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# Expression of Multiple Thyroid Hormone Receptor Isoforms in Rat Femoral and Vertebral Bone and in Bone Marrow Osteogenic Cultures

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**Abstract** Thyroid hormones influence both bone formation and bone resorption. Clinical data and animal studies provide evidence of skeletal site heterogeneity (hip vs. spine) of bone responses to thyroid hormones. In vitro studies also demonstrate direct effects of thyroid hormones on cells of the osteoblast lineage. Transcriptional regulation by thyroid hormone is mediated by ligand-dependent transcription factors called thyroid hormone receptors (TRs). Two genes, c-*Erb*Aα and c-*Erb*Aβ, generate at least four TR isoforms in the rat: TRα$_1$, c-erbAα$_2$, TRβ$_1$, and TRβ$_2$. Although functional TRs have been identified in cells of the osteoblast lineage, it is still not known if TR isoform expression in bone differs depending upon which skeletal site is examined. We have used ribonuclease protection assay and Northern blot analysis to simultaneously examine the expression of TR isoform mRNAs in adult rat femoral and vertebral bone. TRα$_1$, c-erbAα$_2$, and TRβ$_1$ are expressed in both femur and vertebra whole bone. Bone marrow cells from both skeletal sites were also cultured under conditions whereby the osteoprogenitors differentiated into osteoblasts and formed a mineralized extracellular matrix. TRα$_1$, c-erbAα$_2$, and TRβ$_1$ mRNAs are each expressed in both femoral and vertebral osteoblast cultures. The presence of TRα$_1$, c-erbAα$_2$, and β$_1$ proteins was confirmed by Western analysis of nuclear protein extracts from femoral and vertebral cell cultures. These results indicate that the three predominant TR isoforms are highly expressed in bone and osteoblasts from femurs and vertebrae. Whether there are distinct mechanisms of thyroid hormone action mediated by TRα$_1$, c-erbAα$_2$, and TRβ$_1$ at these separate skeletal sites remain to be shown. J. Cell. Biochem. 74:684–693, 1999. 

**Key words:** thyroid hormones; thyroid hormone receptors; osteoblasts; femurs; vertebrae

Bone is a target tissue for thyroid hormone (triiodothyronine; $T_3$), yet the precise mechanisms of action in bone via nuclear thyroid hormone receptors (TRs) have yet to be characterized [Stern, 1996; Klaushofer et al., 1995; Williams et al., 1994]. TRs are members of the steroid/thyroid hormone nuclear receptor superfamily that bind to DNA on similar hormone response element sequences. TRs can bind to thyroid hormone- response elements to either increase or decrease transcription of target genes. Two TR genes (α and β) are expressed in many tissues and their gene products may be alternatively spliced to yield TRα$_1$, c-erbAα$_2$, TRβ$_1$, and TRβ$_2$ isoforms in a tissue-specific manner [Glass and Holloway, 1990]. C-erbAα$_2$ lacks the ability to bind ligand, but may regulate transcription by its ability to inhibit TR-mediated $T_3$-dependent transactivation [Lazar, 1993]. While the molecular and cellular mechanisms of $T_3$ action in bone are unclear, in vitro studies have shown that $T_3$ acts directly on cells of the osteoblast lineage [Britto et al., 1994; Ohishi et al., 1994; Varga et al., 1994; Ishida et al., 1995; Fratzl-Zelman et al., 1997]. Various TR isoforms have been identified in human and rat osteosarcoma cell lines [Williams et al.,

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1994; Allain et al., 1996], in neonatal primary osteoblast cultures [Bland et al., 1997], and in human bone [Abu et al., 1997].

Observations in patients and mature animals emphasize that different anatomic sites in the skeleton respond differently to thyroid hormone. In women with thyroid carcinoma, the high doses of thyroid hormone required to suppress thyroid-stimulating hormone release cause a greater loss of bone mass at the hip than the spine [Diamond et al., 1991]. In adult rats, femoral and vertebral bones are also differentially affected by long-term excessive thyroid hormone administration [Ongphiphadhanakul et al., 1992, 1993; Suwanwalaikorn et al., 1996]. Recently, we reported the development of a culture model to study the nature of osteoblasts derived from different skeletal sites. The in vivo observations of skeletal site heterogeneity in response to thyroid hormone are supported by our in vitro studies. Adult rat bone marrow osteoprogenitors from femurs and vertebrae both develop into functional osteoblasts, yet $T_3$ differentially affects alkaline phosphatase activity and the gene expression of osteocalcin, collagen type I, and insulin-like growth factor-I (IGF-I) in these cultures [Milne et al., 1998a].

