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# A novel promoter regulates calcitonin receptor gene expression in human osteoclasts

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**Abstract**

The calcitonin receptor (CTR) is expressed in a wide variety of tissues and cell types. In bone, its expression is restricted to osteoclasts, the cells that mediate bone resorption. The human CTR (*hCTR*) gene has a complex structural organization that exhibits similarity to the porcine (*pCTR*) and mouse (*mCTR*) CTR genes. In these species, alternative splicing of a single gene generates multiple CTR isoforms that are distributed in both tissue-specific and species-specific patterns. However, the structural organization of the 5′ putative regulatory region and transcriptional mechanisms responsible for tissue-specific expression of the different CTR isoforms are not fully defined. The present studies were undertaken to characterize the structural organization of the 5′-region of the hCTR and identify the regulatory regions involved in osteoclast-specific transcriptional activation. Analysis of mRNA prepared from human osteoclasts using reverse transcription-polymerase chain reaction (RT-PCR) and transient transfection of hCTR promoter-luciferase reporter constructs identified two regions in the 5′-flanking sequence of the *hCTR* gene that regulated CTR gene expression in osteoclasts. Both of these putative promoters were responsive to the osteoclast-inducing cytokine, receptor activator of NF-κB ligand (RANKL) and demonstrated trans-activation by the RANKL-induced transcription factor nuclear factor of activated T cells (NFATc1), consistent with a role in regulating CTR gene expression in osteoclasts.


*Keywords:* Calcitonin receptor; Human; Promoter; Osteoclast

## 1. Introduction

Calcitonin (CT) is a 32-amino-acid peptide hormone that is produced by parafollicular cells of the thyroid gland and mediates calcium homeostasis. CT binding to the calcitonin receptor (CTR) directly inhibits calcium release from bone by osteoclast-mediated bone resorption and enhances calcium excretion by the kidney [1–4]. These activities underlie the utility of CT for treatment of a variety of bone remodeling disorders [5–10].

The CTR is a member of the class II G-protein-coupled receptor family, and contains seven transmembrane domains [11]. CTR is expressed in a variety of tissues and cell types [12–19], including the brain and pituitary (human) [13], lung (porcine) [15], testicular cells (rat) [16], placenta (human) [17,20], kidney (porcine) [18], and osteoclasts (human, rat, and mouse) [19–22], where it has been used as a marker for terminal osteoclast differentiation [23,24]. In addition, the CTR is also expressed in human tumors and human tumor cell lines [14,25–30].

The human CTR (*hCTR*) gene has a complex structural organization and exhibits significant similarity to the porcine (*pCTR*) and mouse (*mCTR*) CTR genes. In the three species, alternative splicing of a single gene generates multiple CTR isoforms, which are functionally distinct in terms of ligand binding specificity and/or signal transduction pathway utilization, and are distributed in both tissue-specific and species-specific patterns [11,14,18,28,30–40]. At least six different isoforms of the hCTR mRNA have been described [11]. Each of

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these cDNAs would be predicted to generate proteins with different structural features.

Utilizing human kidney cells, tissue from human giant cell tumors (GCT) of bone (comprised of 20% to 30% osteoclast-like cells), and a breast cancer cell line (T47D), Hebden et al. identified two exons in the 5′ untranslated region (UTR) sequence of the hCTR, designated exon 1b and exon 1a [41], which are homologous to the mCTR exons 1 and 2 [42]. They further demonstrated that the *hCTR* gene was regulated by two separate promoters, hP1 and hP2, in the T47D breast cancer cell line. Using 5′-RACE (Rapid Amplification of cDNA Ends), Nishikawa et al. [43] identified additional transcribed products in the 5′-UTR of the hCTR equivalent to pCTR exons 1 and 2. They also identified a novel 288 bp hCTR isoform (exon Oc) spliced to exon 3b that they speculated was uniquely expressed in osteoclasts. Analysis of the *mCTR* gene has revealed the presence of three putative promoters one of which (P3) is active exclusively in osteoclasts [19]. It is unclear if there is a regulatory region that is homologous to the mouse P3 in the *hCTR* gene.

The identification of receptor activator of NF kappa B ligand (RANKL) [44,45] as an essential mediator of osteoclast differentiation has focused attention on the signal pathways that are transduced through its receptor, receptor activator of NF-κB (RANK) [46]. RANK activates multiple transcription factors that have essential roles in osteoclast-specific gene expression. These include, for example, nuclear factor of activated T cells (NFATc1) [47], NF-κB [48–50] and c-fos [51]. NFATc1 has been shown to mediate RANKL induction of multiple osteoclast-associated genes, including tartrate-resistant acid phosphatase (TRAP), cathepsin K (Cath K) [52], osteoclast associated receptor (OSCAR) [53], β3-integrin [54], matrix metalloproteinase-9 (MMP-9) [55] and CTR [47,53].

The present studies were undertaken to identify the regulatory regions involved in osteoclast-specific transcriptional activation of the hCTR. Results indicate the presence of two putative promoters that are responsive to RANKL and are transactivated by NFATc1, consistent with their role in regulating CTR gene expression in osteoclasts.

