We have identified two novel type II membrane-bound C-lectins, designated mOCILrP1 and mOCILrP2, of 218 and 217 amino acids, respectively, that share substantial identity with the murine osteoclast inhibitory lectin (OCIL). The extracellular domains of mOCILrP1 and mOCILrP2 share 83 and 75% identity, respectively, with the extracellular domain of mOCIL. When the extracellular domains were expressed as recombinant proteins, each inhibited osteoclast formation in murine bone marrow cultures treated with M-CSF and RANKL with similar potencies to mOCIL (IC_{50} of 0.2 ng/ml). Distinct but highly related genes encoded three OCIL family members, with mOCIL and mOCILrP2 controlled by an inverted TATA promoter, and mOCILrP1 by a TTAAAA promoter. However only mOCIL was robustly regulated by calcitropic agents, while mOCILrP1 was not expressed, and mOCILrP2 was constitutively expressed in osteoblasts. Immunohistochemistry using antipeptide antibodies to the intracellular domain of mOCILrP1/mOCILrP2 and to mOCIL demonstrated that mOCIL and mOCILrP1/mOCILrP2 were concordantly expressed in osteoblasts, chondrocytes, and in extraskeletal tissues. Further, their cellular distribution was identical to that of RANKL. The identification of three distinct genes that were functionally related implies redundancy for OCIL, and their concordant expression with that of RANKL suggests that the RANKL:OPG axis may be further influenced by OCIL family members.

The discovery that osteoclast formation and activity are controlled by M-CSF\(^1\) and members of the TNF ligand and receptor family (1–4) has had a major impact on the understanding of bone biology. The essential requirement of RANKL for osteoclast formation has been established, as has the requirement for its receptor, RANK (5). Mice deficient for RANKL or RANK are osteopetrotic because of failure of osteoclast formation (5–7). The decoy receptor, osteoprotegerin (OPG) is critical for its ability to bind RANKL and inhibit osteoclast formation (3, 8). Evidence in support of the role of OPG was amply provided by genetic experiments whereby OPG→− mice were shown to be severely osteoporotic (9), while transgenic mice overexpressing OPG are osteopetrotic (8).

There are other locally produced factors that can influence these processes, including GM-CSF, TNF, and IL-1α (4, 10, 11). Furthermore, other protein inhibitors of osteoclast formation have been identified that might act independently of the RANK signaling pathway (10, 12, 13). Among these is osteoclast inhibitory lectin (OCIL), a 207-amino acid type II transmembrane C-type lectin, which inhibits osteoclast formation in vitro in cocultures of osteoblastic stromal cells with hemopoietic cells in a lymphocyte-independent manner (14). The production of OCIL by osteoblasts and chondrocytes, its similar tissue distribution to that of RANKL, and the fact that it primarily inhibits osteoclast formation in the early (proliferative) phase, together raise the possibility that OCIL might have a direct action to oppose RANKL in the control of osteoclastogenesis (14).

A large family of proteins containing C-type lectin domains, and we noted the 36% homology between the C-type lectin domains of OCIL and CD-69 (14). In the present work we report the finding of two further proteins that are very closely related to OCIL, but are the products of separate genes. In an effort to evaluate the relative roles of this family of proteins in bone, we have determined their gene structures and also studied their biological actions, tissue distribution, and regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primary mouse osteoblastic cells were prepared from the calvaria of newborn C57BL/6J mice by digestion with collagenase as previously described (10). Cells were routinely grown in αMEM containing 10% fetal bovine serum. Incubation was carried out at 37 °C in a humidified atmosphere equilibrated with 5% CO\(_2\) in air. M-CSF was a gift from Genetics Institute (Cambridge, MA). Recombinant murine RANKL was obtained from Preprotech Inc. (Canton, MA). Recombinant human IL-11 was purchased form R&D Systems Inc. (Minneapolis, MN). All other reagents were of analytical grade obtained from standard suppliers.

