Thyroid hormone stimulation of osteocalcin gene expression in ROS 17/2·8 cells is mediated by transcriptional and post-transcriptional mechanisms

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Abstract

We investigated the mechanism of thyroid hormone regulation of osteocalcin (OC) gene expression in osteoblast-like cells (ROS 17/2·8). Treatment with tri-iodothyronine (T_3) (10^{-8} M) increased OC mRNA levels by ~3-fold after 24 h and reached a maximum, ~5·4-fold, after 48 h. The mRNA levels of other bone-specific genes, alkaline phosphatase and osteopontin, were not affected by T_3 treatment. Interestingly, T_3 induction of OC mRNA varied according to cell density: ~4-fold at $\sim 1 \times 10^5$ cells/dish and 1·5-fold at $40\text{--}60 \times 10^5$ cells/dish. The magnitude of OC mRNA induction by T_3 was ~40% lower than induction by 1,25 dihydroxyvitamin D_3 (1,25 D_3) alone, and the combination of T_3 +1,25 D_3 did not further stimulate OC mRNA levels. T_3 induction of OC mRNA was not affected by treatment with cycloheximide (10 μ g/ml) for 5 h indicating that new protein synthesis is not required for the response. To study the half-life of OC mRNA, ROS 17/2·8 cells were incubated

with actinomycin D. The basal half-life of OC mRNA (means $\pm$ S.E.M.) was $6\cdot4 \pm 0\cdot2$ h which was increased significantly with either T_3 or 1,25 D_3 treatment to $10\cdot9 \pm 0\cdot6$ h and $13\cdot5 \pm 0\cdot4$ h respectively. T_3 modestly up-regulated the rate of OC gene transcription (1·7 $\pm$ 0·2-fold) as determined by run-off assay. T_3 did not induce a reporter construct containing the rat OC gene (rOC) 5'-flanking region (to -1750 bp) or the previously described rOC vitamin D response element, when transfected into ROS 17/2·8 cells. In conclusion, T_3 up-regulates the OC mRNA expression in ROS 17/2·8 cells in a dose-, time- and cell confluence-dependent fashion, and does so by transcriptional and post-transcriptional mechanisms. The greater T_3 induction of OC expression in ROS 17/2·8 cells at low cell density is consistent with findings of thyroid hormone action on bone development.

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Introduction

Thyroid hormone is essential for normal skeletal development, maturation and metabolism. Hypothyroidism impairs skeletal maturation and leads to epiphyseal dysgenesis, resulting in growth retardation and skeletal abnormalities (Allain & McGregor 1993). In adults, thyrotoxicosis is frequently associated with increased bone turnover and decreased bone mass, which is the result of excessive bone resorption relative to formation (Mosekilde & Melsen 1978, Krolner *et al.* 1983).

Thyroid receptors (TRs) have been demonstrated in rat, mouse and human osteoblasts (Rizzoli *et al.* 1986, Sato *et al.* 1987, LeBron *et al.* 1989, Allain *et al.* 1996). TR α and β isoforms are present, in varying proportions, in three osteosarcoma cell lines with phenotypes of fibroblast, preosteoblast and osteoblast like (ROS 25/1, UMR 106

and ROS 17/2·8 respectively) (Williams *et al.* 1994). In addition, the expression of TR β_1 is modulated during osteoblast-like cell differentiation, suggesting an important role in bone development. TR $\alpha 1/\alpha 2$ and TR α/β combined knockout mice have a number of bone defects including: delayed bone maturation, impaired bone mineralization and delayed ossification of epiphysis and diaphysis (Fraichard *et al.* 1997, Gauthier *et al.* 1999, Göthe *et al.* 1999).

Despite the multiple *in vivo* effects of thyroid hormone on bone, only a few genes directly regulated by tri-iodothyronine (T_3) have been identified. In MC3T3 murine osteoblast cells, the collagenase-3 and gelatinase B genes are up-regulated by T_3 and may mediate increased collagen degradation in cultured intact rat calvariae (Pereira *et al.* 1999). For both of these genes, T_3 -induced mRNA accumulation is mediated by

transcriptional mechanisms, and mRNA half-life is not influenced by T₃.

