S'-GCT AAT ACA AATC AGA CTC AAT AGT CG-3'</li> </ul>	I ACA IAG AAG ACC III GCC-3	60	129	Fernandes et al., 1998
<ul> <li>S'-GCC TCT CTG ATG ACA TAT AAT G-3'</li> <li>S'-CTG TCT GCG GCA GTG AGG-3'</li> <li>S'-TTC ATC ACC TGT CAG ACA GG-3'</li> <li>S'-TTA ATG CCA GCA CTT GGG GA-3'</li> <li>S'-TGA ACG GTT TCC TTC ACA GC-3'</li> <li>S'-AGC AAC TGC TTC AGA GC-3'</li> <li>S'-AGC TAA C AG TG CA GT GG GA-3'</li> <li>S'-AGC TCT AAC AGG TGG CCA CTG AG-3'</li> <li>S'-AGC TAA C AG TG GCA CTG AG-3'</li> <li>S'-ACT AAC AGG GG GA A ATC-3'</li> <li>S'-ACT AAC AGG GG GA A ATC-3'</li> <li>S'-ACT AAC GGA GGC TAA GAT GG-3'</li> <li>S'-ACT AAC GGA GGC TAA GAT GG-3'</li> <li>S'-ACT CAA TGG AGG GG AA ATC-3'</li> <li>S'-ACT ACA TGG AGG GG GG GG-3'</li> <li>S'-ACT AAC AGG GGG ATG AGC CCA G-3'</li> <li>S'-ACT ACA TGG ATG ATG GG-3'</li> <li>S'-ACT ACA TGG AGG ACC AGG GC-3'</li> <li>S'-ACT GGG GAG GAT AGG GC G-3'</li> <li>S'-ACT GGC TGA GAC ACC C-3'</li> <li>S'-TGG TGG ATG ACC ATC C-3'</li> <li>S'-GGT CCC TTG ATT ATG TAG-3'</li> <li>S'-GGT CCC TGG AGG ACC ACC C-3'</li> <li>S'-GGT CCC TGG AGG ACC ACC C-3'</li> <li>S'-GGT CCC TGG AGG ACC ACC C-3'</li> <li>S'-GGT CCC TGG AGG CCC AGG C-3'</li> <li>S'-GGT CCC TGG ACG CCC AGC C-3'</li> <li>S'-GGT CCC TGG ACC CCA GCC C-3'</li> <li>S'-GGT CCC TGG ACC CCA GCC C-3'</li> <li>S'-GGT CCC TGG ACG CCC AGC C-3'</li> <li>S'-GGT CCC TGG ACG CCC AGC C-3'</li> <li>S'-GGT CCC TGG ACG CCC AGC C-3'</li> <li>S'-GGT CCC TGG ACG CCC AGG C-3'</li> <li>S'-GGT CCC TGG ACG CCC AGG C-3'</li> <li>S'-GCT CCT TGT AGG ACC ATC C-3'</li> <li>S'-GCT CGT GGC CTC AGG CC-3'</li> <li>S'-GCT CCT TGT AGG ACC ATC C-3'</li> <li>S'-GCT CCT TGC AGG CCC AGG C-3'</li> <li>S'-GCT AAT AGG CCC AGG CCC AGG C-3'</li> <li>S'-GCT CCT AAT AGC CC3'</li> <li>S'-GCT AAT AGT CC-3'</li> <li>S'-GCT AAT AGG CCC AGG CCC AGG C-3'</li> </ul>	CCA '	54	183	Bammler et al., 1994 <sup>c</sup>
<ul> <li>S'-CTG TCT GCG GCA GTG AGG-3'</li> <li>S'-TTC ATC ACC TGT CAG ACA GG-3'</li> <li>S'-TTA ATG CCA GCA CTT GGG GA-3'</li> <li>S'-AGA ACG GTT TCC TTC ACA GC-3'</li> <li>S'-AGT AAC AGG TGG CCA GTG AG-3'</li> <li>S'-ATC AAC AGG TGG CCA GTG AG-3'</li> <li>S'-ATC AAC AGG TGG CCA CTG AG-3'</li> <li>S'-ATC AAC AGG TGG CCA CTG AG-3'</li> <li>S'-ATC TAA GGA GGG GAA ATC-3'</li> <li>S'-ACT AAC AGG GGG CTA GGT CC-3'</li> <li>S'-ACT AAC AGG GGC TAA GAT GG-3'</li> <li>S'-ACT CAA GGA GGC TAA GAT GG-3'</li> <li>S'-ACT CAA GGA GGC TAA GAT GG-3'</li> <li>S'-ACT CAA TGG GGG TAA GAT GG-3'</li> <li>S'-ACT CAA TGG GGG GAG GGG GG-3'</li> <li>S'-ACT ACA TGG GGT AAG GAT GG-3'</li> <li>S'-ACT GGG GGT AAG GAT GG-3'</li> <li>S'-ACT GGG GGT AAG GC TCA G-3'</li> <li>S'-ACT GGG GAT CG GGG GG-3'</li> <li>S'-ACT GGG GAT CG GGG GG-3'</li> <li>S'-ACT GGG CAT GGG GTG G-3'</li> <li>S'-GGT CCA TGG ATG ACC C-3'</li> <li>S'-GGT CCC TGG ACC ATC C-3'</li> <li>S'-GGT CCC TGG ACC ATC C-3'</li> <li>S'-GGT CCC TGG ACC ATC C-3'</li> <li>S'-GGT CCC AAT GGG C-3'</li> <li>S'-GGT GGC CAT GGG CG C-3'</li> <li>S'-GGT GGC CAT GGC CCA ACT CA-3'</li> <li>S'-GGT GGC CAT GGC CTC AAT GG C-3'</li> <li>S'-GGT GCA ATC GGC CCA ATT CG-3'</li> <li>S'-GGT GCA ATT GGC CA-3'</li> <li>S'-GCT GT GGC CTC AAT AGT CC-3'</li> <li>S'-GCT ATA CAA ATC AAT AGT CC-3'</li> <li>S'-GCT AAT AGA CTC AAT AGT CG-3'</li> </ul>	A GAA ACT GTT TTA CTT ACC AG-3'	60	140	This study (BAC end)