**Identification and Cloning of mOCILrP1 and mOCILrP2**—Total RNA was isolated from ST2 mouse stromal cells, primary mouse calvarial osteoblasts, spleen, and liver tissue. First strand cDNA was synthesized from 2 μg of total RNA by incubating for 1 h at 42 °C with 15 units of AMV reverse transcriptase (Promega, Madison, WI) following oligo(dT) priming. For PCR, the sense strand oligonucleotide OCILm32 (5′-GCAACAAAGAACAGAACAGATC-3′), nucleotides for mOCIL, GenBank\(^TM\) AF321553) and the antisense strand oligonucleotide OCILm12 (5′-GGGACCATAGGGGAAGAGTAGTGC-3′), nucleotides for mOCIL, GenBank\(^TM\) AF321553) were used. PCR was run at 94 °C for 5 min, then 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. The resultant PCR fragments were cloned into a pgEM-T (Promega, Madison, WI) vector.
Upon nucleotide sequence analysis, mOCIL and two related but distinct cDNAs (mOCILrP1 and mOCILrP2) were identified. The full-length sequences (mOCILrP1: 1096 bp, GenBank™AY137341; mOCILrP1b: 984 bp, GenBank™AY137342; mOCILrP2: 1026 bp, GenBank™AY137344; mOCILrP2b: 1014 bp, GenBank™AY137345) were identified by 5′- and 3′-RACE using the SMART RACE cDNA amplification kit (Clontech). First-strand cDNA was synthesized from total RNA isolated from mouse bone marrow and spleen cells according to the manufacturer’s instructions. The cDNA was further amplified by PCR using OCILr25 (5′-TCAGGTGGTGTGCTGCCT-CCAAAGG-3′), which is reverse and complementary to primer OCILr25) and UNPR primer as the 3′-anchored primer in a touchdown PCR PCR protocol with denaturation at 94 °C for 1 min, then 5 cycles of 94 °C for 30 s, 72 °C for 1 min, and then 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min.

**A 3′-RACE strategy was also used to obtain the 3′-ends of the mOCILrP1 and mOCILrP2 cDNAs. First-strand cDNA was synthesized from total RNA isolated from mouse bone marrow and spleen cells according to the manufacturer’s instructions. The cDNA was further amplified by PCR using OCILm137 (5′-CCCTTGGAAAGAGGACAGA-CAACACCTGAG-3′), which is reverse and complementary to primer OCILr25) and UNPR primer as the 3′-anchored primer in a touchdown PCR protocol with denaturation at 94 °C for 1 min, then 5 cycles of 94 °C for 30 s, 70 °C for 1 min, then 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min.**

**Isolation and Sequence of mOCIL, mOCILrP1, and mOCILrP2**

**Genomic DNA**—A cDNA fragment corresponding to the extracellular domain of mOCIL was used as a probe to screen a genomic BAC Mouse 1 hybridization library (Genome Systems, Inc.), and positive clones were isolated and screened. After the genomic DNA was digested with HinIII, Southern blot analyses were carried out with the same cDNA probe. The sequences of these BAC clones were determined by nucleotide sequencing of subcloned genomic fragments into pBS or by direct sequencing of the genomic clone DNA using cycle sequencing. Genomic sequences were obtained in GenBank™: mOCIL (AY137338), mOCILrP1 (AY137339 and AY137340), and mOCILrP2 (AY137343).

**Expression of Recombinant Mouse OCILrP1 and OCILrP2—**PCR fragments specifying the extracellular domains of mOCILrP1 (amino acid residues 77–207) and mOCILrP2 (amino acid residues 92–207) were amplified from cDNA clones by PCR using the primers OCILm88 (5′-CTCGAGTCATGTCTGCTTCGGA-3′) and OCILm89 (5′-TCAGAATTCACCTAGTTGCGGA-3′) as sense primers for mOCILrP1 and mOCILrP2, respectively (both oligonucleotides introduce an EcoRI restriction endonuclease site 5′ to mOCIL coding sequence) along with OCILm97 (5′-CTAGCTTAGAAGGACATAGGG-GAAAATCGAAT-3′) as antisense primer for both mOCILrP1 and mOCILrP2. This produces an XhoI site. The PCR fragments were subcloned into the EcoRI and XhoI sites of the plasmid pMAL-c2 (New England Biolabs Inc., Beverly, MA), creating a C-terminal mOCIL fusion with the MBP (maltose-binding protein)-encoding maltE gene. Proteins were expressed in Escherichia coli BL21 cells and purified as previously described (14).

