1,25-dihydroxyvitamin D3 inhibits thyroid hormone-induced osteocalcin expression in mouse osteoblast-like cells via a thyroid hormone response element

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Abstract

Thyroid hormones are important regulators of bone development and metabolism. We have demonstrated that tri-iodothyronine (T3) increased and 1,25-dihydroxyvitamin D3 (1,25D3) attenuated the T3-stimulated expression of osteocalcin (OCN) in the osteoblast-like cell line MC3T3-E1. By means of transfection of promoter–reporter gene constructs we investigated the basal and the regulated transcription of this gene by both hormones. We found that a 0.67 kbp and a 1.3 kbp fragment of the mouse OCN OG2 promoter containing two Runx2 binding sites were significantly more active than a smaller fragment containing only one Runx2 binding site. The longer promoter fragments showed a higher reporter gene expression when the transfected cells were treated with 10−7 M T3. This expression was attenuated by 1,25D3 dose-dependently. These fragments contain a sequence homologue to the recently identified binding site for the 1,25D3 receptor (VDR) in the rat OCN promoter. Deletion of a part of the promoter containing this VDR response element-like sequence (VDRE) resulted in a higher basal expression but abrogated the regulation by T3 and 1,25D3. Electrophoretic mobility shift assays revealed that the deleted sequence was able to bind both in vitro-translated chicken thyroid hormone receptor (TR) and proteins from nuclear extracts that reacted with an antiserum against TR. From these data we conclude that the VDRE-like sequence of the OG2 promoter contains a thyroid hormone response element.

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Introduction

Thyroid hormones are essential for development and maintenance of the skeleton (Mosekilde et al. 1990, Allain & McGregor 1993) and regulate multiple bone cell functions (Klaushofer et al. 1995). The main non-collagenous protein of the bone matrix is osteocalcin (OCN). This protein is expressed by mature osteoblasts and its expression is highly regulated by many local and humoral factors. Interestingly, a species-linked difference between mouse and rat in the regulation of its expression by tri-iodothyronine (T3) and 1,25-dihydroxyvitamin D3 (1,25D3) respectively has been observed. While T3 increases the mRNA levels of OCN in mouse osteoblasts (Varga et al. 1997), OCN expression responds only weakly to T3 in rat osteosarcoma cells (Williams et al. 1995, Gouveia et al. 2001). 1,25D3, however, increases the expression of OCN in human (Kerner et al. 1989) and rat osteoblasts (Chen et al. 1986, Williams et al. 1995), but inhibits its basal expression in mice (Zang et al. 1997). In rat osteoblasts, the stimulation of OCN expression by 1,25D3 is considered to be a direct effect on transcription, whereas the inhibitory effect on basal expression in mouse osteoblasts is mediated by an indirect mechanism. It was shown that 1,25D3 treatment of primary osteoblasts abolishes the binding of the osteoblast-specific activator of transcription Runx2 (Cbfa1, OSF2), which binds to OSE2 (Ducy & Karsenty 1993), a critical...
osteoblast-specific cis-acting element present in both OCN promoters OG1 and OG2 (Zang et al. 1997) and other genes expressed in osteoblasts.

Steroid hormone receptors usually regulate gene expression by binding as a homodimer/heterodimer to the respective response elements. A steroid hormone response element is defined as a DNA sequence which binds selectively the steroid hormone receptor. This sequence consists of two or more repeats (‘sites’), which can be oriented directly or inversely. Deletion analysis of the rat OCN promoter has demonstrated that there is a 25 bp 5' flanking region of the coding sequence able to confer 1,25D3 responsiveness to either the native OCN promoter or a heterologous viral promoter (Demay et al. 1990). Recently, a 3–4–5 rule was postulated, which suggests that the distance between two homologue ‘half-sites’ of such response elements should be three nucleotides for the 1,25D3 receptor (VDR), four nucleotides for the thyroid hormone receptor (TR) and five nucleotides for the retinoic receptors (Umesono et al. 1991). Sequence comparison of the rat 1,25D3 response elements (VDRE) with the mouse OCN OG2 promoter revealed a homologue sequence that differs in 3 bp (Zang et al. 1997). This led us to the hypothesis that this element also confers T3 responsiveness to the mouse OCN promoter.

