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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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Osteopetrosis (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 (microphthalmia), 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-stimulating factor gene (M-CSF1), and a defect in a gene coding for a helix-loop-helix transcription factor is responsible for the microphthalmia phenotype. 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, and has been backcrossed over 20 times to the hybrid C57BL/6J×C3HeB/FeJleo/a F1, 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. In 1985, Marks et al. 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. Many genes involved in inherited human pathologies and localized on 11q13 have an homologous gene present on proximal MMU19 region and vice versa. 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-1), based on its segregation pattern and an allelism test, as well as a putative transporter gene (Roc1), 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
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/FcJ) F1 ocl+ mice were initially obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in our central animal facility in accordance with the general guidelines edited by the Direction des Services Vétérinaires. Heterozygotes animals (ocl+) were identified 3 weeks after birth by DNA genotyping (tail clipping) with a polymorphic marker (D19Mit68) cosegregating with the mutation. Animals suspected to be homozygous at the ocl 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 Génethon 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 (Hœrdt, France), according to the manufacturer’s instructions. BAC end-sequencing reactions were performed with 5 µg 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 MgCl2, and 0.5 U of platinum 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 (D19Car333 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’-CTCAACTTCCGTAGTTAGGATC-3’; D19Car319, 5’-CAGCTCITTATCTCTGTCC-3’ and 5’-CTTCATGCACACAGCAATCC-3’.

**FISH**

BAC DNA was labeled using nick-translation with biotin-14-dATP (Life Technologies). The labeled probe (1 µg/slide) was coprecipitated with 30 µg of mouse Cot-1 DNA (Life Technologies), denatured for 5 min at 70°C in hybridization mixture (50% formamide, 2X SSC, 10% Dextran sulfate), and renatured for 30–60 min at 37°C. The probe was then hybridized on denatured pretreated metaphase chromosomes from mouse SV22-CD cell line2 overnight in a moist chamber at 37°C. The following steps were performed as described previously.8

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>T&lt;sub&gt;A&lt;/sub&gt; (°C)</th>
<th>Product (bp)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
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<td>D19Car&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5'-GGC TGC TTT AGA CTG ATT TG-3'</td>
<td>5'-GGG CTT CAT AGG TAG GAG AC-3'</td>
<td>50</td>
<td>125</td>
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<td>D19Car10</td>
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<td>5'-GCT ACA TAG AAG ACC TTT GGC-3'</td>
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<td>129</td>
<td>Fernandes et al., 1998</td>
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<td>D19Car14 (Gstp1)</td>
<td>5'-GAT CTC CTT TGC CGA TTA CA-3'</td>
<td>5'-TTG CCA TTG TAG ATG GG-3'</td>
<td>54</td>
<td>183</td>
<td>Bammler et al., 1994&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>D19Car51</td>
<td>5'-GGC TCT CTG ATG ACA TAT AAT G-3'</td>
<td>5'-AGA GAA ACT GGT TTA CTT ACC AG-3'</td>
<td>60</td>
<td>140</td>
<td>This study (BAC end)</td>
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<td>D19Car53</td>
<td>5'-CTG TCT GCG GCA GTG AGG-3'</td>
<td>5'-AGA CAC CAT CCA ACA CTT CA-3'</td>
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<td>182</td>
<td>This study (BAC end)</td>
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<td>5'-TTT ATC ACC TGT CAG ACA AGG-3'</td>
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<td>5'-TAC AGA TGG TTT TGA GCC CC-3'</td>
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<td>245</td>
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<td>5'-AGA AGT CTT TCC TTC ACA GC-3'</td>
<td>5'-TGT GTG TGA CTA CAA CTG GC-3'</td>
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<td>435</td>
<td>This study (BAC end)</td>
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<td>D19Car63</td>
<td>5'-CAT TAC CTC AGC TCA TTC GC-3'</td>
<td>5'-GGG ATG GGA ATA TCT GTC AC-3'</td>
<td>58</td>
<td>182</td>
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<td>5'-GCA TCA ACA CCA GGA GCT G-3'</td>
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<td>256</td>
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<td>D19Car75</td>
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<td>5'-GCC TGG AAC TGG TGT TAC AG-3'</td>
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<td>D19Car77</td>
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<td>D19Car87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TAC TCT ATC CAC TGC ATG GG-3'</td>
<td>5'-TCC ATT CCT CCA ACA ACC TG-3'</td>
<td>58</td>
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<td>D19Car89</td>
<td>5'-ATC CAA GGA GGC TAA GAT GC-3'</td>
<td>5'-TCT CAA GTG CTC ATC AAC CG-3'</td>
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<td>D19Car235</td>
<td>5'-ACT ACA TAG CTT ACG GTG AC-3'</td>
<td>5'-CTG CTC TCC TCA GTT CAC G-3'</td>
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<tr>
<td>D19Car237</td>
<td>5'-AGC TGT CTT GGA GCC TCA G-3'</td>
<td>5'-AGC CTA GAG CTG CTC TCT TG-3'</td>
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<td>170</td>
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<td>D19Car239 (Smnp2)</td>
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<td>5'-GAT GAG TTT GAG ATG AGG CG-3'</td>
<td>58</td>
<td>234</td>
<td>Mizuta et al., 1993&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>D19Car241</td>
<td>5'-TGG AGA GAG GAT TCG GCT G-3'</td>
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<td>D19Car243&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-AGC TGG CCT TTG ATT ATG TAG-3'</td>
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<td>58</td>
<td>136</td>
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<td>D19Car245&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-TAT GGA CAC ACT GTG CGC G-3'</td>
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<td>58</td>
<td>149</td>
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<td>D19Car247&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>5'-GCA GAG AAG TGA ATG CTA GG-3'</td>
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<td>D19Car285</td>
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<td>5'-CAG CTA AGC TTT TCA CTG G-3'</td>
<td>58</td>
<td>180</td>
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<td>D19Car293 (Galn)</td>
<td>5'-GTG CAG TAA GGC ACC ATC C-3'</td>
<td>5'-TGG CTA ACA GGG TCA CAA C-3'</td>
<td>58</td>
<td>363</td>
<td>Kofler et al., 1996&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>D19Car301</td>
<td>5'-AAC ATG CTT GGC TGT AGG C-3'</td>
<td>5'-CAC TGC TCT TTA GGC ACC-3'</td>
<td>58</td>
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<td>D19Car303</td>
<td>5'-GCC TGT TGA CTT CTG CTA G-3'</td>
<td>5'-GCT GGT GAA TAA GAT GTT GG-3'</td>
<td>58</td>
<td>180</td>
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<td>D19Car307</td>
<td>5'-GCC ATA AGA GAC CCA GAG C-3'</td>
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<td>MGI:91242</td>
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<td>D19Mit68</td>
<td>5'-CCA ATA CAA ATC AGC CTC AAT AGT CG-3'</td>
<td>5'-AGG TCT TCC CCA TCT TCC TA-3'</td>
<td>60</td>
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<td>MGI:91281</td>
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<td>D19Mit93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-CCT GGC CTC ACC TTT TTA CA-3'</td>
<td>5'-ACA TGC GCT GTG GCT TCT C-3'</td>
<td>56</td>
<td>114</td>
<td>MGI:100754</td>
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</table>