**Osteoclast Formation Assay**—Mouse bone marrow and spleen cells were prepared from adult C57BL/Jb mice, and T cell-depleted spleen cells were obtained as described (10). Cells were cultured for 7 days in culture medium containing 10% fetal bovine serum, 25 ng/ml M-CSF, 100 ng/ml RANKL, and 50 ng/ml RANKL in the absence or presence of recombinant MBP control or recombinant MBP—mOCIL, MBP—mOCILrP1, or MBP—mOCILrP2. The cultures were assessed, respectively, at various concentrations as indicated. Treatments were added on day 0 and with the medium changed on day 3. After 7 days, cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase leukocyte commercial kit (Sigma) as previously described (12).

**Immunohistochemistry**—Skeletal and extraskeletal tissues were obtained from (15). The peptide mOCIL-3 (H-Cys-Val- Thr-Lys-Ala-Ser-Leu-Pro-Met-Leu-Ser-Pro-Thr-Gly-Ser-Pro-Gly-Mal-Glu-NH2) is located in the intracellular domain and is specific to mOCIL, while the OCILrP2 peptide (H-Cys-Val-Gly-Lys-Pro-Glu-Gly-Glu-Lys-Pro-Met-Lys-Thr) is located in the intracellular domain of mOCILrP2; this mOCILrP2 peptide was specific to mOCILrP2 and not to mOCIL. The mOCILrP2 peptide contains 15 amino acids, but has 9 of 15 amino acids in common with mOCILrP1 (see sequences in Fig. 1). The peptides were synthesized, coupled with hemacycin, and used to immunize rabbits, using standard protocols (17). The standard peroxidase-labeled streptavidin-biotin detection method was used according to the manufacturer’s instructions (Dako Corporation, Carpinteria, CA) with minor modifications. The dilutions of the antiserum were utilized in preliminary experiments. Incubation of tissue sections with a 1:200–1:1000 dilution of the primary antiserum was carried out overnight at 4 °C in a humidified chamber. Peroxidase activity was detected with 3′,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Pty. Ltd.) and 0.15% H2O2. Slides were counterstained with hematoxylin and dehydrated and mounted on a coverslip as previously described (15, 16). To confirm specificity of immunostaining with mOCIL or mOCILrP1/mOCILrP2 antisera, sections were preabsorbed with 50 μg/ml of their respective peptides for 60 min prior to addition of antiserum.

**Regulation of Expression of OCIL mRNA in ST2 Mouse Stromal Cells and Bone Marrow Cells**—The OCIL family was identified during the characterization of the OCIL mRNA in ST2 mouse stromal cells and bone marrow cells. The OCIL-related mRNA species were identified during the characterization of the OCIL family. The OCIL family was identified during the characterization of the OCIL mRNA in ST2 mouse stromal cells, primary mouse calvarial osteoblasts, spleen, and liver tissue by RT-PCR.
with primers OCILm32 and OCILm12. PCR products from bone marrow were cloned into pGEM-T, and subsequent nucleotide analysis of 12 clones revealed the presence of mOCIL (six clones) and two other highly related but distinct OCIL sequences (four and two clones). By 5'- and 3'-RACE, the full sequences of these two mOCIL-related cDNAs were determined, which we now call mOCIL-related protein 1 (mOCILrP1) and mOCILrP2, respectively. Alternatively spliced isoforms of mOCILrP1 and mOCILrP2 were also identified by 5'-RACE. A 112-bp truncation was noted in 5'-untranslated region in mOCILrP1, and a 12-bp deletion in the intracellular domain was identified in mOCILrP2: the latter corresponding to a 4-amino acid deletion that maintained the reading frame and we have denoted this isoform mOCILrP2b.