<ul> <li>5'-TTC ATC ACC TGT CAG ACA GG-3'</li> <li>5'-TTA ATG CCA GCA CTT GGG GA-3'</li> <li>5'-TTA ATG CTC AGC TCA GGG CC-3'</li> <li>5'-ATC AAC AGG TGG CCA GTC TC-3'</li> <li>5'-ATC AAC AAG TGG CCA CTG AGA ATC-3'</li> <li>5'-ATC TAC AGA CTC AGA ATC CAG a GG-3'</li> <li>5'-ATC TAC AGG GGG AGT AGG - 3'</li> <li>5'-ATC TAC AGG GGG GGG AG-3'</li> <li>5'-ATC AAG GGG GGC TAA GAT GG-3'</li> <li>5'-ACT CAA GGA GGC TAA GAT GG-3'</li> <li>5'-ACT AAG AGG GGG AG-3'</li> <li>5'-ACT AAG AGG GGG AC-3'</li> <li>5'-ACT AAG AGG GGG AC-3'</li> <li>5'-ACT AAG AGG GGG AC-3'</li> <li>5'-ACT AAG AGG GGT AAGG GG-3'</li> <li>5'-ACT AAG AGG GGT AAG GG-3'</li> <li>5'-ACT AAG AGG GGT AAG GG-3'</li> <li>5'-ACT AGG AGG ATG ATG AGG GG-3'</li> <li>5'-ACT AGG TGG ACT AGG AGC AGG - 3'</li> <li>5'-AGC TGG AGG ACC ATC GG-3'</li> <li>5'-AGC TGG CTT GG ATG ACC ACC C-3'</li> <li>5'-AGC TGG CTT GG ACG ACC C-3'</li> <li>5'-AGC TGG CTT GG ACG ACC C-3'</li> <li>5'-AGC TGG CTT GG ACG ACC C-3'</li> <li>5'-GGT CCC AAT AGG ACC ATC C-3'</li> <li>5'-GCT GGT GGC TCT AGG C-3'</li> <li>5'-GCC AAT AGG ACC ACT CAAT AGT CC-3'</li> <li>5'-GCC AAT AGG CCC AAT AGT CC-3'</li> <li>5'-GCC AAT AGA CCC AGG C-3'</li> <li>5'-GCC AAT AGA CCC AGG C-3'</li> <li>5'-GCC AAT AGA CCC AGG C-3'</li> </ul>	A CAC CAT CCA ACA CGT CA-3'	58	182	This study (BAC end)
<ul> <li>5'-TTA ATG CCA GCA CTT GGG GA-3'</li> <li>5'-AGA ACG GTT TCC TTC ACA GC-3'</li> <li>5'-CAT TAC CTC AGG TCA GTC TC-3'</li> <li>5'-ATC AAC AAG TGG CCA CTG AG-3'</li> <li>5'-AGC TAC AGA GTG AGT TCC AG-3'</li> <li>5'-AGT TAC AGA GTG AGT ATC-3'</li> <li>5'-AGT TAC AGA GTG AGT GG AGT ACC-3'</li> <li>5'-ACT AAC AGG GTG AGT GG-3'</li> <li>5'-ACT CAA GGA GGC TAA GAT GG-3'</li> <li>5'-ACT CAA TGG CTG AGG GTG AC-3'</li> <li>5'-ACT CAA TG GTG AGG GTG AC-3'</li> <li>5'-ACT CAA TG GTG ATT ATG TAG-3'</li> <li>5'-AGC TGG CCT TTG ATT ATG TAG-3'</li> <li>5'-AGC TGG CAA ATC GGG G-3'</li> <li>5'-AAC ATA GGA GCG ACC ACC C-3'</li> <li>5'-AAC ATA GGA GAC ACT CAA CC-3'</li> <li>5'-GTG CAG TGG ACC ACC CAG GC C-3'</li> <li>5'-GTG CAG CAT GGG ACC ATC C-3'</li> <li>5'-GTG CAT GGC TTT TCT GGC 1G-3'</li> <li>5'-GCC TGT TGG ATC ACT GG-3'</li> <li>5'-GCC ATA AGG CCC AGG C-3'</li> <li>5'-GCC AAT AGG CAC ACT GGC C3'</li> <li>5'-GCC AAT AGA CCC AGG C-3'</li> <li>5'-GCC AAT AGA CCC AGG C-3'</li> <li>5'-GCC AAT AGA CCC AGG C-3'</li> </ul>	G TCA ATC ATA AGG GCC AG-3'	58	248	This study (BAC end)
<ul> <li>5'-AGA ACG GTT TCC TTC ACA GC-3'</li> <li>5'-CAT TAC CTC AGG TCA GTC TC-3'</li> <li>5'-CAT TAC CTC AGG TCA GTC ACA GCC TCA GAA</li> <li>5'-AGC TCT CAA CTC CTA GAA ATC-3'</li> <li>5'-GTC TAT CAA CTC CAA GTG AGT TCC AG-3'</li> <li>5'-GTC TAT ATC CAC TGC ATG GAG ATC-3'</li> <li>5'-ACT ACA TGC GTG AGG GTG AG-3'</li> <li>5'-ACT CAA GGA GGC TAA GAT GG-3'</li> <li>5'-ACT CAA TGC GTG AGG GTG AC-3'</li> <li>5'-ACT ACA TGC GTG AGG GTG AC-3'</li> <li>5'-ACT CAA TGC GTG AGG GTG AC-3'</li> <li>5'-ACT CAA TGC GTG AGG GTG AC-3'</li> <li>5'-ACT CAA TG GTG ATG ACA GC-3'</li> <li>5'-AGT GGA GAG GAT TTCG GCT G-3'</li> <li>5'-AGT GGA CAC ACT GTG AGC G-3'</li> <li>5'-TTG GGA CAC ACT GTG CGC G-3'</li> <li>5'-TTG GGA CAC ACT GTG GCG G-3'</li> <li>5'-TTG GGA CAC ACT GTG ACC ACC C-3'</li> <li>5'-TTG GGA CAC ACT GTG ACC ACC C-3'</li> <li>5'-TTG GGC CTA GAC ACT CC-3'</li> <li>5'-GTG GGC TTG ATT ATG GG-3'</li> <li>5'-GTG GGC CTA GGC ACC ACT C-3'</li> <li>5'-GTG GGC CTA GGC ACC ACT C-3'</li> <li>5'-GCC ATA AGG GAC ACT ATT CGG GC-3'</li> <li>5'-GCC ATA AGC CCA GGC CA-3'</li> <li>5'-GCC ATA AGA CTC CATG GC-3'</li> <li>5'-GCC ATA ATC AATT CGG GC3'</li> </ul>	C AGA TGG TTT TGA GCC CC-3'	58	245	This study (BAC end)