The genes in bone and in osteoblasts that are directly responsive to $T_3$ have yet to be identified, and the regulatory mechanism(s) responsible for the skeletal site heterogeneity in gene response remains unexplained. The purpose of this study was to characterize and compare the expression patterns of TRs in rat femur and vertebrae whole bones and in osteogenic cultures derived from the two skeletal sites. Since $T_3$ acts directly on osteoblastic cells, an osteoprogenitor cell culture model developed from bone marrow stromal cells of adult rat femurs vs. vertebrae was used to investigate TR isoform-specific mRNA and protein expression.

## MATERIALS AND METHODS

All procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were sacrificed by exsanguination following injection with xylazine/ketamine. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School in Worcester, MA.

### Cell Culture

Cultures were established to study gene and protein expression of TR isoforms in femoral and vertebral osteoblasts. Unless otherwise stated, all cell culture reagents were purchased from Sigma Chemical Company (St. Louis, MO). The procedures for establishing primary osteoblast cultures from adult rat femoral and vertebral bone marrow cells have been described in detail previously [Milne et al., 1998a,b]. Briefly, femurs and lumbar vertebrae were removed from adult (100–125 g) female Sprague-Dawley rats. The marrow was collected separately from femurs and vertebrae into αMEM medium supplemented with 20% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and antibiotics. Marrow cells were pelleted, resuspended in fresh medium, and seeded (day 0) at $5 \times 10^6$ total marrow cells/ml ($9 \times 10^5$ cells/cm$^2$) onto 100 mm uncoated culture dishes (Corning, Cambridge, MA). On day 1 in culture, dexamethasone ($10^{-8}$ M) and ascorbic acid (50 μg/ml) were added to all dishes. β-glycerophosphate (β-GP; 10 mM) was added to cultures beginning on day 8. Both femoral and vertebral marrow cells maintained under these osteogenic culture conditions exhibited the osteoblast phenotype [Milne et al., 1998a]. After 5 days in primary culture, cells were ~50% confluent, were weakly AP positive, and had not yet begun to form nodules. After 10 days, cultures were ~80% confluent and tightly packed cuboidal cells were observed within colonies. At this time, cells from each skeletal site had high alkaline phosphatase enzyme activity and significant levels of osteocalcin gene expression. Cultures from this time point (day 10 primary culture) were chosen for the isolation of RNA to study TR isoform gene expression in osteoblasts. Total RNA was prepared from the cultures by the method of Chomczynski and Sacchi [1987]. Cultures were also harvested on days 5, 10, and 15 for nuclear protein extraction to analyze TR isoforms protein expression as a function of osteoblast differentiation. Mineralization in both cultures began on day 12 and numerous mineralized nodules were observed by day 15.

### RNA Isolation From Femur and Vertebra Bone

We incorporated aspects of several methods for RNA extraction [Chirgwin et al., 1979; Chomczynski and Sacchi, 1987; Han et al., 1987;

Nemeth et al., 1989] and modified the guanidine thiocyanate method used for isolating RNA from tissue with high RNase activity [Chirgwin et al., 1979]. All reagents were from Fisher Scientific, unless otherwise noted. Femurs of 6-week-old male and female Sprague Dawley rats were used to study the gender-specific expression of TRs. Femurs and lumbar vertebrae of female adult rats were used to study skeletal-site specific TR expression. The bones were removed quickly, trimmed of muscle and connective tissue, chilled in liquid nitrogen, and then pulverized with a mortar and pestle prechilled in liquid nitrogen. RNA was extracted from the bone powder by homogenization in guanidine thiocyanate solution (GT solution) containing 4.5M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 25 mM EDTA, pH 7.0, and 8% final concentration of β-mercaptoethanol (β-ME).

Ice-chilled 20% N-laurylsarcosine (50 µl/ml GT Soln) and 2M sodium acetate, pH 4.0 (100 µl/ml GT Soln) were added to the homogenate and mixed thoroughly. Phenol, buffer-saturated, pH 4.3, was added at 1 ml/ml GT solution and ice-cold chloroform/isoamyl alcohol (49:1) was then added (220 µl/ml GT Soln). The contents were centrifuged at 12,000*g* 4°C for 20 min. The upper (aqueous) phase was transferred to a fresh tube, an equal volume of 100% isopropanol (-20°C) was added and the RNA was precipitated at –20°C for 15 min. The sample was centrifuged at 12,000*g* for 15 min, -10°C. The supernatant was removed entirely and the RNA pellet was completely dissolved in pellet solution (6M guanidine hydrochloride, 25 mM EDTA, pH 7.0, and 720 µl β-ME/100 ml pellet solution). The dissolved RNA was precipitated with an equal volume of cold isopropanol, then centrifuged at 12,000*g* for 15 min. The resulting RNA pellet was redissolved and reprecipitated two more times. The final RNA pellet was washed with ethanol, and dissolved in RNAse-free water. A final volume of 1 ml water to 1 g of original bone material gave an approximate concentration of 1 mg RNA/ml. Each RNA sample was prepared from one femur or three vertebrae. Concentrations and purity of the final RNA samples were calculated from $A_{260}$ and $A_{280}$ readings.