Using an electrophoretic mobility shift assay (EMSA) we demonstrate that this VDRE-like sequence of the OG2 promoter binds the TRα1. We further show by deletion analyses of transfected OG2 promoter–reporter–vector constructs that this sequence is important for the basal and the T3-regulated expression of OCN. Furthermore, we also found that 1,25D3 inhibited the T3-regulated expression of OCN, and we present evidence for an involvement of the VDRE-like sequence in this attenuation process.

### Material and methods

#### Cell culture

MC3T3-E1 cells (kindly donated by Dr Kumegawa, Meikai University, Department of Oral Anatomy, Sakado, Japan), and MC3T3-E1 clone 30 (kindly donated by Dr Franceschi, Department of Periodontics, University of Michigan, USA), and rat osteosarcoma cells (ROS 17/2·8) were cultured in αMEM (Sigma), supplemented with 4·5 g/l glucose, 5% fetal calf serum (FCS) (Sigma) and 30 µg/ml gentamycin (Sigma) at 37 °C under 5% CO₂ in humidified air. They were sub-cultured twice a week using 0·001% pronase E (Roche) and 0·02% EDTA in Ca²⁺- and Mg²⁺-free PBS. To prevent a potential phenotypic drift during repeated sub-cultures the cells were not used more than 4 weeks after thawing.

#### RNA extraction and Northern analysis

For Northern analysis cells were seeded at a density of about 20 000 cells/cm² and cultured in the medium described above. After 6 days of culture a medium change was performed and 24 h later cells were treated with or without 10⁻⁷ M T3 or 10⁻⁸ M 1,25D3 for 48 h. RNA was isolated using TRIZOL reagent (Sigma) according to the supplier’s suggestions. The total amount of RNA was estimated by measuring the absorption at 260 nm with a spectrophotometer. Northern hybridisation was performed by fractionating 10 µg total RNA on a 1% agarose gel containing 2·2 M formaldehyde. After electrophoresis the gel was transferred to a nylon filter (NEN, Brussels, Belgium) with 20 × SSC (1 × SSC is 0·15 M sodium chloride and 0·015 M sodium citrate). After baking the filter for 2 h at 80 °C, hybridisation was done overnight in 10% SDS, 1 M sodium phosphate buffer pH 7·4, after 1 h pre-hybridisation in the same solution. For estimation of the amount of hybridised mRNA, the filters were exposed and evaluated in an InstantImager (Packard Instrument Company, Meridien, CT, USA). As hybridisation probe we used the mouse OCN cDNA (Celeste et al. 1986). As a control we hybridised the same Northern blots with an EcoRI cDNA fragment of the human ribosomal cDNA. The probes were labelled by multi-prime labelling according to the supplier’s suggestions (Roche).

#### Semiquantitative RT-PCR

The RNA was isolated as described above and 1 µg RNA was reverse transcribed by Moloney murine leukaemia virus reverse transcriptase (Invitrogen) with random primers. For PCR we used as primers for Runx2 5'-GAG GGC ACA AGT TCT ATC TGG A and 5'-GGT GGT CCT CCG CTA TCT C and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 5'-CTG CAC CAC CAA CTG
CTT AGC C and 5'-GTC CAC CAC CCT GTT GCT GTA G. Amplification was performed with RedTaq DNA-Polymerase (Sigma) with an initial denaturation step at 94 °C for 5 min and thereafter 1 min at 94 °C, 2 min at 57 °C and 2 min at 72 °C (40 cycles) or 1 min at 94 °C, 2 min at 54 °C and 2 min at 72 °C (25 cycles) respectively. The PCR fragments were resolved by 1% agarose gel electrophoresis.