<ul> <li>S'-CAT TAC CTC AGC TCA GTC TC-3'</li> <li>S'-ATC AAC AAG TGG CCA CTG AG-3'</li> <li>S'-AGC CTC CAA CTC CTA GAA ATC-3'</li> <li>S'-GTC TAC AGA GTG AGT TCC AG-3'</li> <li>S'-ACT ACA TGG AGG GTG AGG GG 3</li> <li>S'-ACT CAA GGA GG TAA GAT GC-3'</li> <li>S'-ACT CAA GGA GG TAA GAT GG-3'</li> <li>S'-ACT CAA GGA GG TAA GAT GC-3'</li> <li>S'-ACT CAA TC GTG ATG AGG GG 3</li> <li>S'-ACT CCA ATC GTG ATG AGG GG 3'</li> <li>S'-ACT CCA ATC GTG ATG GC 1CA G-3'</li> <li>S'-ACT CCA ATC GTG ATG AGG GG 3'</li> <li>S'-ACT CCA ATC GTG ATG AGG 40-3'</li> <li>S'-AGC TGG CTT GGA GC TCA G-3'</li> <li>S'-AGC TGG CTT GGA GC TCA G-3'</li> <li>S'-AGC TGG CTT GGA GC CC G-3'</li> <li>S'-CTA GC TGG ATA ACT CC-3'</li> <li>S'-GGT CTC TCT TGG AGG C-3'</li> <li>S'-GGT GCA ATC GGC CTA GGC C3'</li> <li>S'-GGT GCA ATC AGA CCC GAG C-3'</li> <li>S'-GCC TGT TGA CTT TT TCT GC-3'</li> <li>S'-GCC TGT TGA CTT AGA CCC 3'</li> <li>S'-GCC ATA ATC AGA CCC AGG C-3'</li> <li>S'-GCC ATA CAA ATC AGA CCC AGT CA-3'</li> </ul>	T GTG TGA CTA CAA CTG GC-3'	58	435	This study (BAC end)
<ul> <li>S'-ATC AAC AAG TGG CCA CTG AG-3'</li> <li>S'-AGC CTC CAA CTC CTA GAA ATC-3'</li> <li>S'-GTC TAC AGA GTG AGT TCC AG-3'</li> <li>S'-ACT ACA TGC ATG GAG GGG GG-3'</li> <li>S'-ACT ACA TGG GGG GGC TAA GAT GC-3'</li> <li>S'-ACT ACA TG GGA GGC TAA GAT GC-3'</li> <li>S'-ACT ACA TG GGA GGC TAA GAT GC-3'</li> <li>S'-ACT ACA TG GGA GG TAA GAT GC-3'</li> <li>S'-ACT ACA TG GGA GGC TAA GAT G-3'</li> <li>S'-ACT ACA TG GGA GG TAA GAT G-3'</li> <li>S'-ACT ACA TG GGA GGC TAA GAT G-3'</li> <li>S'-ACT CAA TG GGA GG TAA GAT G-3'</li> <li>S'-AGC TGG CTT GGA GC TCA G-3'</li> <li>S'-AGC TGG CTT GGA GC TCA G-3'</li> <li>S'-AGC TGG CTT GGA GC TCA G-3'</li> <li>S'-AGC TGG CTT GGA GC CC CA G-3'</li> <li>S'-GGT CTC TCT TGG ATT ACT ACT CC-3'</li> <li>S'-GGT GCA ATT GGA GC C3'</li> <li>S'-GGC TGC CTA GAC ACT C-3'</li> <li>S'-GGT GCA ATT GGA GC C3'</li> <li>S'-GGC TCT TGT GG ACG C-3'</li> <li>S'-GGT GCA ATT GGA GC C3'</li> <li>S'-GCC TGT TGG ATT AGT CC-3'</li> <li>S'-GCC TGT TGG ATT ACT CC-3'</li> <li>S'-GCC TGT TGG ATT ACT CC-3'</li> <li>S'-GCC TGT TGG CTT AGT CCA GG C-3'</li> <li>S'-GCC TGT TGG CTT ACT CC-3'</li> <li>S'-GCC TGT TGG CTT ATT ACT CC-3'</li> <li>S'-GCC TGT TGG CTT ACT CC-3'</li> <li>S'-GCC TGT TGG CTT ACT CC-3'</li> <li>S'-GCT TGT TGG CTT ACT CC-3'</li> <li>S'-GCT TGT TGT ACT CC-3'</li> <li>S'-GCT TGT TGG CTT ACT CC-3'</li> <li>S'-GCT ATA CGA ATT CGA GG C-3'</li> <li>S'-GCC TGT TGT AGA CCT ATT CC-3'</li> <li>S'-GCT ATA CAA ATT AGA CTT ATT AGT CG-3'</li> </ul>	G ATG GGA ATA TGT CTC AG-3'	58	182	This study (BAC end)
<ul> <li>5'-AGC CTC CAA CTC CTA GAA ATC-3'</li> <li>5'-GTC TAC AGA GTG AGT TCC AG-3'</li> <li>5'-TAC TCT ATC CAC TGC ATG GG-3'</li> <li>5'-ACT ACA TAG CAG GGG GG GG-3'</li> <li>5'-ACT ACA TAG CTG AGG GTG AC-3'</li> <li>5'-ACT ACA TGG AGG GCT CA G-3'</li> <li>5'-AGC TGT CTT GGA GC TCA G-3'</li> <li>5'-AGC TGG AGG GAT GGG GC GC-3'</li> <li>5'-AGC TGG AGG GAT TCG GG-3'</li> <li>5'-AGC TGG AGG GAT TCG GG-3'</li> <li>5'-AGC TGG AGG GAT CG G-3'</li> <li>5'-AGC TGG AGA ACT GG-3'</li> <li>5'-TAT GGA CACT GTG ACG G-3'</li> <li>5'-TAT GGA CACT GTG ACG G-3'</li> <li>5'-TAT GGA CAC ATC GGA GC G-3'</li> <li>5'-GGT CTC TCT TGG AAC CC-3'</li> <li>5'-GGT CAC ATG GGC ACC C3'</li> <li>5'-GGC TAG AGG ACC ATC C-3'</li> <li>5'-GGC TAG AGG ACC ATC C-3'</li> <li>5'-GGC TCT GGC TCT AGC ACC C-3'</li> <li>5'-GGC TAG ATA ACT CC-3'</li> <li>5'-GCC ATA AGA CTT GG-3'</li> <li>5'-GCC ATA CAA ATC AGA CCAG G-3'</li> <li>5'-GCC ATA CAA ATC AGA CTC AAT AGT CG-3'</li> </ul>	A TCA ACA GCA GGA GCT G-3'	58	256	This study (BAC internal)