Total RNA preparations (2 µg aliquots) were evaluated on 1% agarose gels to verify the isolation of intact RNA. For Northern analyses, the total RNA was enriched for mRNA by use of a commercial kit (PolyATtract mRNA isolation System II, Promega, Madison, WI). For RPA analysis, a phenol-chloroform extraction step was added to remove trace proteins, which are known to interfere with RPA.

### Probe Preparation

Labeled antisense RNA transcripts (riboprobes) were synthesized from linearized cDNA templates using the Riboprobe II in vitro transcription kit (Promega). The probes were radiolabeled using [$\alpha$-$^{32}$P] UTP (8,000 Ci/mmol; NEN, Boston, MA). An antisense riboprobe complementary to rat cyclophilin RNA (pTRI-cyclophilin-rat; Ambion, Austin, TX) was used as the internal standard for RPA. The full-length cyclophilin transcript was 165 nucleotides (nt) and protected a fragment of 103 nt. The TR$\alpha_1$ cRNA was 369 nt in length and generated a protected fragment of 289 nt. The c-erbA$\alpha_2$ cRNA was 396 nt in full-length and protected a fragment of 310 nt. The TR$\beta_1$ cRNA was 489 nt and protected a 459 nt fragment. The TR$\beta_2$ cRNA was 618 nt in length and protected a 512 nt fragment of mRNA. The TR$\alpha_1$ [Thompson et al., 1987], c-erbA$\alpha_2$ [Lazar et al., 1988], TR$\beta_1$ [Weinberger et al., 1986], and TR$\beta_2$ [Hodin et al., 1989] probes were designed to be specific for the region of the mRNA in which the nucleotide sequences differ between the four isoforms, so that there was no cross-hybridization of riboprobes.

### Northern Analysis

Polyadenylated mRNA (5 µg/lane) from whole bone RNA preparations was size-fractionated on 1.2% agarose/1.8% formaldehyde gels and transferred to nitrocellulose membranes, following procedures described previously [Milne et al., 1998a]. The membranes were hybridized with [$^{32}$P]-labeled riboprobes at 62°C for 18 h, followed by final stringency washes at 0.1 × SSC/0.1% SDS at 75°C. Membranes were exposed to BioMax MS film (Kodak, Rochester, NY) with intensifying screen at –80°C for 48 h.

### RNase Protection Assay

A high-speed hybridization ribonuclease protection assay kit (Hybspeed RPA: Ambion) was used for all RPA reactions. For each assay, high specific activity labeled TR riboprobe ($1 \times 10^5$ cpm), cyclophilin probe ($4 \times 10^4$ cpm), sample RNA (25 µg total RNA), and yeast tRNA (25 µg)

were co-ethanol precipitated at –20°C for 30 min. Each hybridization reaction step was then followed according to the Hybspeed RPA protocol. The fragments protected from RNase A/$T_1$ digestion were separated and detected on 5% or 6% polyacrylamide/8 M urea/1 X TBE denaturing gels. Dried gels were exposed to XAR film (Kodak) or Biomax MS film at –80°C for 10 to 24 h.

### Nuclear Protein Preparation and Western Analysis

Femoral and vertebral bone marrow cells were harvested on days 5, 10, and 15 of primary culture. Nuclear proteins were isolated and analyzed following procedures of Williams et al. [1994]. Nuclear proteins (100 µg/lane) were resolved on 10% SDS-polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C in TBS-T (10 mM Tris HCl [pH 7.8], 150 mM NaCl, 0.05% Tween-20) containing 3% (w/v) nonfat milk powder (Price Chopper) and 5% BSA (blocking buffer). The membranes were rinsed twice in TBS-T and incubated overnight at 4°C with primary antibody diluted 1:300 in blocking buffer. The primary rabbit polyclonal antibodies used are specific to each of the thyroid receptor isoforms analyzed and were all purchased from Affinity BioReagents (Golden, CO). Polyclonal antibody clone TRα-1–403 reacts specifically with TR$\alpha_1$, but not c-erbA$\alpha_2$ or TR$\beta_1$. Clone TRvα-2–431 reacts specifically with c-erbA$\alpha_2$ protein, while clone TRβ-62 reacts specifically with TR$\beta_1$ [Falcone et al., 1992; Macchia et al., 1992]. Following primary antibody incubation and four 5-min washes in TBS-T, membranes were incubated with secondary antibody (goat anti-rabbit IgG-horseradish peroxidase conjugated; Kodak) at 1:50,000 in TBS-T for 1 h at room temperature. The membranes were again washed four times, and the TR proteins were detected by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