Vectors

The 0·16 kbp and the 1·3 kbp promoter fragment of the OG2 OCN promoter (Ducy & Karsenty 1995) were kindly provided by G Karsenty (Department of Molecular Genetics, University of Texas, Houston, TX, USA). All constructs were first cloned into the KpnI/XhoI site of the pGL3 basic luciferase reporter vector. Due to an influence of T3 on the luciferase expression all constructs were sub-cloned into the KpnI/HindIII site of the SEAP2 basic vector (secreted form of the human placental alkaline phosphatase) (Clontech, Palo Alto, CA, USA). A 670 bp promoter fragment (pOG2–0·67) extending from +13 to 657 was constructed by PCR using the primers 5'-TGG TCG ACT TGT CTG T-3' and 5'-CCA AGA CCT GGC CCA G-3' (Ducy & Karsenty 1995). The deletion was introduced by amplification of the pOG2–0·67 using two additional primers, 5'-CCC ACT GGA TGA GCA GAG CTG CCC TGG GGT TTG GCT CCC-3' and 5'-GGG AGC CAA ACC GGG CAG CTC TGC TCA TCC AGT GGG-3' (Ducy & Karsenty 1995). The oligonucleotides were commercially synthesised. We used the 40 bp fragment 5'-TCG ACC CTG AAC TGG GCA AAT GAG GAC ATT GGT ACT TGT CTG T-3' and 5'-CCA AGA CCT GGC CCA G-3' (Ducy & Karsenty 1995) containing the homologue sequence to the VDRE from rat. As a positive control we used the rat growth hormone (rGH) 5'-TRE AAA GGT AAG A TCA GGG ACG TGA CCG CAG (Williams & Brent 1995). The probes were labelled by polynucleotide kinase and [γ-32P]ATP and used at a concentration of 50 fM. The assay was performed in a volume of 20 µl with about 5 µg nuclear extracts (Dignam et al. 1983) from MC3T3-E1 cells, in 20 mM Hepes, 80 mM KCl, 0·1% Nonidet NP-40, 1 mM dithiothreitol, 2 µg/ml poly dI/dC. Alternatively, we used 4 µl in vitro-translated TR from the chicken–erbA(α) cDNA using the in vitro translation system from reticulocytes (Promega). The protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide: bisacrylamide 80:1) run in 0·25× Tris–borate–EDTA. Gel shift immunoassay experiments were performed by incubating the protein/DNA complex with 1 µl anti-thyroid hormone receptor antibody (ABR, Golden, CO, USA) for 30 min. The gels were exposed to the InstantImager.

Transfection experiments

Transfection experiments were performed in 48-well plates (Nunc, Roskilde, Denmark). Cells were seeded at a density of 36 000 cells/cm² and cultured overnight. On the next day medium was removed and cells were transfected with both 0·25 µg OCN promoter–vector construct and 0·25 µg cDNA of the chicken TRα (kindly provided by M Zenke, IMP, Vienna, Austria) in DOSPER (Roche), with a ratio of 1:5 in 120 µl serum-free medium. On the next day the medium was changed to the culture medium described above. Two days later medium was changed again to the same medium with or without 10−7 M T3. Forty-eight hours later 50 µl supernatant were removed and analysed for SEAP activity according to the supplier's suggestions (Roche). Chemoluminescence was measured in a TopCounter (Packard) and statistical analysis (ANOVA) was performed using the Statview 4·5 software package (Abacus Concepts Inc., Berkley, CA, USA).

EMSA

Oligonucleotides were commercially synthesised. We used the 40 bp fragment 5'-TCG ACC CTG AAC TGG GCA AAT GAG GAC ATT GGT ACT TGT CTG T-3' and 5'-CCA AGA CCT GGC CCA G-3' (Ducy & Karsenty 1995) containing the homologue sequence to the VDRE from rat. As a positive control we used the rat growth hormone (rGH) 5'-TRE AAA GGT AAG A TCA GGG ACG TGA CCG CAG (Williams & Brent 1995). The probes were labelled by polynucleotide kinase and [γ-32P]ATP and used at a concentration of 50 fM. The assay was performed in a volume of 20 µl with about 5 µg nuclear extracts (Dignam et al. 1983) from MC3T3-E1 cells, in 20 mM Hepes, 80 mM KCl, 0·1% Nonidet NP-40, 1 mM dithiothreitol, 2 µg/ml poly dI/dC. Alternatively, we used 4 µl in vitro-translated TR from the chicken–erbA(α) cDNA using the in vitro translation system from reticulocytes (Promega). The protein/DNA complexes were separated in a 4% polyacrylamide gel (acrylamide:bisacrylamide 80:1) run in 0·25× Tris–borate–EDTA. Gel shift immunoassay experiments were performed by incubating the protein/DNA complex with 1 µl anti-thyroid hormone receptor antibody (ABR, Golden, CO, USA) for 30 min. The gels were exposed to the InstantImager.