<ul> <li>5'-GTC TAC AGA GTG AGT TCC AG-3'</li> <li>5'-TAC TCT ATC CAC TGC ATG GG-3'</li> <li>5'-ACT ACA TGC CAC TGC ATG GG-3'</li> <li>5'-ACT ACA TGG GGG GCT GCA GG 3'</li> <li>5'-AGT TCT GGA GCC TCA G-3'</li> <li>5'-AGT CCA ATC GTG ATG ACG GC3'</li> <li>5'-AGC TGG AGG GAT TCG GGC GC-3'</li> <li>5'-TGA GGA GAG GAT TCG GG GC G-3'</li> <li>5'-TAT GGA CAC ACT GTG ATG AGG -3'</li> <li>5'-TAT GGA CAC ACT GTG ATG AGC -3'</li> <li>5'-GGT CTC TCG ATA ACT CC-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GGT CAC ATA GGA GAC ATC C-3'</li> <li>5'-GGT GCA ATC CAG GAG C-3'</li> <li>5'-GCC TGT TGA CTT TT TCT GG-3'</li> <li>5'-GCC TGT TGA ATC AGA CC-3'</li> <li>5'-GCC ATA AGA CTT CTT AGT CG-3'</li> <li>5'-GCC ATA CAA ATC AGA CTC AAT AGT CG-3'</li> </ul>	5'-CCC TGG AAC TGG TGT TAC AG-3'	60	233	This study (BAC internal)
<ul> <li>5'-TAC TCT ATC CAC TGC ATG GG-3'</li> <li>5'-ACT CAA GGA GGC TAA GAT GG-3'</li> <li>5'-ACT ACA TGG CGG GGC GAG GGT GC-3'</li> <li>5'-ACT ACA TGG CGT GA GGC TCA G-3'</li> <li>5'-AGC TGG CGA GGC TCA G-3'</li> <li>5'-TGG AGA GAG GAT TCG GCT G-3'</li> <li>5'-TGG TGG CCT TG ATT ATG TAG-3'</li> <li>5'-TGG TGG CCT TG ATT ATG TAG-3'</li> <li>5'-TGG TGG CCT TGG ATG ACG GC-3'</li> <li>5'-TGT GGA CAC ACT GTG CGG G-3'</li> <li>5'-TGT GGA CAC ACT GGG CG G-3'</li> <li>5'-TGT GGA CAC ACT GGG CG G-3'</li> <li>5'-TGT GGA CAC ACT GGG CG G-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GGT GCA ATG CTT GGA CCA GGC C-3'</li> <li>5'-GCC TGT TGA CCA GGG C-3'</li> <li>5'-GCC TGT TGA CCA GGC CA-3'</li> <li>5'-GCC ATA ATA CAA TC AGA CTC AAT AGT CG-3'</li> </ul>	TGT	58	199	This study (BAC internal)
<ul> <li>5'-ACT CAA GGA GGC TAA GAT GC-3'</li> <li>5'-ACT ACA TAG CTG AGG GTG AC-3'</li> <li>5'-AGC TGT CTT GGA GCC TCA G-3'</li> <li>5'-TGG AGA GAG GAT CG GCT CA G-3'</li> <li>5'-TGG AGA GAG GAT CG GCT TAG G-3'</li> <li>5'-TGG TGG CCT TTG ATT ATG TAG-3'</li> <li>5'-TAG GGA CAC ACT GTG ATT ATG TAG-3'</li> <li>5'-TAT GGA CAC ACT GTG ATT ATG TAG-3'</li> <li>5'-TAT GGA CAC ACT GTG ATT ATG TAG-3'</li> <li>5'-TAG TGG CAT TGG ATA ACT CC-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GGT CAC ATA GGA GAC ATC C3'</li> <li>5'-GCC ATA AGA GAC CAT GG-3'</li> <li>5'-GCC ATA AGA CTC ATG CA-3'</li> <li>5'-GCC ATA AGA CTC AAT AGT CA-3'</li> <li>5'-GCC AATA GAA ATC AGA CTC AAT AGT CG-3'</li> </ul>	C ATT CCT TCC ACA ACC TG-3'	58	303	This study (BAC end)
<ul> <li>5'-ACT ACA TAG CTG AGG GTG AC-3'</li> <li>5'-AGC TGT CTT GGA GCC TCA G-3'</li> <li>5'-CAT CCA ATC GTG ATG ACA GC-3'</li> <li>5'-TGG AGG GAG GAT TCG GCT G-3'</li> <li>5'-TAT GGA CAC ACT GTG GCT G-3'</li> <li>5'-TAT GGA CAC ACT GTG CGC G-3'</li> <li>5'-TAT GGA CAC ACT GTG CGC G-3'</li> <li>5'-TAT GGA CAC ACT GTG GCC G-3'</li> <li>5'-TAT GGA CAC ACT GTG GGC G-3'</li> <li>5'-TAT GGC TGC TTG ATT ATG TAG-3'</li> <li>5'-TAT GGC CAT GGA ACT CC-3'</li> <li>5'-GTG CAG TAG GCT GGC G-3'</li> <li>5'-GTG CAT GGC TGT AGG C-3'</li> <li>5'-GTG CAT GGC TTT TCT GG-3'</li> <li>5'-GCC TGT TGG CAT GGC CA-3'</li> <li>5'-GCC ATA AGA GCC CAG GG C-3'</li> <li>5'-GCC ATA GAA ATC AGA CCC AGT GG-3'</li> </ul>	T CCT GAC CTC TAC AAA CG-3'	58	404	This study (BAC end)
<ul> <li>5'-AGC TGT CTT GGA GCC TCA G-3'</li> <li>5'-CAT CCA ATC GTG ATG ACA GC-3'</li> <li>5'-TGG AGA GAG GAT TCG GCT G-3'</li> <li>5'-TGT GGA CAT GTG ATT ATG TAG-3'</li> <li>5'-AGC TGG CCT TTG ATT ATG TAG-3'</li> <li>5'-TAT GGA CAC ACT GTG CG G-3'</li> <li>5'-TAT GGA CAC ATC ATA ACT CC-3'</li> <li>5'-GTG CTT GGC TGG ATA ACT CC-3'</li> <li>5'-GTG CTT GGC TGG CGG G-3'</li> <li>5'-GTG CTT GGC TT GG CGC G-3'</li> <li>5'-GCT AGA GCA CCA GG C-3'</li> <li>5'-GCT AAGA GCA CCA GG C-3'</li> <li>5'-GCT GCT GGC TAC ATC AAT AGT CG-3'</li> <li>5'-GCT ATA CAA ATC AGA CTC AAT AGT CG-3'</li> </ul>	5'-CTG CTC TCC TCA GTT CAC G-3'	58	229	This study (BAC end)