The deduced protein sequences derived from mOCILrP1, mOCILrP2 cDNA sequences and their homology are compared with mOCIL in Fig. 1. The predicted structures of mOCILrP1, mOCILrP2, and mOCILrP2b are similar to that of mOCIL and are typical of a type II membrane protein. Each protein is predicted to contain a 113-amino acid C-lectin type motif, and the extracellular domains share five cysteine residues, and two potential N-linked glycosylation sites in common (Fig. 1).

mOCILrP1 has a open reading frame encoding a 207-amino acid protein, with a predicted 142 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 44 amino acid cytoplasmic domain. mOCILrP2 is predicted to be a 217 amino acid protein, composed of a 141 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 55 amino acid cytoplasmic domain; mOCILrP2b differs from mOCILrP2 by having a 51 amino acid cytoplasmic domain, with amino acids Val138–Cys139 deleted. The extracellular domains of mOCILrP1 and mOCILrP2 are well conserved sharing 83 and 74% amino acid identity (92 and 89% nucleotide identity), respectively, with the extracellular domain of mOCIL, and 79% between mOCILrP1 and mOCILrP2 (91% nucleotide identity). In contrast, the sequence identity in the intracellular domains of mOCILrP1 (41%) and mOCILrP2 (33%) with mOCIL are reduced, although clusters of amino acid identity are maintained (Fig. 1).

Gene Structure of OCIL and Related Proteins—To isolate the murine OCIL gene, a genomic BAC Mouse I library was screened with a cDNA probe corresponding to the extracellular domain of mOCIL, and 8 positive BAC clones were identified. Two BAC clones (db. 20149 and db. 20152) were selected for further analysis as a result of different hybridization profiles from Southern blotting of HindIII-digested murine DNA.

Sequencing of 8622 bp of the BAC clone (db. 20152) was completed and based on identity with the cDNA sequence for mOCIL, identified as the mOCIL gene, containing 5 exons (Fig. 2). The 5'-flanking region adjacent to exon 1 (identified by 5'-RACE strategies) contained an A/T-rich motif, AATAAA, 23 nucleotides upstream from the first exon and was assumed to be an inverted TATA promoter (Fig. 3). Other genes expressed by bone cells such as RANKL (18), bone sialoprotein (19, 20), cathepsin K (21), and TRAP (22) use inverted TATA promoters. The intron-exon splice junctions of the mOCIL gene are shown in Table 1 (top).

Nucleotide sequence analysis of the second BAC clone (db. 20149) revealed it to contain the genes for mOCILrP1 and mOCILrP2. Both genes contained six exons, and the positions of three introns are conserved with those for mOCIL (Fig. 2); the exon-intron splice junctions of the mOCILrP1 and mOCILrP2 genes are shown in Table 1 (middle and lower), respectively. Like mOCIL, mOCILrP1 and mOCILrP2 contained an A/T-rich motif, AATAAA, 23 nucleotides upstream from the first exon and was assumed to be an inverted TATA promoter, while mOCILrP1 contains a TATAAAA box 22-nucleotides upstream from the first exon (Fig. 3). A TATAAAA box has been demonstrated as a promoter for connexin 26 and glutamate dehydrogenase (23–25). mOCILrP1 also contained a putative CAAT box in the 5'-flanking sequence (~70 to ~66, from the transcriptional start site). Both mOCILrP1 and mOCILrP2 demonstrated alternative mRNA splicing. An intra-exonic splice donor site was located within exon 1 of mOCILrP1, and this resulted in a deletion of 112 nucleotides of the 5'-untranslated region (Fig. 3).
### Table I

The nucleotide sequences of the intron-exon splice junctions of the murine OCIL, OCILrP1 and OCILrP2 genes

The splice donor and acceptor splice sequences of the murine OCIL gene are presented. All splice donor and acceptor junctions identified conformed to the GT-AG consensus rule for splice junctions, except that of the splice donor site for exon 2 of mOCILrP2; the consensus sequences for eukaryotic 5' splice donor sites and 3' splice acceptor sites were proposed to be (C/A)AG I gtragt and yxnyag yxnyagIG, respectively, where intronic sequence is indicated in lowercase letters. The exonic nucleotide sequences are represented in uppercase letter and the intronic sequences in lowercase letters. Exons are numbered from the 5' transcriptional start site for each of the OCIL family genes. Exon and intron sizes are indicated in base pairs and were determined by RT-PCR and gene sequences. The amino acids interrupted are indicated, and n/a denotes not applicable.