A key osteoblast protein shown to be regulated by thyroid hormone is osteocalcin (OC), or bone gla protein, a small protein that contains 45–60 amino acid residues. It is the major non-collagenous protein (10%) of the bone extracellular matrix and is synthesized almost exclusively by mature osteoblasts during the late stages of differentiation and during the onset of matrix mineralization (Aronow *et al.* 1990). The function of OC is not completely understood; however, it is believed to play a role in bone formation and remodeling. OC knockout mice have increased bone density, suggesting that OC is an inhibitor of bone formation and that it may be required for bone mineral maturation (Ducy *et al.* 1996, Boskey *et al.* 1998). OC has also been shown to play a role in bone resorption (Glowacki & Lian 1987, McSheehy & Chambers 1987).

The close correlation of the serum concentration of OC and thyroid status has been shown in a number of clinical studies (Yoneda *et al.* 1988, Kojima *et al.* 1992, Lenger *et al.* 1997). In hyperthyroid patients, there is a linear correlation between serum levels of OC and either free thyroxine (T₄) or free T₃. Hypothyroid patients have reduced levels of serum OC, which are rapidly normalized after T₄ treatment. Rats treated with excessive levels of thyroid hormone have a dose-dependent increase in the levels of OC mRNA in the femur (Ross & Graichen 1991, Kung & Ng 1994). *In situ* hybridization experiments document that T₄ treatment increases expression of OC mRNA in femoral osteoblasts, but not in vertebral osteoblasts (Kung & Ng 1994), pointing towards a differential response of vertebral and femoral osteoblasts to thyroid hormone. Rats treated with high dose T₄ for 28 days have reduced bone mineral density in the femur but not in the spine, corresponding to the regions of thyroid hormone-induced OC mRNA expression (Gouveia *et al.* 1997). This is also in agreement with clinical findings of reduced femoral rather than vertebral bone mineral density in thyrotoxicosis (Diamond *et al.* 1991).

Despite all of the clinical and experimental data showing a correlation between OC and T₃ levels, the mechanism of OC regulation by T₃ in osteoblasts is not well understood. We investigated these mechanisms in ROS 17/2.8 cells, a rat osteosarcoma cell line that expresses an osteoblast-like phenotype. T₃ increases OC mRNA levels by both transcriptional and post-transcriptional mechanisms.

Materials and Methods

Cell culture

The rat osteosarcoma cell lines ROS 17/2.8 were grown in complete media (CM; Ham's F-12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 5% (v:v) fetal bovine serum (FBS; Gibco-BRL)), in a humidity controlled incubator, at 37 °C and 5% CO₂. The pH of

the media was kept between 7.2 and 7.6. Stock solutions of T₃ (10⁻⁴ M in 0.02 NaOH), all-trans retinoic acid (RA; 10⁻⁴ M in methanol), 9-cis-RA retinoic acid (9-cis-RA; 10⁻⁴ M in ethanol) and 1,25 dihydroxyvitamin D₃ (1,25D₃; 10⁻⁵ M in isopropanol) (all from Sigma Chemical Co., St Louis, MO, USA), were diluted in serum-free media (SF; Ham's F-12 medium supplemented with insulin (5 µg/ml), transferrin (5 µg/ml) and Na₂SeO₃ (5 ng/ml) (Sigma Chemical Co.)) as previously described (Williams *et al.* 1994). Cells were grown in SF media for 24 h prior to hormone treatment studies.