<ul> <li>5'-CAT CCA ATC GTG ATG ACA GC-3'</li> <li>5'-TGG AGA GAG GAT TCG GCT G-3'</li> <li>5'-TGT GG CCT TTG ATT ATG TAG-3'</li> <li>5'-AGC TGG CCT GTG GAC AGC G-3'</li> <li>5'-CTA GCC TGC CTA GAC AAC C-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GGT CTT GG ATA ACT C-3'</li> <li>5'-GGT GTA GGC ACC AGC G-3'</li> <li>5'-GGT GTA GGC CCA GAG C-3'</li> <li>5'-GGT GCT GGC TGT AGG C-3'</li> <li>5'-GGT GCA TGG TAC ATG CAT GG-3'</li> <li>5'-GGT GCA ATC AGA CTC AAT AGT CG-3'</li> </ul>	A CTA GAG CTT CTC TCT GG-3'	58	170	This study (BAC end)
<ul> <li>5'-TGG AGA GAG GAT TCG GCT G-3'</li> <li>5'-AGC TGG CCT TTG ATT ATG TAG-3'</li> <li>5'-TAT GGA CAC ACT GTG CGC G-3'</li> <li>5'-CTA GCC TGC CTA GAC AAC C-3'</li> <li>5'-GGT CTC TCT TGG ATA ACT CC-3'</li> <li>5'-GTG CAG TAA GGG ACC ATC C-3'</li> <li>5'-GCC ATG AGG ACC CCA GAG C-3'</li> <li>5'-GCC ATA AGG GAC CCA GAG C-3'</li> <li>5'-GCA ATA CAA ATC AGA CTC AAT AGT CG-3'</li> </ul>	T GAG TTG GAG ATG AAG CG-3'	58	234	Mizuta et al., 1993 <sup>d</sup>
5'-AGC TGG CCT TTG ATT ATG TAG-3' 5'-TAT GGA CAC ACT GTG CGC G-3' 5'-CTA GCC TGC CTA GAC AAC C-3' 5'-GGT CTC TCT TGG ATA ACT CC-3' 5'-GTG CAG TAA GCG ACC ATC C-3' 5'-AAC ATG CTT GGC TGT AGG C-3' 5'-CCC TGT TGG CGT AGG C-3' 5'-GCC ATA AGA CTC CTT TCT GG-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	A TGA CTG TGA CTT CAA CTC-3'	58	113	GenBank AA209103
5'-TAT GGA CAC ACT GTG CGC G-3' 5'-CTA GCC TGC CTA GAC AAC C-3' 5'-GGT CTC TCT TGG ATA ACT CC-3' 5'-GTG CAG TAA GCG ACC ATC C-3' 5'-AAC ATG CTT GGC TGT AGG C-3' 5'-CCC TGT TGA CGA CTC AGG C-3' 5'-GCT ATA AGA GAC CCA GAG C-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	5'-TAT ACT CTC ATA TGT GGC AGC-3'	58	136	This study (BAC internal)
5'-CTA GCC TGC CTA GAC AAC C-3' 5'-GGT CTC TCT TGG ATA ACT CC-3' 5'-GTG CAG TAA GCG ACC ATC CC-3' 5'-CAC ATG CTT GGC TGT AGG C-3' 5'-CCC TGT TGA CGT TT TCT GG-3' 5'-GCT ATA AGA GAC CAT GGG C-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	TGC	58	149	This study (BAC internal)
5'-GGT CTC TCT TGG ATA ACT CC-3' 5'-GTG CAG TAA GCG ACC ATC C-3' 5'-AAC ATG CTT GGC TGT AGG C-3' 5'-CCC TGT TGA CTT TCT GG-3' 5'-GCT ATA AGA GAC CCA GGG C-3' 5'-GGT GCA CAT GGA TAC AGT CA-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	A GAG AAG TGA ATG CTA GG-3'	58	147	This study (BAC internal
5'-GTG CAG TAA GCG ACC ATC C-3' 5'-AAC ATG CTT GGC TGT AGG C-3' 5'-CCC TGT TGA CTT TCT GG-3' 5'-GCC ATA AGA GAC CCA GAG C-3' 5'-GGT GCA CAT GTA CAG C-3' 5'-GGT GCAA ATC AGA CTC AAT AGT CG-3'	A ATC TTC ACT GGC TCA CC-3'	58	180	This study (BAC end)
5'-AAC ATG CTT GGC TGT AGG C-3' 5'-CCC TGT TGA CTT TTT TCT GG-3' 5'-GCC ATA AGA GAC CCA GAG C-3' 5'-GGT GCA CAT GTG TAC ATG CA-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	g ctg aca ggg tca caa c-3'	58	363	Kofler et al., 1996 <sup>e</sup>
5'-CCC TGT TGA CTT CTT TGC GG-3' 5'-GCC ATA AGA GAC CCA GAG C-3' 5'-GGT GCA CAT GTG TAC ATG CA-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	CCT	58	199	This study (BAC end)
5'-GCC ATA AGA GAC CCA GAG C-3' 5'-GGT GCA CAT GTG TAC ATG CA-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	ATG	58	258	This study (BAC end)
5'-GGT GCA CAT GTG TAC ATG CA-3' 5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	T GGT GAA TCA GAT GTT GG-3'	58	180	This study (BAC end)
5'-CCA ATA CAA ATC AGA CTC AAT AGT CG-3'	AGA	58	152	MGI:91242
	G GTC TCC CCA TCT TCC TA-3'	60	136	MGI:91281
D19Mit93 <sup>a</sup> 5'-CCT GGC CTC ACC TTT TTA CA-3' 5'-ACA	A TGC GCT GTG GCT CTC-3'	56	114	MGI:100754
<sup>a</sup> PCR assays generating a polymorphic product (C57BL/6 $\neq$ Mus spretus).				
·Biochem J 298:385–390, 1994. <sup>d</sup> Nucl A cide Res 21, 1761–1766, 1003				