<sup>a</sup>PCR assays generating a polymorphic product (C57BL/6 * Mus spretus).
<sup>b</sup>C57BL/6 alleles.
<sup>e</sup>Gene 182: 71–75; 1996.
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 pd(T)$_{15}$ 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′-ATCTCTAAGCCGAAGTGTAGC-3′ and 5′-TCGGTTTCCTCCTGGAATGC-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, Groningen, The Netherlands). DNA adaptor-ligated products were purified using the High Pure PCR product purification kit and analyzed by gel electrophoresis in 2% agarose gel. After sequencing of plasmid DNAs with IRD700-labeled reverse primer (MWG Biotech, Ebersberg, Germany), 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)F1ocl+× (B6C3-a/a F1 oc+/× Mus spretus)F1ocl+. 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 hybrid, in order to generate vigorous 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. 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://wwwinformatics.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 osteopetrotic phenotype was 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.
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 content-based 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 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). Based on the sequence of these five mouse ESTs, we

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.

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.
protein, it is very likely that the gene we isolated, as being
similarity of amino acid sequence with human OC116-predicted
Thus, based on the MMUOC116 gene location and the high
is also present in exon 2, and the UAG stop codon is in exon 20.
Heinemann et al., we identified 20 exons ranging from 79 bp
ESTs described previously, we were able to predict the intron/
Based on these results and the sequence data of the five mouse
somes and described the genomic organization of this gene.
fluorescent in situ hybridization on human metaphase chromo-
products derived from wt or mutant mice (data not shown). While the
revealed a major size difference between the RT-PCR products
4
3 was identified in the genomic DNA of
starting in the middle of intron 1 and extending 62 bp into exon
1, 32 bp upstream from the 1579-bp genomic deletion (Figure
junctions (data not shown) or an alternative splice site in intron
The human OC116 gene was isolated by Li et al. and reported
as putative novel human osteoclast-specific vacuolar proton pump subunit.13 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.18 demonstrated that although the vacuolar H+ -ATPase proton pump was still active in oc/oc mice and present throughout the cytoplasm, it was no longer found on the oste-
oclast 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 11q13.11 This rare form of human osteopetrosis is lethal within the first decade in the absence of bone marrow transplantation,9,10 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.16 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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2. Baron, B., Metezeau, P., Kelly, F., Bernheim, A., Berger, R., Guernet, J. L., and Goldberg, M. E. Flow cytometry isolation and improved visualization of sorted

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.


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