Dose-response, time-response and cell-density studies

Cells were plated in a density of 10⁶ cells/100 mm culture dishes (Falcon; Becton Dickinson, NJ, USA) and allowed to grow for 72 h in 12 ml CM. Cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) (Gibco-BRL) and incubated for 24 h in 12 ml SF media. For the dose-response studies, cells were treated with T₃ (10⁻¹¹ to 10⁻⁸ M), RA (10⁻¹¹ to 10⁻⁶ M), 9-cis-RA (10⁻¹¹ to 10⁻⁶ M) or 1,25D₃ (10⁻¹¹ to 10⁻⁸ M) for 24 h. For the time-response studies, cells were treated with T₃ (10⁻⁸ M) for varying periods of time (6, 18, 24, 48 and 72 h) and the cell culture media were changed every 24 h. For the cell-density studies, cells were plated in varying densities (10⁵, 2 × 10⁵, 4 × 10⁵, 6 × 10⁵, 8 × 10⁵, 10⁶, 2 × 10⁶, 4 × 10⁶ and 6 × 10⁶ cells/dish). Cells were also kept in SF media for 24 h and prior to T₃ treatment (10⁻⁸ M) for 24 h.

Effect of protein inhibitor on OC mRNA synthesis

A total of 10⁶ cells were plated in 100 mm dishes, grown in CM for 72 h, washed twice with Dulbecco's PBS and maintained in SF media for 24 h. They were next exposed to 10 µg/ml cycloheximide (CHX; Sigma Chemical Co.) for 5, 10 or 18 h while being treated with T₃ (10⁻⁸ M), 1,25D₃ (10⁻⁸ M) or T₃+1,25D₃ for 24 h. After CHX treatment, cells were again washed twice with PBS and returned to the hormone-supplemented SF media until completing 24 h of hormone treatment. Total RNA was then extracted for Northern blot analysis.

Determination of OC mRNA half-life

Cells were plated as described above and kept in SF media for 24 h before being treated with T₃ (10⁻⁸ M), 1,25D₃ (10⁻⁸ M) or both hormones for 24 h. After hormone treatment, cells were washed twice with Dulbecco's PBS and transferred to SF media with actinomycin D (AD; 5 µg/ml; Sigma Chemical Co.). Cells were harvested every 3 h for a total of 9 h. Total RNA was isolated and analyzed by Northern blot. The percentage of remaining mRNA was calculated by comparing the amount of OC mRNA at each time-point to the amount of OC

mRNA present at the time-point zero of AD treatment, after normalization to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA or 18S ribosomal RNA expression.

RNA isolation and Northern blot analysis

Total RNA was extracted from cultured cells using TRizol (Gibco-BRL), according to the manufacturer's instructions. Northern blots were performed as previously described (Ausubel *et al.* 1997). Briefly, electrophoresis of 30–40 µg RNA per lane was performed in a 2.2% agarose-formaldehyde gel. The RNA was transferred in 20 × SSPE (3 M NaCl, 0.2 M NaH₂PO₄ H₂O, 0.02 M EDTA, pH 7.4) from the gel to a nylon membrane (Gene Screen Plus; DuPont, Boston, MA, USA), which was prehybridized for 4 h and hybridized for 24 h at 42 °C in a hybridization oven (Bellco Glass Inc., Vineland, NJ, USA). Approximately 1.2 ng purified ³²P-labeled cDNA probes for alkaline phosphatase (ALP), OC, osteopontin (OP), GAPDH (gifts from Dr Akiko Ida-Klein, VA Medical Center, Los Angeles, CA, USA), β-actin or 18S rRNA (Ambion, Austin, TX, USA) were added per ml hybridization solution. The specific activity of the probes was approximately 1.7×10^{-9} c.p.m./µg cDNA. Hybridized filters were then washed once with 2 × SSPE for 15 min at room temperature, once with 2 × SSPE with 2% SDS for 45 min at 65 °C, and once with 0.1 × SSPE for 15 min at room temperature. Membranes were then exposed to film for 24 h or less to phosphorimager screens. Hybridization signal intensity was measured by densitometry of autoradiograms using the NIH image software (Scientific Computing Resource Center, National Institutes of Health, Bethesda, MD, USA) or by PhosphorImager analysis of the radioactive signal captured by phosphor screens (Molecular Dynamics, Sunnyvale, CA, USA).