Table 1. Primers for PCR amplification of physical and genetic mapping markers

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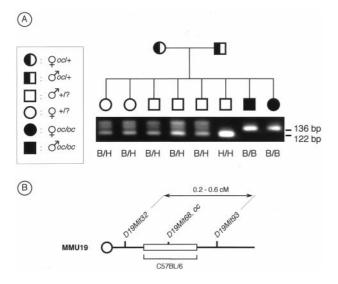


Figure 2. (A) Genotyping of an  $oc/+ \times oc/+$  cross with the polymorphic microsatellite D19Mit68. Mutant oc/oc mice show the cosegregation of the osteopetrotic phenotype with C57BL/6 alleles (B/B). (B) Schematic representation of the C57BL/6 minimal region cosegregating with D19Mit68 and the oc locus. Recombinant oc/oc and oc/+ animals excluded D19Mit32 and D19Mit93 as proximal and distal boundaries, respectively. The genetic distance between these two boundaries is derived from the EUCIB data.

# BAC Sequencing and Clone Assembly

BAC DNA was prepared according to a standard alkaline lysis protocol, and purified twice on a CsCl gradient. BAC DNA (20 µg) was mechanically disrupted using a hydroshear device (Gene Machines, San Carlos, CA), generating genomic DNA fragments an average of 3 kb in size. BAC DNA fragments were repaired with T4 DNA polymerase, and ligated to BstXI adaptors (Invitrogen, Groningen, The Netherlands). DNA adaptor-ligated fragments were purified by electrophoresis on a 0.7% agarose (FMC Bioproducts, Rockland, ME) preparative gel. After electrophoresis, fragments were excised from the gel, purified on Qiaquick columns (Qiagen, Courtaboef, France), and ligated to pcDNA2.1 vector (Invitrogen). Recombinant plasmids were transformed into Escherichia coli DH10B cells by electroporation (Electromax; Life Technologies). Ampicillin-resistant bacterial clones were picked with a Flexy robot (Proteigene, Saint-Marcel, France) and inoculated in 96-well arrays. Clones corresponding to 12 96-well dishes ( $8 \times BAC$  length) were end-sequenced on a Licor 4200 system (Li-Cor Inc., Lincoln, NE). Sequence assembly was performed using the Phred-Phrap software.<sup>7</sup>

# Genomic DNA Amplification and Sequencing

Genomic DNA (50–100 ng) (prepared from mouse tail) was used as a matrix for amplification with the following upstream and downstream primers: 5'-ATCCTAAGCCGAAGTTGAGC-3' and 5'-TCCGTTTCCTCCTGGATGC-3'. Polymerase chain reaction products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Meylan, France). After sequencing with IRD700-labeled reverse primer (MWG Biotech, Ebersberg, Germany), the analysis was performed as described above.

### Reverse Transcription (RT)-PCR

We extracted total RNA from kidney with Extract All reagent (Eurobio, Les Ulis, France), and cDNA was generated using Expand reverse transcriptase (Roche) and p(dT)<sub>15</sub> oligonucleotide (Roche) in a 40-µL reaction volume under conditions recommended by the manufacturer. Polymerase chain reaction was performed with the following upstream and downstream primers: 5'-ATCCTAAGCCGAAGTTGAGC-3' and 5'-TC-CGTTTCCTCCTGGATGC-3'. Polymerase chain reaction products were purified using the High Pure PCR product purification kit, and subcloned into pGEMT-Easy vector (Promega) according to the manufacturer's protocol. Recombinant plasmids were transformed into JM109 competent bacteria. Plasmid DNAs were prepared using the Wizard Plus SV miniprep kit (Promega, Charbonnieres, France), digested with Not I (two sites flanking the cloning site of the vector), and analyzed by gel electrophoresis in 2% agarose gel. After sequencing of plasmid DNAs with IRD700-labeled SP6 and M13u (universal) primers (MWG), the analysis was performed as described above. The GenBank accession number is AF188702.

### Results

#### Genetic Mapping of the oc Mutation

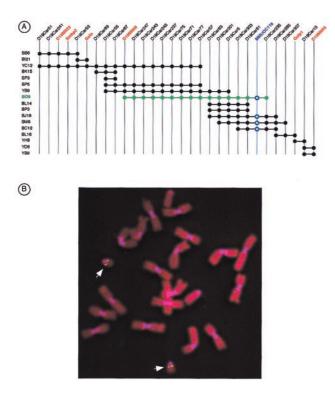
In the present study, we have been able to refine the genetic localization of the *oc* gene in the pericentromeric region of mouse chromosome 19 using an interspecific intercross of the type: (B6C3-*a/a* F1 *oc/*+ × *Mus spretus*)F1*oc/*+ × (B6C3-*a/a* F1 *oc/*+ × *Mus spretus*)F1*oc/*+ × (B6C3-*a/a* F1 *oc/*+ × *Mus spretus*)F1*oc/*+. Close to 400 F2 progeny were generated, and using eight microsatellite markers (*D19Mit22*, *D19Mit31*, *D19Mit32*, *D19Mit42*, *D19Mit51*, *D19Mit68*, *D19Mit93*, and *D19Mit109*), polymorphic between C57BL/6, C3H, and *Mus spretus*, 24 +/? recombinants and 20 *oc/oc* recombinant animals were typed in more details (data not shown). Backcrossing the original mutant stock to B6C3-*a/a* F1 hybrids<sup>17</sup> in order to generate vigourous breeding stock introduced a C3H genetic background that was detected on MMU19 in heterozygotes *oc/*+ mice.

The analysis of these recombinants led to the definition of a minimal candidate region, with *D19Mit32* representing the centromeric boundary and *D19Mit93* the telomeric one. High-resolution genetic maps of mouse chromosomes have been generated in the European Collaborative Interspecific Mouse Backcross (EUCIB) project based on close to a thousand progeny produced by an interspecific backcross between C57BL/6 and *Mus spretus*.<sup>6</sup> In that project, the genetic distance between *D19Mit32* and *D19Mit93* ranges from 0.2 to 0.6 cM in the BSS and BSB crosses, respectively (http://www.informatics.jax.org/menus/map\_menu.shtml).

Finally, the systematic genotyping of over 200 oc/oc mice generated from the (B6C3-a/a F1 oc/+) × (B6C3-a/a F1 oc/+) cross showed, invariably, B/B homozygous alleles for *D19Mit68* segregating with the osteopetrotic phenotype (an example of the pedigree is shown in **Figure 2**). Thus, a defined region bearing the C57BL/6 background in which the *oc* mutation appeared could be delimited by *D19Mit32* and *D19Mit93* and centered on *D19Mit68*.

# Physical Mapping of the Candidate Region

Based on our genetic mapping results, a screening of YAC and BAC libraries was undertaken. From a first set of two YACs and three BACs, end probes were isolated and sequenced in order to derive new STS markers and build up a set of overlapping clones.