| Exon | Exon size (bp) | 5' Splice junctions | Intron size (bp) | 3' Splice junctions | Amino acids interrupted |
|------|---------------|---------------------|------------------|---------------------|------------------------|
| OCIL | 1             | GAA G TG G          | 2342             | gagattggcgctctcatattatatatatccag | GT AAA ATT |
|      | 2             | TTG TCA G           | 917              | ccatgatccctgtctctcatatttttag | CA ACA AAG |
|      | 3             | GAT GAG CT G        | 464              | tgtattttctgtctcatattttcag | AAT TTC CTA |
|      | 4             | AAG GAG CT G        | 982              | gcagcttactgtctctctctccag | G ATT CCC |
|      | 5             | n/a                 | n/a              | n/a                 | n/a |
| OCILrP1 | 1       | GTGTAAGAGG          | -6000            | tttatatgtcatagtgttgctctgcag | G TTT GTG |
|     | 1b          | TCTAAAGAT           | -6000            | tttatatgtcatagtgttgctctgcag | G TTT GTG |
|      | 2           | CAT TCT T           | 705              | aattcgacttttaattcatattctttcacag | AT AAA ATT |
|      | 3           | TTA GCA G           | 894              | ctatgattcactttcatattttcacatttttag | TA ACA AAG |
|      | 4           | AAG GAG CT G        | 481              | tattcttgacttttttctgtatattttcag | AAT TTC CTA |
|      | 5           | AAC AAC AT          | 974              | tgtgagcttaactttttagtttttctcttcag | G ATT CCC |
|      | 6           | 454                 | n/a              | n/a                 | n/a |
| OCILrP2 | 1       | GAA AAA CT          | 5165             | cattttatgtatgtggcttttcag | G TTT GTA |
|     | 2           | CAA TGT TGT         | 707              | aattcgacttttaattcatattttcacag | AAA ATT GTC |
|     | 2b          | ACT GGA GGT         | 719              | aattcgacttttaattcatattttcacag | AAA ATT GTC |
|      | 3           | TTG TCA A           | 927              | tcatgattcacttttaattcatattttcacag | CA AAA AAG |
|      | 4           | GAG GAG CT G        | 479              | tggttctgttttttctgtatattttcag | G TTT TTC AAG |
|      | 5           | AAC AAC AT          | 978              | tgtgagcttaactttttagtttttctcttcag | G ATT CCC |
|      | 6           | 452                 | n/a              | n/a                 | n/a |
Our previous study of the tissue distribution of OCIL inhibits osteoclast formation in cultures of adherent spleen cells treated with soluble RANKL and M-CSF (14). To determine if mOCILrP1 and mOCILrP2 exert similar biological action as mOCIL, recombinant proteins for mOCIL, mOCILrP1, and mOCILrP2 were not conserved.

**mOCIL, mOCILrP1, and mOCILrP2 Inhibit Osteoclast Formation**—As we have shown previously, the recombinant extracellular domain of mOCIL inhibits osteoclast formation in cultures of adherent spleen cells treated with soluble RANKL and M-CSF (14). To determine if mOCILrP1 and mOCILrP2 exert similar biological action as mOCIL, recombinant proteins for mOCILrP1 and mOCILrP2 fused to maltose-binding protein were prepared. Mouse marrow cells were cultured in the presence of RANKL and M-CSF for 7 days and multinucleate TRAP-positive osteoclasts (MNC) were counted at the end of the incubation period. This is representative of three independent experiments. Bars represent mean ± S.E., n = 4 for each treatment. *, p < 0.05, **, p < 0.01, ***, p < 0.001 versus MBP alone.

2). An intra-exonic splice donor site in exon 2 of mOCILrP2 results in exclusion of 12 bp, deleting four amino acids (Fig. 1, mOCILrP2 amino acids 36–39) although the reading frame is maintained.