Nuclear run-off transcription analysis

Nuclei of control and T_3 -treated (10^{-8} M for 24 h) ROS 17/2.8 cells were prepared as previously described (Ausubel *et al.* 1997) with modifications (Mosavin & Mellon 1996). Transcription assays were performed on 200 µl frozen nuclei from SF- and T_3 -treated cells thawed at room temperature and transferred to two 15 ml conical polypropylene centrifuge tubes. The radiolabeled RNAs were extracted using TRizol (Gibco-BRL) and the SF and T_3 RNA samples were normalized for c.p.m. prior to hybridization. The labeled RNA probes were hybridized to slot blot membranes containing 20 µg NaOH denatured OC and GAPDH cDNAs, which were isolated by enzymatic digestion from their vectors, pGEM-4Z and pBlue-script respectively. The prehybridization and hybridization were performed as described for Northern blot; however, hybridization was performed for 72 h. The GAPDH signal was used for normalization.

Chloramphenicol acetyltransferase and luciferase fusion genes

For experiments using the OC promoter, the plasmid POC_{2.0}CAT (a gift from Dr Marie B Demay, Massachusetts General Hospital, Boston, MA, USA), which contains the rOC promoter sequence from −1750 to −8 fused to the chloramphenicol acetyltransferase (CAT) gene (Demay *et al.* 1989), was employed. A 53 bp oligonucleotide containing the rat OC 1,25D₃ responsive element (VDRE) and a critical sequence for 1,25D₃ transactivation (Sneddon *et al.* 1997) was synthesized (Life Technologies, Grand Island, NY, USA) and inserted into the Bgl/II site of pGL3 basic vector (Promega, Madison, WI, USA), which contains luciferase (LUC) as gene reporter, to yield rOCVDRE-LUC. This region is identical to the OC promoter sequence, −458 to −410, previously reported (Sneddon *et al.* 1997). Orientation and copy number of the oligonucleotide in each construct were determined by digestion and confirmed by DNA sequencing.

Transfections

ROS 17/2.8 cells maintained in CM were reseeded (10^5 cells/well of 12-well plates) 24 h before transfection and kept, until harvesting, in Ham's F-12 medium supplemented with 5% charcoal-stripped FBS and 1 × penicillin-streptomycin-glutamin (Life Technologies). Transfections were carried out by Effectene Transfection Reagent (Qiagen, Valencia, CA, USA), a non-liposomal lipid-based reagent, with 0.2 µg test plasmid and 0.1 µg control plasmid/well. After 18 h of cell incubation with transfection complexes, the cells were washed with Dulbecco's PBS, 1 ml fresh media plus hormone (T_3 , 1,25D₃ or T_3 +1,25D₃, all at 10^{-8} M) was added and again after 24 h of hormone treatment. Following 48 h of hormone treatment, cells were harvested and cell lysates prepared using 120 µl Reporter Lysis Buffer (Promega) per well. All test plasmids were co-transfected with a control plasmid, pxGH5, which expresses human growth hormone (hGH). The hGH secreted by transfected cells to the medium was assayed by hGH-TGES 100T Kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). CAT activity was assessed by liquid scintillation counter as described in the protocol of the CAT Enzyme Assay System (Promega). LUC activity was measured using the Luciferase Assay System (Promega) and the TD-20/20 Luminometer (Promega). Each sample was assayed in duplicate and normalized by hGH, yielding relative CAT or LUC activities (CAT or LUC activity counts/hGH counts).

Statistical analysis

The expression of mRNA and CAT is shown as means ± s.e.m. Densitometric values were normalized by

the expression of GAPDH or β -actin (18S rRNA for cell density studies) and expressed as arbitrary units (density of tested mRNA/density of control). For all experiments, fold-induction is relative to the control values, which were considered to be 1. One-way analysis of variance (ANOVA) was used to compare more than two groups and was followed by the Student–Newman–Keuls test in order to detect differences between groups. The Student's *t*-test (unpaired) was used to analyze the effect of T_3 at the transcriptional level (run-off assay). To calculate the mRNA half-life, OC mRNA decay was analyzed by linear regression of the percent RNA remaining at each time-point of AD treatment. For all tests, $P \leq 0.05$ was considered statistically significant. GraphPad InStat was used for statistical analysis and Prism software for linear regressions (GraphPad Software Inc., San Diego, CA, USA).