## RESULTS

Total RNA samples extracted from rat femurs and vertebrae were subjected to agarose gel electrophoresis and stained with ethidium bromide to evaluate the effectiveness of the bone tissue isolation method. The resulting sharp 28S and 18S ribosomal RNA bands indicated that this RNA isolation method preserved the integrity of the samples without evidence of degradation (Fig. 1). The procedure also gave consistently good yields of total RNA from both femurs and vertebrae (0.5–1.0 mg RNA/g whole bone).

Since the transcripts for TR genes have been reported to be in low abundance in many tissues [Williams et al., 1994; Chamba et al., 1996; Hodin et al., 1990], we examined the expression of TR genes using the extremely sensitive RNase protection assay (RPA). The assays were initially performed with bone RNA preparations from individual male or female adult rats obtained from two different commercial sources in order to determine possible sample-to-sample variability in the detection of TR transcripts. The femurs from both male and female adult rats possessed detectable levels of TR$\alpha_1$ mRNA, and the vertebral sample was also positive for TR$\alpha_1$ expression (Fig. 2A). In addition, c-erbA$\alpha_2$ transcripts were observed in femur and vertebral samples from both sexes (Fig. 2B). The signal for c-erbA$\alpha_2$ in vertebrae was very faint. This was most likely due to some loss of sample

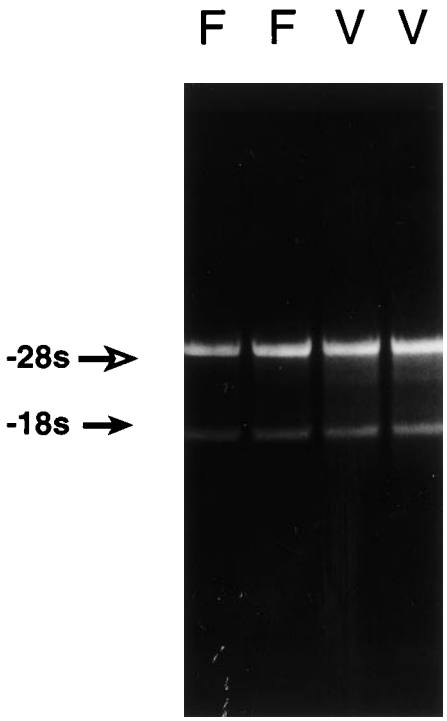


**Fig. 1.** The ethidium bromide staining pattern of total RNA isolated from the femurs and vertebrae of adult rats. The RNA extraction procedures are described in Materials and Methods. Each lane contains 2 µg total cellular RNA prepared from femurs (F) or vertebrae (V). The RNA samples (each containing 5 µg of ethidium bromide) were denatured by heating at 65°C for 5 min and subjected to electrophoresis on a 1% agarose gel.