The conservation of protein sequence identity and the genomic organization between these proteins implies that mOCIL, mOCILrP1, and mOCILrP2 may result from recent gene duplication. Supporting this proposition was the high nucleotide sequence identity (~90%) between the three genes, which was confined to exons 2–5 and intervening introns of mOCIL with the corresponding regions (exons 3–6) of mOCILrP1 and mOCILrP2. Consistent with the low amino acid identity in the intracellular domains between these family members (Fig. 1), exon 1 of mOCIL shared little nucleotide identity with exons 1 and 2 of either mOCILrP1 or mOCILrP2, mOCILrP1, and mOCILrP2 appeared closest due to their genomic organization and since exon 2 of mOCILrP1 and mOCILrP2 shared 70% nucleotide identity; exon 1 of mOCILrP1 and mOCILrP2 shared little identity. Meanwhile intron 1 sequences of mOCIL, mOCILrP1, and mOCILrP2 were not conserved.

The sequence identity between mOCIL from its two related molecules, mOCILrP1 and mOCILrP2, and the fact that the peptide region used for antibody production was conserved between these family members (Fig. 1, mOCIL sequence Cys108 to Asn125), our original localization studies would not have discriminated between the different family members. Only anti-peptide antibodies directed against intracellular regions may be able to distinguish between mOCIL from its two related molecules, mOCILrP1 and mOCILrP2 (Fig. 1). Further, the sequence identity between mOCILrP1 and mOCILrP2 may not allow antibody discrimination of these two proteins. Rabbit polyclonal antipeptide antibodies to the intracellular domain of mOCIL and mOCILrP1/mOCILrP2 were prepared, specificity established, and used to determine expression of these proteins in skeletal and extraskeletal tissues from fetal, newborn, and adult C57BL/6J mice. We provide an example of localization of mOCIL and mOCILrP1/mOCILrP2 in day 1 mouse long bone (Fig. 5). Both mOCIL and mOCILrP1/mOCILrP2 showed the same localization, being expressed by osteoblasts, chondrocytes in the growth plate, and skeletal muscle overlaying the bone (Fig. 5, A, E and B, F). Staining was specific since preabsorption with the peptides for the intracellular domains of mOCIL (Fig. 5C) or mOCILrP1/mOCILrP2 (Fig. 5D) abolished signals for their respective antibodies. In other experiments where mOCIL peptide was used for preabsorption and detection was for mOCILrP1/mOCILrP2, or vice versa, the peptide did not block antibody recognition, indicating specificity of the antibodies. In all other tissues examined (Table II) no difference in localization of mOCIL to mOCILrP1/mOCILrP2 was observed, suggesting that these proteins may be coordinately expressed.

**Regulation of mRNA Expression**—By Northern analysis with a mOCIL riboprobe, we observed that IL-1, IL-11, or 1,25-dihydroxyvitamin D3 treatment of murine calvarial osteoblasts in culture resulted in increased production of mOCIL mRNA (14). However, this probe detects each of the three mOCIL family members. To determine the relative contribution of mOCIL, mOCILrP1, and mOCILrP2 in response to calcitropic agents, we examined mRNA levels in ST2 cells following a 24 h treatment with PGE2, 1,25-dihydroxyvitamin D3, and dexamethasone, a treatment regimen that maximally regulates expression of transcripts that hybridize with the mOCIL probe (Fig. 6A). By Northern analysis mRNA transcripts detected by the mOCIL probe were enhanced 3.5-fold (Fig. 6A). Semiquantitative RT-PCR using primers specific for mOCIL, mOCILrP1, or mOCILrP2 established that: 1) mOCIL mRNA was increased 20-fold, 2) mOCILrP1 mRNA was not detected (even after 80 cycles of PCR), and 3) mOCILrP2 mRNA was increased 2.5-fold (Fig. 6A). In osteoclast formation assays using murine bone marrow cultures under the stimulus of 1,25-dihydroxyvitamin D3, mOCIL mRNA levels decreased after 3 days of treatment, while both mOCILrP1 mRNA and mOCILrP2 mRNA were constitutively expressed and remained unaltered over the duration of the experiment (Fig. 6B). These data indicated that in the osteoblast, mOCIL is the only family member responsive to osteotropic stimuli. Further, given that mOCILrP1 mRNA was not detected in osteoblast cell lines or primary osteoblasts, the detection of mOCILrP1/mOCILrP2 in osteoblasts by immunohistochemistry (Fig. 5, B and F) would imply that this antibody was only detecting mOCILrP2 in the osteoblast.