Results

T_3 stimulated OC mRNA levels in a dose-dependent fashion (Fig. 1). T_3 induction of 2.4- to 3.0-fold ($P < 0.05$) was seen at 24 h with T_3 concentrations of 10^{-9} and 10^{-8} M respectively. The expression of two other bone-specific genes, ALP and OP, were not modulated by T_3 treatment at any hormone concentration. T_3 (10^{-8} M) stimulated OC mRNA expression in a time-dependent manner (Fig. 2). Significant induction of OC mRNA, approximately 2.5-fold ($P < 0.02$), was detected at 24 h. The maximum effect, approximately 5.4-fold ($P < 0.001$), was reached after 48 h of T_3 exposure and remained constant after 72 h of T_3 treatment. The broad OC mRNA band is typically observed in Northern blots, due to variability in the length of the poly(A) tail of the OC transcript (observed range of 580–660 nucleotides) (Pan & Price 1985).

A factor that may have led to variability of T_3 responsiveness of OC in previous studies is the extent of cell to cell contact. Previous studies have shown that an increase in ROS 17/2.8 cell density inhibits expression of proliferation-specific genes (e.g. histone H4 and H2B genes) and increases expression of bone-related genes, including OC (van den Ent *et al.* 1993). We tested the effect of cell density on the T_3 induction of OC mRNA. Increased basal expression of OC mRNA was seen with increased cell density, while the magnitude of T_3 induction decreased (Fig. 3). Among the control cells (–), the OC mRNA expression was 5- and 9.8-fold higher ($P < 0.001$) in cells that were plated at 40×10^5 and 60×10^5 cells/dish respectively, when compared with cells that were plated at 10^5 cells/dish (Fig. 3B). At lower cell densities, however, T_3 significantly induced OC mRNA expression (Fig. 3B and C). In cells that were initially plated at 10^5 and 4×10^5 cells/dish (approximately 10–30% confluent at the end of the experiment),

Figure 1 Expression of OC, ALP and OP mRNA in ROS 17/2.8 cells. SF-cultured cells were treated with T_3 (10^{-11} to 10^{-8} M) for 24 h. (A) Densitometric analysis shows the T_3 -induced changes in OC mRNA expression normalized to GAPDH mRNA. Data are given as fold-induction relative to the control (–). Values are means $\pm$ S.E.M. ($n=3$). * $P < 0.05$ vs control (–). (B) Northern blot of 30 μ g total RNA/lane hybridized with OC, OP, ALP and GAPDH 32 P-labeled cDNA probes.

T_3 induced OC mRNA by approximately 4-fold. This T_3 -mediated induction was progressively and significantly reduced as cell density was increased (Fig. 3B and C), falling to 1.5-fold induction in cells that were plated at 6×10^6 cells/dish (overly confluent cells).

Since it is known that TRs may form heterodimers with retinoic acid receptors (RAR), retinoid x receptors (RXR) or vitamin D nuclear receptors (VDR), we next determined if the combination of T_3 with RA, 9-cis-RA or 1,25D₃ would influence the effect of T_3 treatment on OC mRNA expression (data not shown). Treatment with T_3 alone resulted in ~ 3 -fold induction of OC mRNA expression ($P < 0.05$ vs SF). RA and 9-cis-RA alone induced the OC mRNA approximately 2-fold (not a statistically significant increase), but did not further stimulate the T_3 -induced expression of OC mRNA. 1,25D₃ stimulated OC mRNA expression at a higher magnitude than T_3 (5.1-fold vs 3.0-fold, $P < 0.05$) and the

Figure 2 Time-course of OC mRNA expression in ROS 17/2.8 cells. SF-cultured cells were treated with 10^{-8} M T_3 for 5, 18, 24 and 72 h. (A) Densitometric analysis shows the T_3 -induced changes in OC mRNA expression. Data are given in arbitrary density units (OC/ β -actin) and as means $\pm$ S.E.M. ($n=3$). P value by ANOVA is <0.0001 ; $*P<0.05$ vs control (-) of each time-point. (B) Northern blot of 30 μ g total RNA/lane hybridized with OC and β -actin 32 P-labeled cDNA probes. β -actin mRNA expression was used for normalization.

combination of T_3 and 1,25D $_3$ treatments did not further stimulate the OC mRNA expression greater than 1,25D $_3$ alone (6.2-fold vs 5.1-fold, $P>0.05$).