## 2. Materials and methods

### 2.1. Phylogenic sequence analysis

The mouse and human genomic sequences encompassing the hCTR gene were identified by BLAST searches (www.ncbi.nlm.nih.gov/BLAST). Sequence manipulation and alignment of 15 kb of the mouse and human promoter regions were completed using Pustell matrix (available in MacVector). We specifically compared a 5000-bp sequence upstream of the human Oc genome [43] to the mouse genome by VISTA (www.gsd.lbl.gov/vista/index.shtml) to identify regions of high identity and conserved regulatory elements. Putative transcription factor binding sites in the upstream 4728 bp of the *hCTR Oc* gene were identified using MATCH based on the TRANSFAC4.0 matrices (transfac.gbf.de/TRANSFAC/) with a core match of 1.0. NFAT: AP1 composite sites were identified using Model Inspector (Genomatix software GmbH).

### 2.2. Human bone marrow culture

Human bone marrow cells were obtained from the proximal femur at the time of total joint arthroplasty. The study protocol was approved by the New England Baptist Hospital Institutional Review Board (IRB), and informed consent was obtained from all patients prior to surgery. Samples were collected in syringes containing 1000 U/ml of preservative-free heparin. The bone marrow cells were diluted in α-MEM (Invitrogen, Carlsbad, CA) and mononuclear cells were isolated by centrifugation over a HISTOPAQUE 1077 density gradient (Sigma, St. Louis, MO). Cells were seeded and non-adherent cells were isolated after overnight culture in the presence of 200 pg/ml human GM-CSF (R&D Systems, Flanders, NJ). The non-adherent cells ($5\times10^5$ cells/well) were cultured in the presence of 100 pg/ml human GM-CSF for 6 days (phase 1) and further cultured with 30 ng/ml human M-CSF and 30 ng/ml human soluble RANKL (sRANKL) (R&D Systems, Flanders, NJ) for 7 days (phase 2) in 0.3 ml of α-MEM containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) in 48-well plates (Corning, Corning, NY). TRAP staining was performed to identify osteoclast-like cells using a histochemical technique [56]. After 14 days, human osteoclast-like cells were generated in replicate 6-well plates for RNA extraction.

Table 1
Sequences of primers used for RT-PCR

| Name | Primer sequences |
|---|---|
| E1F | TTAGGGGGAAAGAAGAGGAGTCG |
| E2F | CCCTGCGGCTGACATCTCCTGC |
| OcF | GAGAGTGGGTGGGAGATAATGT |
| E5R | CTGGGCAGAACTGATAGGACA |

### 2.3. RNA preparation and PCR amplification

Total RNA isolated from human tissues including the brain, kidney, mammary gland, placenta and liver, were purchased from Stratagene (La Jolla, CA). Human osteoclast-like cells were prepared, as described above. GCTs of bone were obtained from discarded surgical tissue from the Beth Israel Deaconess Medical Center, with approval by the Beth Israel Deaconess Medical Center IRB. The human ovarian tumor cell line (BIN-67) was used as in previous studies [28]. The total RNA from osteoclast-like cells, GCT of bone and the human ovarian tumor cell line were isolated by using the TRIzol Reagent (Invitrogen, Carlsbad, CA) and purified following manufacturer's instructions.

cDNA was transcribed from 1 μg of total RNA by a reverse transcription kit (Invitrogen, Carlsbad, CA) using a mixture of specific primers to hCTR and Oligo dT primers. To identify the isoforms expressed in different tissues and cell types, three sets of primers encompassing the sequences between exon 1 and exon 5 were designed. Primer sequences are listed in Table 1. cDNA was denatured at 94 °C, 30 s, annealing at 54 °C, 30 s and extension temperature at 72 °C, 2 min for 55 cycles using the PCR SuperMix containing Taq polymerase enzyme (Invitrogen, Carlsbad, CA). The PCR products were purified by gel extraction using a QIAGEN gel extraction kit and subcloned into pPCR-Script AMP SK(+) plasmid (Stratagene, La Jolla, CA) for DNA sequence analysis.

### 2.4. Promoter-reporter construction

Two CTR fragments of 1.1 kb and 4.0 kb overlapping a 5.017-kb region containing the potential hCTR osteoclast-specific promoters (P3 and POc) were amplified from human genomic DNA (Promega, Madison, WI) by PCR (Fig. 1). The sequences of the primers, designed to contain restriction enzyme sites (*Nhe*I and *Xho*I), are shown in Table 2.

The two PCR products were digested with *Nhe*I and *Xho*I (New England Biolabs, MA). The pGL3-Basic luciferase reporter vector (Promega, Madison, WI) was prepared by digestion with *Nhe*I and *Xho*I (New England Biolabs, MA). The CTR fragments were ligated to the linearized pGL3-Basic Vector with a T4 ligase kit (Promega, Madison, WI). The resulting promoter-reporter constructs were designated pGL3 hP3-1.1 and pGL3 hPOc-4.0. Plasmids were transformed into JM109 cells (Promega, Madison, WI). The JM109 cells were grown and plasmids were isolated by EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA). The pBJ5-human NFATc1 expression plasmid (pSH102) was a generous gift from G. R. Crabtree.