RIA of OCN

To estimate the concentration of OCN protein in the culture media we used a commercial RIA kit (Biomedical Technologies Inc., Stoughton, MA, USA). According to the supplier’s description of the RIA, there is no cross-reaction with bovine OCN and the RIA did not detect any measurable OCN in the culture media. Cells were seeded at a density of 50 000 cells/cm² in 96-well culture plates in 1,25-dihydroxyvitamin D3 inhibits osteocalcin expression · F VARGA and others

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αMEM supplemented with 5% FCS and cultured overnight. On the next day cultures were untreated or treated with $10^{-7}$ M T3 or $10^{-8}$ M 1,25D3. At day 4 the culture media were removed and frozen. We performed single measurements of 10 µl culture media from each well. The results are given as ng OCN/ml culture media.

**Results**

As previously shown, T3 increases the expression of OCN in MC3T3-E1 osteoblast-like cells after a 24 h T3 treatment. This stimulation is time- and dose-dependent and depends on the cell density (Varga et al. 1997, 1999). The basal expression is inhibited by 1,25D3 (Zhang et al. 1997) and both this inhibition and the stimulation by T3 was transmitted to the protein level (Fig. 1A). Recently, several clones of the original MC3T3-E1 cell line were derived (Wang et al. 1999). MC3T3-E1 clone 30, in contrast to the original MC3T3-E1 cell line studied previously (Varga et al. 1997), expresses Runx2 at moderate levels (Fig. 1B) but just after seeding no OCN could be detected by Northern blot (Fig. 2). In this MC3T3-E1 clone, T3 also increased the expression of OCN (Fig. 2).

Sequence comparison of the mouse OG2 promoter with that of the rat revealed a homologue sequence to the rat VDRE that contained a putative TRE. This element consists of a conserved 'half-site' while the second one that is 4 bp distant includes one mutation (Fig. 3A, notice the arrow) but should also bind TRα1, although with reduced affinity (Judelson & Privalsky 1996). We used this VDRE-like sequence with several base pairs flanking the 5' and 3' direction to perform EMSAs using either *in vitro*-translated TR (Fig. 4A) or nuclear extracts from MC3T3-E1 cells (Fig. 4B). The oligonucleotides containing the VDRE-like sequence bound both the *in vitro*-translated TR and the TR present in nuclear extracts of MC3T3-E1 cells. The latter was found to react with an antiserum against TRα1 as demonstrated by a decrease of the electrophoretic mobility (Fig. 4B). Surprisingly the binding of the antibody seemed also to increase the affinity of the TR.

For transfection experiments we constructed several shortened fragments of the OG2 promoter and a 0.67 kbp fragment with a deletion of the 40 bp containing the VDRE-like sequence (Fig. 3B). After transfection of the 0.16 kbp OG2 fragment into original MC3T3-E1 cells we found a 2-fold increase of the reporter gene transcription compared with the promoter-free (basic) vector (Fig. 5). The longer fragment (0.67 kbp) containing the VDRE-like sequence showed a ~7-fold increase while the 1.3 kbp fragment displayed only a 4-fold increase of the reporter gene transcription. The deletion mutant (6TRE), however, showed the highest basal activity (Figs 5 and 6).
In the original MC3T3-E1 cells, treatment of these transfections with T3 for 48 h resulted in no increase of the reporter gene transcription, either of the basic vector, indicating no influence of T3 on the assay system, or of the smallest fragment, indicating that there was no response element in this short promoter fragment (Fig. 5). Both the 0·67 kbp fragment and the 1·3 kbp fragment responded with a 2·5- or 3-fold increase of the transcription after 48 h of treatment with T3. The promoter fragment which lacks that part of the promoter containing the VDRE-like sequence ( δTRE) showing the highest basal expression, did not respond to T3 treatment, indicating that the deleted sequence is important for repression of the basal as well as the T3-stimulated OCN expression (Fig. 5).