CHX treatment was used to determine if *de novo* protein synthesis was required for T_3 stimulation of OC expression. Since 1,25D $_3$ is known to directly regulate OC gene transcription via the VDR, we also compared T_3 , 1,25D $_3$ and T_3 +1,25D $_3$ treatments in the presence of CHX. The induction of OC mRNA expression by T_3 was unaffected by 5 h of CHX treatment, while the 1,25D $_3$ - and the 1,25D $_3$ + T_3 -mediated induction was increased, reaching ~ 13 -fold ($P<0.001$) (Fig. 4). It is noteworthy that, after 10 h of CHX, T_3 did not significantly induce OC expression, while 1,25D $_3$ was still able to up-regulate OC mRNA (8.6-fold, $P<0.001$). After 18 h of CHX, neither T_3 nor 1,25D $_3$ stimulated OC expression.

To analyze if T_3 up-regulation of OC mRNA was due to a post-transcriptional effect, we assessed whether T_3 treatment modified OC mRNA stability. In addition, we also compared the effects of T_3 with those of 1,25D $_3$ alone and in combination with T_3 (Table 1). The baseline half-life of OC mRNA was $\sim 6.4 \pm 0.2$ h. T_3 treatment stabilized the OC mRNA, increasing the mRNA half-life to 10.9 ± 0.6 h. 1,25D $_3$ treatment prolonged OC mRNA half-life to 13.5 ± 0.4 h. The combination of T_3 with 1,25D $_3$ treatment resulted in an OC mRNA half-life of 11.9 ± 1.3 h.

Figure 3 Effect of cell density on T_3 induction of OC mRNA expression. ROS 17/2.8 cells were plated in varying cell densities and treated with 10^{-8} M T_3 for 24 h. (A) A Northern blot of 20 μ g total RNA/lane hybridized with OC 32 P-labeled cDNA probe is shown. (B) Densitometric analysis of T_3 - and cell density-induced changes in OC mRNA expression. Data are given as arbitrary units (OC/18S rRNA) and as means $\pm$ S.E.M. ($n=3-4$). $*P<0.05$ in relation to control (-) cells in each cell density. (C) Fold-induction of OC expression by T_3 ; $*P<0.05$ vs $40-60 \times 10^5$ cells/100 mm dish and $\Delta P<0.05$ vs $8-20 \times 10^5$ cells/100 mm dish. P value by ANOVA is ≤ 0.002 in (B) and (C).

We determined the effects of T_3 on the rate of OC gene transcription by performing nuclear run-off assay. Transcriptionally active nuclei were isolated from ROS 17/2.8 cells maintained for 24 h in the absence (control cells, -) or presence of 10^{-8} M T_3 . The nascent transcripts were hybridized to filter-bound OC cDNA. T_3 treatment for 24 h stimulated the rate of OC transcription 1.7-fold compared with control cells ($P<0.05$, $n=4$) (Fig. 5).

Although the magnitude of transcriptional stimulation by T_3 was modest, a reporter construct containing 1.8 kb

Figure 4 Effect of T₃ and 1,25D₃ treatment on OC mRNA expression in the presence of CHX. Cells were cultured in SF medium for 24 h before CHX (10 µg/ml) plus T₃ and/or 1,25D₃ (VD) (both at 10⁻⁸ M) treatment for 5, 10 or 18 h. Cells were then washed twice with PBS and returned to hormone treatment for a total of 24 h. RNA (30 µg total RNA/lane) from ROS 17/2.8 cells was analyzed by Northern blot using ³²P-labeled cDNA probes specific for OC and GAPDH. GAPDH mRNA expression was used for normalization. (A) Densitometric analysis of mRNA expression is shown as arbitrary units (OC/GAPDH), means ± S.E.M.; *P<0.05 vs control (open bars). (B) Northern blot shows the expression of OC and GAPDH mRNAs.