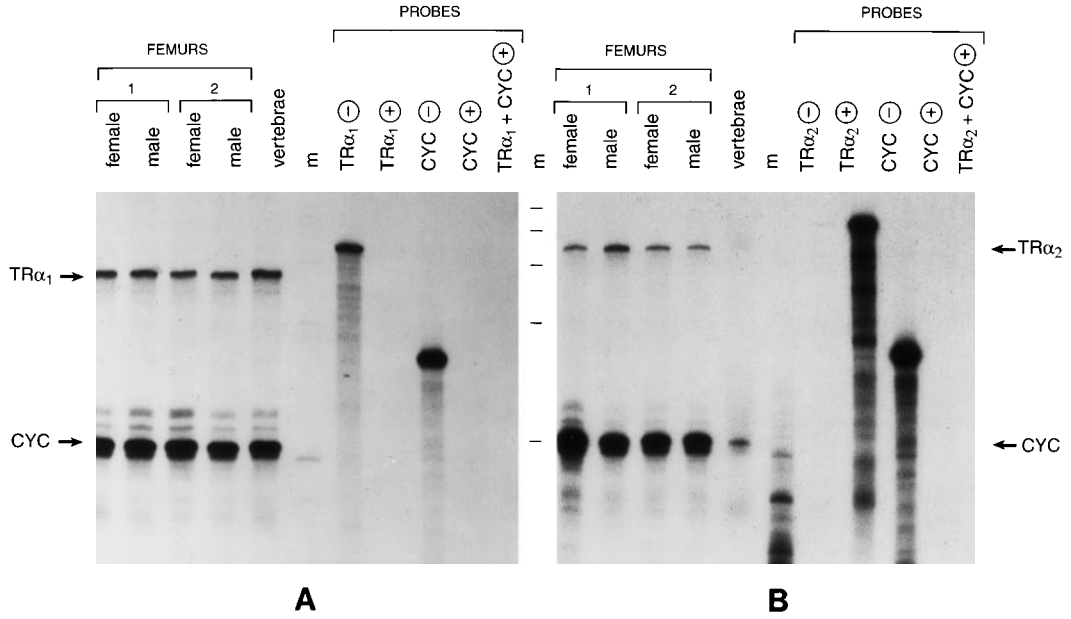


**Fig. 2.** TR$\alpha_1$ and c-erbA$\alpha_2$ transcripts in the femurs of adult female and male rats. Total RNA was analyzed for the presence of TR$\alpha_1$ **(A)** or c-erbA$\alpha_2$ (TR$\alpha_2$) **(B)** mRNA by RPA, as described in Materials and Methods. Each lane marked "femur" represents analysis of 25 µg RNA from the femur of an individual animal. Samples labeled (1) and (2) were from animals obtained from two different commercial sources; Charles River and Harlan, respectively. The lane marked "vertebrae" indicates analysis of RNA (25 µg) pooled from several adult female rats. The riboprobe specific for rat cyclophilin (CYC) mRNA was included in all assays as the internal standard. Full-length riboprobes (⊖) and riboprobes digested with RNase A/$T_1$ mixture (⊕) in the presence of yeast RNA (50 µg) are shown on the right side of each autoradiogram. Full-length TR$\alpha_1$ riboprobe was 369 nt and protected a fragment of 289 nt. Full-length TR$\alpha_2$ riboprobe was 396 nt and protected a fragment of 310 nt. Full-length CYC riboprobe was 165 nt and protected a 103 nt fragment. These assay products were resolved on 6% denaturing polyacrylamide gels. Markers (M) were 500, 400, 300, 200, and 100 nt in length. The TR$\alpha_1$ gel was exposed to XAR film for 10 h. The TR$\alpha_2$ gel was exposed to the more sensitive MS film for 15 h.

RNA, since the cyc internal control signal was also faint.

We then used RPA with TR isoform-specific riboprobes to examine total RNA extracted from femoral- and vertebral-derived bone marrow stromal cells, which had been cultured to differentiate into osteoblasts. We have shown previously that both femoral and vertebral marrow cells grown in medium containing 20% FBS supplemented with dexamethasone, ascorbic acid, and β-GP have high levels of the enzyme alkaline phosphatase, express genes of the osteoblast phenotype (osteocalcin and collagen type I), develop bone nodules which mineralize, and respond to $T_3$ in vitro [Milne et al., 1998a,b]. Shown in Figure 3, riboprobes for both TR$\alpha_1$ and c-erbA$\alpha_2$ detected transcripts present in RNA derived from whole femur, whole vertebrae, femoral and vertebral osteoblast cultures, and liver tissue. As has been reported [Thompson et al., 1987; Chamba et al., 1996; Hodin et al., 1990], the level of expression of TR$\alpha_1$ and c-erbA$\alpha_2$ was low in rat liver. We observed that TR$\alpha_1$ transcripts were at comparable levels between whole femur and whole vertebrae RNAs and between femoral and vertebral osteoblast culture RNAs (Fig. 3A). This was also true for c-erbA$\alpha_2$ mRNA levels (Fig. 3B).

As shown in Figure 4, RPA analysis also detected mRNA for the TR$\beta_1$ isoform in femur, in vertebrae, and in both femoral and vertebral osteoblast cultures. Although a positive signal was detected in both whole bone tissue and in osteoblast cultures, TR$\beta_1$ mRNA expression levels were lower in cultured cells compared to whole bone. In agreement with previous studies [Chamba et al., 1996; Hodin et al., 1990], TR$\beta_1$ mRNA was also abundantly present in rat liver tissue. Femurs, vertebrae, and osteoblast cultures were each negative for TR$\beta_2$ expression (data not shown).