It was found that 1,25D3 inhibited the basal expression of OCN in mouse osteoblasts (Fig. 1A; Zhang et al. 1997). This prompted us to test whether 1,25D3 could also attenuate the T3-stimulated OCN expression. Figure 2 shows that 1,25D3 dose-dependently attenuated the T3-induced OCN expression as similarly found in the original MC3T3-E1 cells (data not shown). Transfection of the 0·67 kbp fragment into MC3T3-E1 clone 30 and treatment with T3 resulted in a 4-fold increase of the reporter gene transcription and as found with Northern blot 1,25D3 dose-dependently attenuated this transcription (Fig. 6A). Transfection of the δTRE construct into MC3T3-E1 clone 30 yielded also an increase of the basal transcription as found with original MC3T3-E1 cells (Figs 5 and 6B). Treatment of these transfected cultures with T3 or with both T3 and 1,25D3 resulted in a tendency to decrease the basal transcription (Fig. 6B) but the effects were statistically not significant.

Discussion

By means of transfection experiments we have shown that the part of the mouse OG2 promoter...
containing a sequence which is a homologue to the VDRE of the rat OCN promoter was important for the T3 regulation of the mouse OCN expression but also for the inhibition by 1,25D3. We have further shown that this part of the promoter also bound the TR. In addition, we found that the longer promoter fragment with 0.67 kbp, containing both Runx2 binding sites, had a much higher basal activity than the truncated one (0.16 kbp) containing only the proximal Runx2a binding place. But surprisingly, this seemed to be independent of Runx2 because the transfected promoter–reporter gene constructs showed a comparable basal and T3-dependent expression whether transfected into the Runx2-expressing MC3T3-E1 clone 30 or into the original MC3T3-E1 cells that express no Runx2. One explanation of this contradictory result could be that, although not detected by PCR, the original MC3T3-E1 cells express enough Runx2 protein to enable basal expression of the reporter gene expression. This explanation, although not very likely, could also explain the higher expression of the constructs containing two Runx2 binding sites. Otherwise, we have to postulate other transcription factors (Ducy & Karsenty 1995) or homologues of Runx2 or yet unidentified splicing variants.
Recently, it was published that the functional Runx2 sites found in the OG2 promoter, contribute differentially to the basal OCN expression (Frendo et al. 1998). The Runx2a site next to the transcription start located between /p1\_137 and /p1\_131 was described as being more important for bone-specific transcription from the OG2 promoter than the more distally located Runx2b site at /p1\_608 to /p1\_602. A mutation of the Runx2a binding site results in a strong decrease of the reporter gene transcription but the mutation of the Runx2b site results only in a marginal decrease.

Similar results were found in transgenes carrying these mutated reporter gene constructs (Frendo et al. 1998). The difference between our findings and those of Frendo et al. (1998) in the contribution of the Runx2a and Runx2b binding sites of the OG2 promoter to the basal reporter gene transcription could be due to other binding sites for transcription factors upstream from the Runx2a site, and deletion of this upstream region could abolish the interaction with Runx2 protein resulting in a reduced basal expression from this short promoter fragment.

In the rat OCN promoter a VDRE was identified which could be also found in the mouse OCN OG2 promoter, although with three nucleotides changed. Deletion of this VDRE-like sequence of the OG2 promoter resulted in a reporter gene expression between the basal and T3-stimulated expression in both cell lines studied. The TR, unlike other classical steroid receptors, is able to bind to the TREs in the absence of the hormone and mediates a decrease of basal expression. In the presence of its ligand, TR binds specifically to the TRE of genes and induces their expression (Brent et al. 1989, Graupner et al. 1989). In common with the fact that an oligonucleotide containing the rat VDRE-like sequence bound in vitro-translated TRα1 we can conclude that this DNA fragment behaves like a typical TRE (Williams & Brent 1995).