of the rat OC gene (rOC) 5'-flanking region, POC_{2.0}CAT, was transfected into ROS 17/2.8 cells to determine the T₃ response (data not shown). T₃ treatment did not stimulate the OC promoter, while 1,25D₃ treatment resulted in a 6.6-fold (*P*<0.001) induction of CAT expression. No further induction was observed when cells were treated with T₃+1,25D₃ (6.8-fold, *P*<0.001 vs con-

trol). Due to the similarity in response elements for TR and VDR (Schrader *et al.* 1994a), we also tested the possibility that T₃ could transactivate the OC promoter through the previously described rOC VDRE. 1,25D₃ treatment resulted in a 2.1-fold (*P*<0.05) increase of the rOCVDRE-LUC reporter construct activity, but T₃ had no effect, with or without 1,25D₃ (data not shown).

Table 1 OC mRNA half-life. Values are presented as means $\pm$ S.E.M. (n=4)

	Slope (%/h)	R ²	P	Half-life (h)
SF	-7.8 $\pm$ 0.3	0.98	0.001	6.4 $\pm$ 0.2
T ₃	-4.8 $\pm$ 0.3	0.93	0.007	10.9 $\pm$ 0.6*
1,25D ₃	-3.7 $\pm$ 0.1	0.95	0.005	13.5 $\pm$ 0.4*
T ₃ +1,25D ₃	-4.2 $\pm$ 0.5	0.90	0.013	11.9 $\pm$ 1.3*

*P<0.01 compared with SF.

Figure 5 Run-off transcription analysis of OC in ROS 17/2.8 cells treated with T₃. (A) ³²P run-off transcripts from nuclei of control (-) and T₃-treated cells at 24 h were hybridized to 20 µg denatured double-stranded OC and GAPDH cDNAs for 72 h. (B) Densitometric analysis is shown as fold-induction in relation to control (SF) and as means $\pm$ S.E.M. (n=4), *P=0.02 by Student's t-test (unpaired).

Discussion

A significant finding of the present investigation was that T₃ significantly increased OC mRNA half-life. The T₃-dependent control of mRNA stability is an important mechanism of thyroid hormone regulation of gene expression. Genes such as cytochrome P450 in HepG2 cells (Peng & Coon 1998), β 3-adrenoceptor in adipocytes (el Hadri *et al.* 1996) and apolipoprotein-AI in Hep G2 cells (Vandenbrouck *et al.* 1995) are all up-regulated by T₃, predominantly by an increase in the mRNA half-life (100%, 36% and 300% respectively), while transcriptional effects of T₃ were not observed by run-off assay.

We additionally determined whether the effects of thyroid hormone on OC gene expression required new protein synthesis. Since 1,25D₃ is known to have direct and indirect effects on OC gene expression (Stein *et al.* 1996), we also used 1,25D₃ and 1,25D₃+T₃ to compare to the effects of T₃ alone. T₃ up-regulated OC mRNA expression after 5 h of CHX treatment but not after 10 h, a time at which the effects of 1,25D₃ on OC mRNA were still detectable. This is an indication that T₃ stimulation of OC mRNA is mediated through short half-life proteins, such as TR and potentially RNA binding proteins. It is interesting that in GH₁ cells, exposure to T₃ induces down-regulation of TR (Raaka & Samuels 1981) by mechanisms that involve ubiquitination and selective proteolysis by the proteasomes (Dace *et al.* 2000). On the other hand, exposure to 1,25D₃ stabilizes VDR in CHX-treated ROS 17/2.8 cells, and the corresponding VDR half-life increases from 2 h to 15 h (van den Bemd *et al.* 1996). Although receptor availability is a reasonable explanation for our findings, it is possible that other mechanisms may be involved. In GC cells, for example, T₃ stimulates the type 1 5'-deiodinase (D1) gene by mechanisms not sensitive to CHX, whereas the T₃ effects on GH mRNA synthesis are CHX sensitive (Maia *et al.* 1995).