**Figure 3.** (A) BAC and YAC STS-based content contig map of the candidate region for the *oc* locus. Bxx and Yxx represent BAC and YAC references, respectively. Positive PCR assays with each genomic clone are represented by a dot. Open circles identify PCR assays corresponding to MMUOC116. (B) FISH hybridization on SV22CD mouse metaphase chromosomes using the BO9 BAC DNA as a probe.

A more in-depth study of the physical map of this region of the mouse genome will be published separately (manuscript in preparation). Five YACs and 12 BACs were finally retained for

further characterization and were assembled in an STS contentbased contig shown in Figure 3A. To ascertain the localization of the YAC and BAC genomic clones at the same time as testing for chimerism, fluorescent in situ hybridization was systematically performed on mouse SV22-CD metaphase chromosomes; an example of such hybridization using BAC BO9 DNA as a probe is shown in Figure 3B. Aside from the three microsatellite MIT markers, D19Mit32, D19Mit68, and D19Mit93, three genes conserved between HSA11q13 and MMU19 were positioned on this contig (Gstp1, Smbp2, and Galn). Twenty-one new STSs were generated from sequence data obtained either from BAC/YAC clone insert-end or internal sequencing. Oligonucleotide sequences for these PCR assays as well as the annealing temperature and the size of the product generated from a C57BL/6 DNA template are presented in Table 1. Five of these new STSs, present on BACs BP5 and/or BO9, display polymorphic PCR products between C57BL/6 and Mus spretus, which appeared to cosegregate with oc/oc animals like D19Mit68 (data not shown). We then generated a 1.1-Mb contig based on a set of overlapping BAC/YAC clones including both proximal and distal boundaries of the candidate region where oc had been located.

# A Mouse Homologue of the Human 116-kDa V-ATPase Subunit Gene (MMUOC116) Is Mutated in oc/oc Mice

The physical and genetic mapping results guided our choice in the selection of two BACs of 120 kb (BP5) and 220 kb (BO9) for shotgun cloning and sequencing. Sequence comparison using BLAST-based software<sup>1</sup> between one of the contigs of BAC BO9 against several database (nr GenBank, dbEST, dbSTS, ...) identified five mouse ESTs (GenBank Accession Nos. AI663350, AI549720, AI180721, AI649394, AI607442) displaying sequence identity with one sequence contig of the BO9 clone. This set of ESTs showed a high degree of similarity to the cDNA sequence of a new human osteoclast-specific 116-kDa vacuolar proton pump subunit (OC-116KDa) (Genbank Accession No. U45285).<sup>15</sup> Based on the sequence of these five mouse ESTs, we

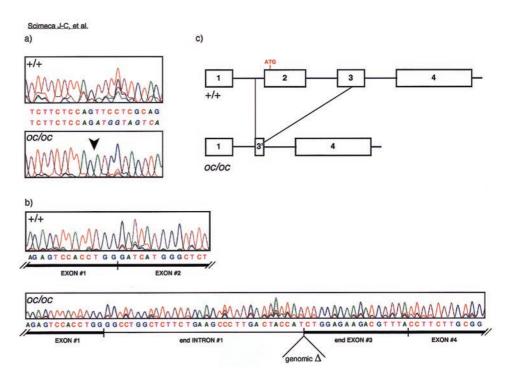


Figure 4. DNA sequence comparison of mouse OC116 gene in wt and in oc/oc mice. (a) The distal border of the deletion (exon 3 into intron1) in normal (+/+) and mutant (oc/oc) genomic DNA. (b) The expected exon 1/exon 2 junction in +/+ mice cDNA, and one of the alternative RT-PCR products derived from oc/oc mice. (c) A schematic representation of the 1.6-kb deletion in genomic DNA present in oc/oc mutants compared with the wild type.

MMUOC116 HSAOC116	1	NGSMFRSEEVALVQLILPTESAYNCVSGLGELGLVEFRDLNESVSAFQRR MGSMFRSEEVALVQLELPTAAAYTCVSRLGELGLVEFRDLNASVSAFQRR
MMUOC116 HSAOC116	51 51	FVVDVRRCEELEKTFTFLREEV ORAGLTLAPPEOTIPAPPPRDLLRIQEE FVVDVRRCEELEKTFTFLOEEVRRAGLVLEPPKCRLPAPPPRDLLRIQEE
MMUOC116 HSAOC116		T DRLAQELRDVRGNQQALRAQLHQLRLHSAVLGQHH SP PVAA DHTBGE FS T BRLAQELRDVRGNQQALRAQLHQLCLHAAVLRQGH BP QUAA AHTDGA -S
MMUOC116 HSAOC116	$   \begin{array}{r}     151 \\     150   \end{array} $	E TTPLLPG TRGPH SOLKVNFVAGAVEP YKAMALERLLWRACRGFLIASFR E TPLLQA PGGPH OLLWVNFVAGAVEP YKAMALERLLWRACRGFLIASFR
MMUOC116 HSAOC116	$\begin{array}{c} 2 & 0 & 1 \\ 2 & 0 & 0 \end{array}$	ETEGOLEDPVTGEPATWMTFMISYWOEQIGQKIRKITDCFHCHVFPMLEQ ELEQPLEHPVTGEPATWMTFMISYWGEQIGQKIRKITDCFHCHVFPmLQQ
MMUOC116 HSAOC116		EEAR RALQQLQQQSQELQEVLGET DRFLSQVLGRV QLLPP WQVQ HKM EEARLGALQQLQQQSQELQEVLGET BRFLSQVLGRV LQLLPP GQVQ HKM
MMUOC116 HSAOC116	$\begin{array}{c} 301\\ 300 \end{array}$	kavyl Lnqcsvntthkcliae vwch ardlp v q galqss seegysava kavyl Lnqcsvsthkcliae awcs vrdlp 1 q galrds smeegysava
MMUOC116 HSAOC116		HRIPCODNPPTLIRTNRFTSSPQGIVDAYGVGRYREVNPAPYTIITFPFL HRIPCRDNPPTLIRTNRFTSSPQGIVDRYGVGRYQEVNPAPYTIITFPFL
MMUOC116 HSAOC116	$\begin{array}{r} 401\\ 400\end{array}$	FAVNFGDVGHGLLMFLFALANVLTENRPAVKAAQNEIWQTFFC <mark>GRYLLLL</mark> FAVMFGDVGHGLLMFLFALAMVLAENRPAVKAAQNEIWQTFFF <mark>R</mark> GRYLLLL
MMUOC116 HSAOC116	$451 \\ 450$	nglfswytgfiynecfsrattiffsgwsvaamanqsgwsdeylsquimut Nglfswytgfiynecfsratsiffsgwsvaamanqsgwsdaplaqhimut
MMUOC116 HSAOC116	$501 \\ 500$	LEPNITGVFLGPYÞFGIDÞIWSLAPNHLSFLNSFKNKMSVILGVTHMAFG Löpnvtgvflgpyþfgidþiwslapnhlsflnsfknkmsvilgv <mark>v</mark> hmafg
MMUOC116 HSAOC116		VELSIFNHVHFGQAHRLLLETLPELIFLLGLFGYLVFLIVYKWVNVSAAS VULCVFNHVHFGQAHRLLLETLPELIFLLGLFGYLVFLVIYKWLCVAAR
MMUOC116 HSAOC116	601 600	ASSAPSILIHFINMFLFSONPANHLLHGQEVVQYVLVVLALATVPILLL Ass-psilihfinmflpshspankllyproevvqatuvvlalamvpilll
MMUOC116 HSAOC116	651 649	GTPLYLROHRHRRNTORRPAGOODEDTOKLASPDASTLENSWSPDEEK GTPLHL – HRHRRLERRPADROEENKAGLIDLPDASV – NEWSSDEEK
MMUOC116 HSAOC116	701 695	AGSPIDEE – TERVPSEFNHQAIHTIEFCLGCISNTASYLRLWALSLAHA AGGLODEE EAELVPSEVLNHQAIHTIEFCLGCUSNTASYLRLWALSLAHA
MMUOC116 HSAOC116	745	QLSEVLWAMVWRIGLOGOREMOVANVVLVPWFAAFAVLTVAILLVMEGLS Qlsevlwamvwriglgegremovanvvlvpepaafavmtvaillvmegls
MMUOC116 HSAOC116		AFLHALRLHWVEFQNKFYSGTGYKLSPFTFIVDSD AFLHALRLHWVEFQNKFYSGTGYKLSPFTFAATDD