**DISCUSSION**

In the present study, we have identified two new C-lectins, OCILrP1 and OCILrP2, that are related to CD69, and form with OCIL, a new family of highly related molecules. OCIL, OCILrP1, and OCILrP2 are predicted to be type II membrane-bound proteins with a short intracellular domain. Each contains within their extracellular region, a hallmark C-lectin
domain (carbohydrate recognition domain) that shares ~35% homology with CD69. Apart from this similarity, each of the OCIL family members shares high nucleotide and amino acid sequence identity, genomic structure, and biological function.

Analysis of each of the genes revealed that the OCIL gene was composed of five exons, whereas the mOCILrP1 and mOCILrP2 genes both contained six exons. Each of the three genes share common intron-exon boundaries over exons 2–5 of mOCIL, which is the highest shared nucleotide identity (~90%) between the OCIL family members. The genes are divergent in their 5′-exons that encode the intracellular domains for their respective proteins. Both mOCIL and mOCILrP2 genes appear to be under the control of an inverted TATA promoter. Similar inverted TATA promoters (AATAAA) have been described in genes expressed by osteoblasts and osteoclasts, including RANKL, TRAP, cathepsin K, and bone sialoprotein (BSP) (18–22). The presence of these inverted TATA promoters may imply some tissue specificity or regulatory control for these genes. However, the expression of mOCIL and mOCILrP2 were not restricted solely to the osteoblast. In contrast, mOCILrP1 did not appear to be regulated by a classical TATA promoter, but contains a TTAAA box, a sequence that acts as a promoter for connexin 26 and glutamate dehydrogenase (23–25). In contrast to mOCIL and mOCILrP2, osteoblastic cells did not express mOCILrP1. The three genes display low nucleotide sequence identity in their 5′-flanking region. Taken together, these features support the proposition that the genes for mOCIL, mOCILrP1, and mOCILrP2 probably arose as a result of gene duplication events from a common ancestral gene. Consistent with this notion, the screening strategy used to identify genomic BAC clones for OCIL also recovered a common genomic BAC clone for mOCILrP1. The presence of these inverted TATA promoters may imply some tissue specificity or regulatory control for these genes.

![OCIL, A Family of Osteoclast Inhibitors](Image 264x521 to 563x737)

**Fig. 5. Immunohistochemistry for mOCIL and mOCILrP1/mOCILrP2 in mouse long bone.** mOCIL (A) and mOCILrP1/mOCILrP2 (B) protein expression was observed in proliferating chondrocytes (PC), hypertrophic chondrocytes (HC), and overlying skeletal muscle (SM). Controls of sections of long bone that have been preabsorbed with peptides for mOCIL (C) or mOCILrP1/mOCILrP2 (D). Panels E and F show high power views of panels A and B, respectively, in which osteoblasts are denoted (arrow). Original magnification: (A–D) ×100; (E and F) ×400.