Northern analysis of poly $(A)^+$ RNA prepared from femurs and vertebrae provided information about the sizes of the mRNAs for TR$\alpha_1$, c-erbA$\alpha_2$, and TR$\beta_1$ in bone (Fig. 5). The TR$\alpha_1$ probe hybridized to a predominant 5.3-kilobase (kb) band found in both femurs and vertebrae (Fig. 5A). A 2.8-kb transcript was detected with

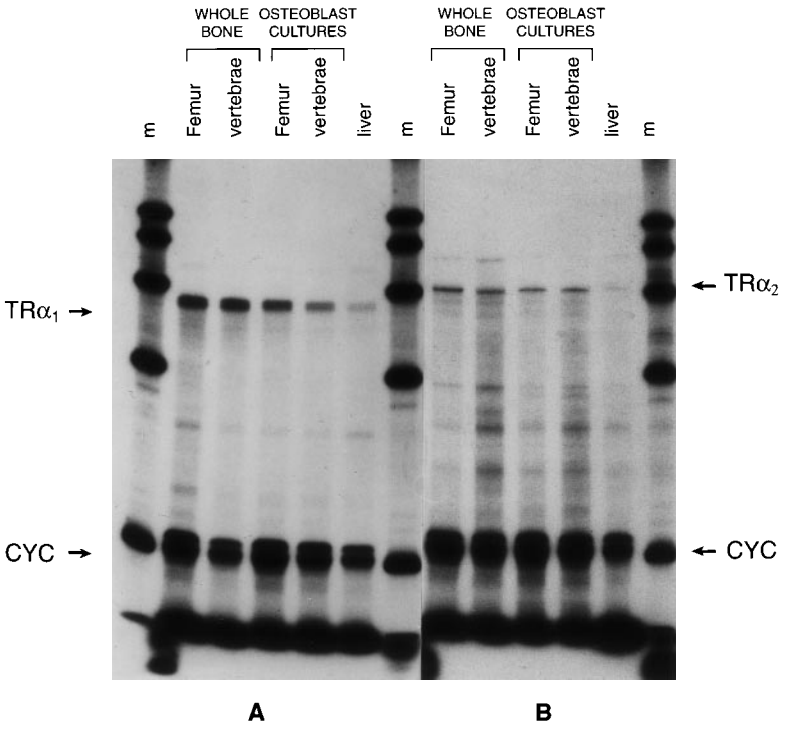


**Fig. 3.** Comparison of TR$\alpha_1$ and TR$\alpha_2$ transcripts in adult femur vs. vertebrae whole bone and in femoral vs. vertebral osteoblast cultures. Total RNA (25 µg per sample) was assayed for the presence of TR$\alpha_1$ **(A)** and TR$\alpha_2$ **(B)** transcripts by RPA, as described in Materials and Methods. Liver total RNA (25 µg) was also assayed. The CYC transcript was used as an internal control. These assay products were resolved on 5% denaturing polyacrylamide gels.

the c-erbA$\alpha_2$ riboprobe and was present in bone RNA from both skeletal sites (Fig. 5B). The TR$\beta_1$ riboprobe hybridized to 2.5-, 4.1-, 5.6-, and 7.2-kb transcripts in bone (Fig. 5C). All four TR$\beta_1$ mRNA species were found in femurs, while the vertebrae expressed predominantly the 5.6-kb mRNA. These transcript sizes for TR$\alpha_1$, c-erbA$\alpha_2$, and TR$\beta_1$ correspond closely to TR mRNAs previously detected in rat heart, kidney, brain, and liver [Hodin et al., 1990], as well as in osteoblastic osteosarcoma cells [William et al., 1994). A 7.0-kb TR$\beta_1$ transcript was also previously observed by Williams et al. [1994] in rat osteosarcoma cell lines, and may represent a splice variant specific to osteoblasts since it has not been identified in other rat tissues [Williams et al., 1994]. The 6.2-kb mRNA that is characteristic of TR$\beta_2$ expression in pituitary [Hodin et al., 1989] was not observed in Northern blots of poly(A)$^+$ RNA from whole bone (data not shown).

To determine whether the TR isoform mRNAs present in the primary osteoblast cultures were translated into proteins, femoral and vertebral bone marrow cells were maintained under conditions which result in sequential osteoblast differentiation. Nuclear protein extracts were prepared at three different time points during the culture period. Western blot analysis using specific polyclonal antibodies revealed the presence of TR$\alpha_1$, c-erbA$\alpha_2$, and TR$\beta_1$ proteins in both femoral and vertebral cultures at each time point examined (Fig. 6). The TR$\alpha_1$ protein was observed as a band of 48 kDa, with higher molecular weight proteins also present (Fig. 6A). The TR$\alpha_2$ antibody also detected multiple protein bands, with a predominant doublet of 58 and 55 kDa (Fig. 6B). The TR$\beta_1$ protein was visualized as a doublet of 65 and 63 kDa with lower molecular weight proteins also present (Fig. 6C). In control experiments, no detectable protein bands were visualized when the primary antibody was omitted. Expression levels of the TR isoform proteins did not differ appreciably between days 5, 10, and 15 as the cells differentiated in culture.