Figure 5. Amino acid comparison of mouse vs. human OC116 predicted protein sequences. Black boxes represent identical residues, while grey boxes correspond to similar ones.

designed oligonucleotide primers to perform RT-PCR experiments on mRNAs derived from kidneys of wt and *oc/oc* mice.

A set of primers chosen between exon 1 and exon 4 of OC116 revealed a major size difference between the RT-PCR products generated from wt or mutant mice (data not shown). While the corresponding bands of these RT-PCR products were gel purified, cloned, and sequenced, genomic sequencing was performed on DNA extracted from wt and oc/oc mice. A 1579-bp deletion starting in the middle of intron 1 and extending 62 bp into exon 3 was identified in the genomic DNA of oc/oc mice (Figure 4a,c). This deletion removes the translation start site present at the beginning of exon 2. The sequence analysis of RT-PCR products derived from oc/oc mice showed either exon 1/exon 4 junctions (data not shown) or an alternative splice site in intron 1, 32 bp upstream from the 1579-bp genomic deletion (Figure 4b). Thus, while RT-PCR products between exon 1 and 4 are accurately spliced in kidneys of wt mice, a large genomic deletion removing exon 2 and most of exon 3 results in aberrant mRNA transcripts in mutant animals.

The predicted protein encoded by the mouse homologue of OC116 displays 84% identities and 91% similitudes at the amino acid level with its human homologue (**Figure 5**). A recent study by Heinemann et al.<sup>12</sup> localized the OC116 gene at 11q13 by fluorescent in situ hybridization on human metaphase chromosomes and described the genomic organization of this gene. Based on these results and the sequence data of the five mouse ESTs described previously, we were able to predict the intron/exon structure of MMUOC116. Similarly to what was found by Heinemann et al., we identified 20 exons ranging from 79 bp (exons 1 and 3) up to 221 bp (exon 4). The translation start site is also present in exon 2, and the UAG stop codon is in exon 20. Thus, based on the MMUOC116 gene location and the high similarity of amino acid sequence with human OC116-predicted protein, it is very likely that the gene we isolated, as being

mutated in oc/oc mice, is the mouse homologue of the human OC116 gene.

#### Discussion

We have isolated the mouse homologue of a new human osteoclast-specific vacuolar proton pump subunit gene that appears to be deleted over 1.6 kb at its 5' end in osteosclerosis (oc/oc) mutants. This result was achieved by a positional cloning strategy starting from the segregation of the oc mutation in an interspecific genetic cross to define proximal and distal recombination boundaries, followed by physical mapping over several hundreds of kilobases, and finishing by the sequence of a 200-kb genomic area.

The human OC116 gene was isolated by Li et al. and reported as putative novel human osteoclast-specific vacuolar proton pump subunit.<sup>15</sup> We hypothesize that the 1.6-kb deletion present in oc/oc mice inactivates the normal expression of the MMUOC116 gene and, consequently, could be deleterious for the vacuolar proton pump localization and expression on apical membranes of osteoclast cells. Indeed, previous work by Nakamura et al.<sup>18</sup> demonstrated that although the vacuolar H<sup>+</sup>-ATPase proton pump was still active in oc/oc mice and present throughout the cytoplasm, it was no longer found on the osteoclast apical membranes. One of the features of oc/oc mice osteoclasts is the absence of ruffled border formation, leading to a defect in bone resorption. Whether the mislocalization of the vacuolar proton pump to the outer membrane of osteoclasts is a cause or a consequence of this lack of ruffling remains to be discovered.

Recently, Heaney et al. described the mapping of human autosomal recessive osteopetrosis to a 14-cM interval on 11q13.<sup>11</sup> This rare form of human osteopetrosis is lethal within the first decade in the absence of bone marrow transplantation,<sup>9,10</sup> and its phenotype presents many similarities with the osteosclerosis mutation. Based on the present work, it is very likely that mutations affecting OC116 gene expression could be responsible for this form of human osteopetrosis. Sequencing of the entire OC116 genomic sequence of such affected individuals is presently in progress in our laboratory.

Finally, Li et al have recently presented an abstract at the 21st American Society for Bone and Mineral Research Meeting, showing that knockout -/- mice for the OC116 gene were growth retarded, developed severe osteopetrosis with deficiencies in bone remodeling and tooth eruption, and died at about 4 weeks of age.<sup>16</sup> All these phenotypic features are highly similar to what has been described for *oc/oc* mice, and strongly suggest that the genomic DNA deletion we observed in the MMUOC116 gene is sufficient to account for this phenotype.

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