**TABLE II**

| Tissue distribution of mOCIL and mOCILrP1/mOCILrP2 in skeletal and extraskeletal tissues from embryonic (day 15), newborn (day 1), and adult (weeks 5–8) mice by immunohistochemistry |
|---|---|---|---|
| Tissues | Fetal (day 15) OCIL3/rP2 | Newborn (day 1) OCIL3/rP2 | Adult (5–8 weeks) OCIL3/rP2 |
| Extraskeletal tissues | | | |
| Brain | ++++ | ++++ | ++++ |
| Heart | ++++ | ++++ | ++++ |
| Skin | ++++ | ++++ | ++++ |
| Kidney | ++/+ | +/+ | +/+ |
| Lung | ++++ | ++++ | ++++ |
| Liver | ++++ | +/+ | +/+ |
| Small intestine | ++++ | ++++ | ++++ |
| Skeletal tissues/cells | | | |
| Long bone | | | |
| Chondrocytes (growth plate) | ++++ | ++++ | ++++ |
| Osteoblasts | na | ++++ | ++++ |
| Osteocytes (mature) | na | –/– | –/– |
| Perichondrium/periosteum | ++++ | ++++ | ++++ |
| Narrow/megakaryocytes | na | ++++ | ++++ |
| Calvarial bone | | | |
| Osteoblasts | ++++ | ++++ | ++++ |
| Periosteum | nd | ++++ | ++++ |
or mOCILrP2 as described under samples analyzed for OCIL expression by Northern, were reversed-transcribed and subjected to semiquantitative RT-PCR for mOCIL, mOCILrP1, mOCILrP2, and GAPDH mRNA, as described under "Experimental Procedures." Relative mRNA levels were normalized for loading variability by comparison with 18S rRNA levels in the same filters. The OCIL/18S RNA ratio, normalized to control levels (1.0), is indicated at the right of the Northern blot. For RT-PCR analysis, an aliquot of the same RNA samples analyzed for OCIL expression by Northern, were reversed-transcribed and subjected to semiquantitative RT-PCR for mOCIL, mOCILrP1, or mOCILrP2 as described under “Experimental Procedures.” Relative product yield following amplification were normalized to GAPDH levels, and mOCIL/GAPDH ratios, normalized to control levels, are shown at the right. B, expression of mOCIL, mOCILrP1, and mOCILrP2 mRNAs during osteoclast formation in murine bone marrow culture. Murine bone marrow cells were cultured for 8 days in the presence of 10 nM 1,25-dihydroxyvitamin D3, PGE2 and dexamethasone, while mRNA levels for mOCILrP1 or mOCILrP2 were unchanged. Such findings suggest that calcitropic agents principally regulate OCIL mRNA but not OCILrP1 or OCILrP2 in osteoblastic cells. It remains to be determined whether mOCIL is the principally regulated OCIL family member in other tissues by growth factors, cytokines or hormones. The unique biological functions of each of the mOCIL family members may be apparent with better understanding of transcriptional or post-transcriptional regulation of each individual member.

We established an apparent widespread tissue distribution for mOCIL by in situ hybridization and immunohistochemistry using an antibody raised against a peptide in the extracellular domain of OCIL (14). However, the reagents used in our original localization studies were unable to distinguish between mOCIL, mOCILrP1, or mOCILrP2. Using antibodies to peptides specific for mOCIL and to mOCILrP2 (that may also recognize mOCILrP1) we were unable to discriminate the tissue distribution of mOCIL from that of mOCILrP2. The tissue distribution of mOCIL and mOCILrP2 expression was identical to that we have previously described for RANKL (16). This colocalization may result from RANKL, mOCIL, and mOCILrP2 using a common inverted TATA promoter ensuring concordant expression for these proteins. Further, since OCIL can counteract the osteoclast inductive ability of RANKL, OCIL may function to suppress RANKL actions at other sites. We have demonstrated that fibroblastic cells from extraskeletal sites have the capacity to support osteoclast formation under appropriate stimulus (29), and OCIL along with OPG produced by these cells may act as tonic inhibitors of this process in osteoblasts. We established that mOCIL mRNA was robustly regulated by calcitropic agents, mOCILrP2 mRNA levels demonstrated only minor variation in response to 1,25-dihydroxyvitamin D3, PGE2 and dexamethasone, while mOCILrP1 was not expressed. Similarly, mOCIL mRNA expression was down-regulated in marrow cultures during in vitro osteoclast formation, while mRNA levels for mOCILrP1 or mOCILrP2 were unchanged. Such findings suggest that calcitropic agents principally regulate OCIL mRNA but not OCILrP1 or OCILrP2 in osteoblastic cells. It remains to be determined whether mOCIL is the principally regulated OCIL family member in other tissues by growth factors, cytokines or hormones. The unique biological functions of each of the mOCIL family members may be apparent with better understanding of transcriptional or post-transcriptional regulation of each individual member.
normal physiology in extraskeletal tissues, but permit the development of multinucleated cells reminiscent of osteoclasts in certain pathologies (29).

The finding of OCIL and its related molecules raises the possibility for the development of novel antiresorptives to combat osteoporosis, cancer-induced bone loss, and bone loss associated with inflammatory diseases such as rheumatoid arthritis and periodontal disease. The identification of the receptor(s) associated with inflammatory diseases such as rheumatoid arthritis, osteoporosis, cancer-induced bone loss, and bone loss as a possibility for the development of novel antiresorptives to combat certain pathologies (29).

Acknowledgment—We thank Prof. T. J. Martin for constructive comments.

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