## DISCUSSION

This study characterizes for the first time the expression of multiple thyroid hormone receptor isoform mRNAs in femurs and vertebrae of adult animals. We have also characterized the

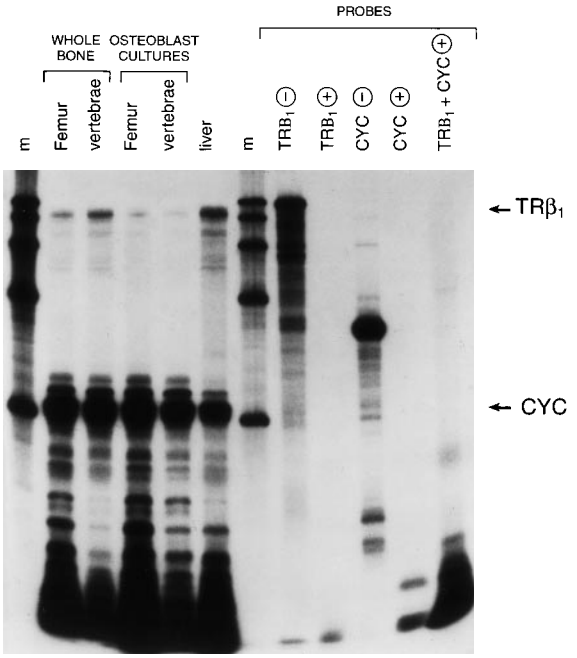


**Fig. 4.** Detection of $TR\beta_1$ transcripts in adult femur vs. vertebrae whole bone and in femoral vs. vertebral osteoblast cultures. Total RNA (25 µg per sample) was analyzed for the presence of $TR\beta_1$ mRNA by RPA. Liver total RNA was also assayed for comparison. The full-length $TR\beta_1$ riboprobe (⊖) was 489 nt in length and gave a protected a fragment of 459 nt. These assay products were resolved on a 6% denaturing gel. Markers (M) were 500, 400, 300, 200, and 100 nt. This gel was exposed to XAR film for 24 h.

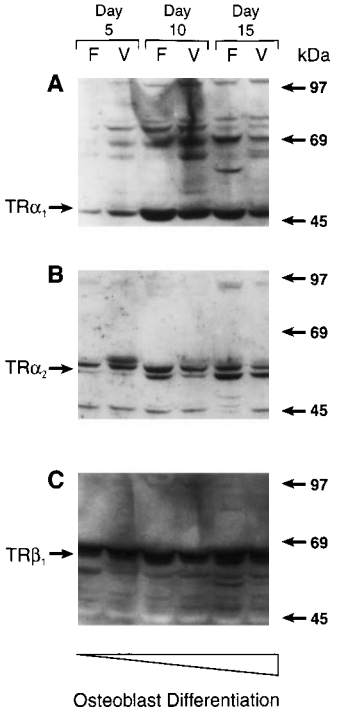


**Fig. 6.** TR isoform protein expression in differentiating femoral (F) and vertebral (V) osteoblast cultures. Nuclear proteins were extracted on the days after culture indicated and analyzed by Western blotting (100 µg protein/lane). The membranes were incubated with antibodies specific for $TR\alpha_1$ **(A)**, $TR\alpha_2$ **(B)**, or $TR\beta_1$ **(C)** isoforms. Osteoblasts derived from both skeletal sites expressed $TR\alpha_1$ (48 kDa), $TR\alpha_2$ (58 and 55 kDa), and $TR\beta_1$ (65 and 63 kDa) proteins.