T₃ regulates genes by inducing transcription alone (e.g. SERCA1 (Thelen *et al.* 1994); D1 and GH (Raaka & Samuels 1981); collagenase-3 and gelatinase B (Pereira *et al.* 1999)) or in combination with mRNA stabilization (e.g. malic enzyme (Back *et al.* 1986) and uncoupling protein-1 (UCP-1) (Bianco *et al.* 1988, Rehnmark *et al.* 1992)). In addition to stabilizing OC mRNA, T₃ treatment enhanced the rate of OC transcription, ~1.7-fold, as determined by nuclear run-off assay. T₃ induction of ~1.8 kb of the 5'-flanking region of the OC gene was tested in ROS 17/2.8 cells (Demay *et al.* 1989). This sequence is sufficient (i) to provide basal promoter activity (Towler *et al.* 1994), (ii) to direct osteoblast-specific expression and (iii) to confer full 1,25D₃-mediated transactivation of the OC gene (Demay *et al.* 1989). Nevertheless, T₃ treatment did not induce CAT activity, while 1,25D₃, as expected, stimulated the promoter activity approximately 6.6-fold under the same conditions. T₃ also did not induce the 53 bp sequence containing the rOC VDRE, a critical sequence for 1,25D₃ transactivation (Sneddon *et al.* 1997). The element conferring T₃ induction may be located outside of the 1.8 kb 5'-flanking region studied, or the element may be too weak to map in a transient transfection system. VDR interacts in solution with TR and T₃ treatment can enhance the response compared with 1,25D₃ alone (Schrader *et al.* 1994b). TR has been shown to repress 1,25D₃-mediated transactivation (Raval-Pandya *et al.* 1998), and VDR can antagonize T₃ induction (Yen *et al.* 1996), although these effects were not observed in our system.

Multiple levels of gene regulation by hormones have been shown in a number of systems, and the level of

regulation can vary depending on the extent of cell differentiation. For example, 1,25D₃ regulates OC expression in ROS 17/2.8 cells primarily by increasing the mRNA half-life, but stimulates transcription in cells that express a less mature osteoblast phenotype (Shalhoub *et al.* 1998). Nuclear hormone receptor co-activators and co-repressors expressed during development may modulate some of these effects.

T₃ induction of OC gene expression was dependent on cell density. Basal OC gene expression was increased in conditions of high cell density, whereas OC mRNA induction by T₃ was decreased. A previous study showed that an increase in ROS 17/2.8 cell density inhibits expression of proliferation-specific genes and increases expression of mature bone-related genes, including OC (van den Ent *et al.* 1993). A similar effect of cell density on T₃ stimulation of gene expression was found for the Na-K-ATPase $\alpha 3$ gene. T₃ induction was present at 4×10^6 cardiac myocytes/plate but not when twice as many were plated (Chin *et al.* 1998). In a related study, cell confluence changed the response of OC to T₃ in ROS 17/2.8 cells; however, maximal induction by T₃ was seen at both low and high cell densities (Williams *et al.* 1995). In our hands, the maximal induction of OC mRNA expression by T₃ was achieved in cells that were approximately 20–30% confluent. In 100% confluent cells the T₃-mediated induction of OC expression decreased ~50% and was abolished in overly confluent cells. In several aspects, this reciprocal regulatory relationship is analogous to the proliferation/differentiation transition stage observed during development of the bone cell phenotype in normal diploid osteoblasts.

In conclusion, in ROS 17/2.8 cells, T₃ does not interact with 1,25D₃ to regulate the OC gene but up-regulates the OC mRNA expression in a dose-, time- and cell confluence-dependent fashion and requires ongoing protein synthesis to sustain the increase in OC expression. Moreover, our results indicate that T₃ regulates OC expression at the transcriptional and post-transcriptional levels. The greater T₃ induction of OC expression in ROS 17/2.8 cells at low cell density is consistent with thyroid hormone action during bone development.

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