The VDRE-like sequence, which is a homologue to the VDRE in the rat OCN promoter that is important for 1,25D3-regulated OCN expression in rat osteoblasts, could also be interpreted as a TRE consisting of two ‘half-sites’ separated by four nucleotides. However, while the 3’ ‘half-site’ is well conserved the second one located 4 bp distant contains one base pair which reduces binding of TRs (Judelson & Privalsky 1996). This could explain the relatively weak interaction found in the EMSA. Interestingly, addition of the antibody resulted in an obvious increase in binding of the TR isolated from nuclear extracts. Interaction of a receptor with its response element depends on the affinity of both partners. Change of affinity could be achieved by either changing the nucleotide sequence of the response elements (Katz et al. 1995, Judelson & Privalsky 1996, Staal et al. 1996) or change of the conformation of the receptor protein as often found in mutated receptors. This opens a field for a wide variation of modulation for such an interaction. Variation of the nucleotide composition and the distance of ‘half-sites’ of the response elements determines not only the type of the binding receptor (Kato et al. 1995, Klinge et al. 1997) but can also change the conformation of the receptor protein and from that the change of the antigenic determinants as found with a set of antibodies against the VDR (Staal et al. 1996).
a change could also modulate the set of accessory proteins interacting with the steroid receptors (Seol et al., 1995, Hollenberg et al., 1996). The binding of accessory proteins could also change the conformation and therefore the affinity as recently described for the oestrogen receptor (Romine et al. 1998). The antibody used in our experiments could substitute for such a protein. This would explain the increase in the binding affinity of TR to the TRE as found in the EMSA.

Interestingly, T3 did not induce a reporter gene construct containing the rat OCN gene 5′-flanking region (−1750 bp) or the previously described rat OCN VDRE when transfected into ROS 17/2.8 cells in spite of the high homology between the rat VDRE and the putative TRE in mouse (Gouveia et al. 2001). Multiple Runx2 binding sites are required for basal and 1,25D3-responsive transcription (Javed et al. 1999). The different distribution of the Runx2 binding sites (Ducy & Karsenty 1995) and hence a different preferred formation of complexes of steroid receptors with Runx2 could be responsible for this species-dependent difference in the regulation of OCN expression. But as mentioned, instead of Runx2 other accessory proteins could be important.

The difference of the nucleotide sequence could also be responsible for the species difference in the regulation of OCN expression between rat and mouse as it has been found that no VDR binds to the VDRE-like sequence in mouse OG2 promoter. Moreover, it was found that 1,25D3 inhibited OCN expression in mouse osteoblasts by an indirect mechanism (Zhang et al. 1997). These authors found that mutation of the Runx2a site resulted in an abrogation of the 1,25D3 inhibition of the basal expression of a reporter gene construct. We found that 1,25D3 also attenuated T3-induced OCN transcription as found with Northern blots in both the original MC3T3-E1 cell line (data not shown) and MC3T3-E1 clone 30. Transfection of OG2−0.67 kb reporter gene constructs into the latter cell line resulted in an increase of the transcription and 1,25D3 dose-dependently attenuated this transcription. Deletion of the sequence containing the postulated TRE (OG2−dTRE) resulted in an abrogation of the dose-dependent regulation by both T3 and 1,25D3. Furthermore, both hormones reduced the basal expression, although not significantly. These data suggest a different mechanism between the basal and the T3-regulated OCN expression because T3 did not regulate Runx2 expression (data not shown). While a direct interaction of TR and VDR by binding is discussed controversially, a regulatory cross-talk was found (Yen et al. 1996). Depending on the sequence of the response elements, binding of VDR/retinoid X receptors heterodimers to TREs can modulate T3-mediated transcription. Not surprisingly, the inverse mechanism that TRs can also repress VDR-mediated transcription was also found with reporter gene constructs of the rat OCN and rat 24-hydroxylase promoter (Raval-Pandya et al. 1998). Further studies will reveal these regulatory interactions between 1,25D3 and T3 in the regulation of OCN expression and the obscure species difference.

In summary, we have shown that a part of the mouse OG2 promoter containing a rat VDRE-like sequence was able to bind the TRα1 and functions as a TRE in a reporter gene assay. 1,25D3 attenuated the transcription from this promoter possibly by a mechanism different from its effect on basal transcription. We further found that this regulation could be abolished by deletion of that part of the promoter containing the postulated TRE. Being aware that deletion of this sequence could change the conformation of the DNA and influence the regulatory behaviour of the promoter, our data suggest that T3 regulates the expression of OCN by increasing its transcription via binding to a TRE, located in the OG2 promoter.

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