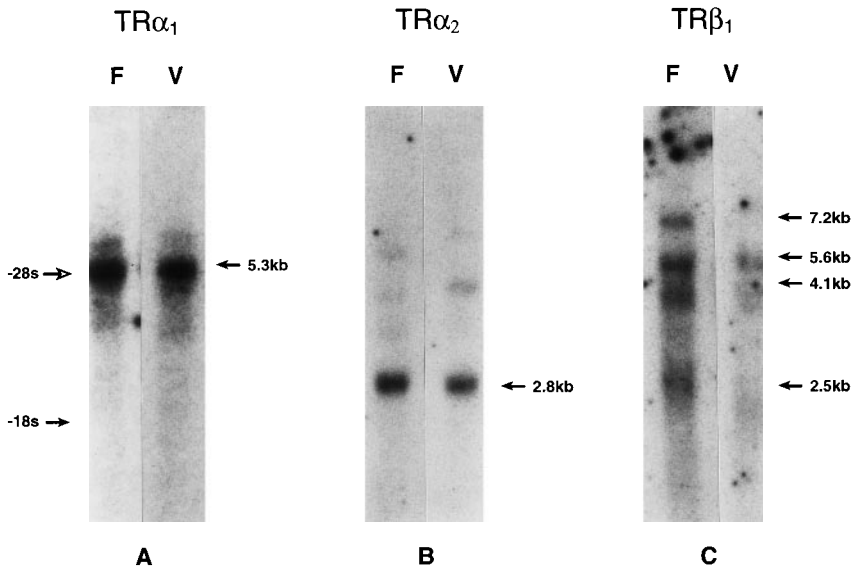


**Fig. 5.** Northern analysis of TR isoform mRNAs in whole bone. Poly (A)+ RNA (5 µg/lane) prepared from femurs (F) and vertebrae (V) was separated by electrophoresis on a 1.2% agarose/1.8% formaldehyde gel. The RNA was transferred to nitrocellulose membrane and hybridized with [$^{32}$P]-labeled riboprobes specific for either $TR\alpha_1$ **(A)**, $TR\alpha_2$ **(B)**, or $TR\beta_1$ **(C)**. Membranes were washed at high stringency (75°C, 1 h, in 0.1 × SSC/0.1% SDS) and subjected to autoradiography for 48 h at –70°C, using MS film. The positions of 28S (4.8 kb) and 18S (1.9 kb) ribosomal RNA are shown.

presence of mRNA and nuclear protein for the multiple TR isoforms in differentiating femoral and vertebral osteoblast cultures. Thyroid hormone is necessary for normal skeletal development and regulates bone turnover in the adult. Numerous studies have documented direct effects of $T_3$ on cells of the osteoblast lineage, yet the genes in osteoblasts that are directly activated (or repressed) by TRs have not been identified. Defining the molecular mechanisms by which thyroid hormone acts in bone is a complex task because multiple forms of the $T_3$ receptor exist, with specific roles for each of these isoforms not yet determined definitively in any tissue. Studies of TRα vs. TRβ knockout mice indicate differential functions of these genes in several tissues [Hsu and Brent, 1998]. Mice devoid of $TR\alpha_1$ and $TR\beta_1$ and $TR\beta_2$ have decreased bone growth, delayed ossification, and decreased bone mineral content [Gothe et al., 1998]. Since thyroid hormones appear to differentially affect bone metabolism at the hip and spine [Suwanwalaikorn et al., 1996], we sought to determine if there is a predominance of a specific TR isoform at either of the skeletal sites that could provide an explanation for the different thyroid hormone responses.

Herein, we investigated the expression of the various TR isoforms in femur vs. vertebral whole bone by use of the sensitive RNase protection assay. We were able to detect transcripts for $TR\alpha_1$, c-erbA$\alpha_2$, and $TR\beta_1$ in bone tissue derived from both male and female adult rats. In addition to cells of the osteoblast lineage, whole bone tissue contains numerous other cell types including osteoclasts, adipocytes, cartilage cells, and lympho-hematopoietic cells. In fact, the presence of TRs in osteoclasts and chondrocytes as well as osteoblasts of human bone has been previously demonstrated by immunocytochemistry [Abu et al., 1997]. We have reported previously that $T_3$ ($10^{-8}$ and $10^{-7}$ M) markedly increases IGF-I gene expression in osteogenic cells derived from vertebrae, but not femurs, and the hormone causes a decrease in alkaline phosphatase enzyme expression in femoral, but not vertebral cultures [Milne et al., 1998a]. Therefore, to characterize TR isoform expression in osteoblasts of adult bone from these two anatomical sites, we established cultures using marrow cells derived from femurs and vertebrae. $TR\alpha_1$, c-erbA$\alpha_2$, and $TR\beta_1$ mRNAs were each expressed in femurs and in vertebrae.

However, it has frequently been observed that there is a discrepancy between the levels of TR isoform mRNA and protein expression [Williams et al., 1994; Allain et al., 1996; Chamba et al., 1996; Strait et al., 1990]. Thus, we used Western blot analysis of extracted nuclear proteins from femoral vs. vertebral osteoblast cultures and were able to demonstrate expression of protein for $TR\alpha_1$, c-erbA$\alpha_2$, and $TR\beta_1$ isoforms in both femoral and vertebral cells. With this information, we conclude that the skeletal site heterogeneity observed in response to thyroid hormone treatment is not caused by differential protein expression of the distinct thyroid receptor isoforms. Earlier analysis of TR isoforms in osteoblast cultures suggested that $T_3$ responses were associated with differing patterns of TR gene expression and stages of osteoblast phenotype expression [Williams et al., 1994]. These studies made use of the osteosarcoma cell lines ROS 25/1, UMR 106, and ROS 17/2.8 that express a fibroblastic, pre-osteoblastic, and mature osteoblastic phenotype, respectively. In this report we have utilized nontransformed, differentiating osteoblast cultures derived from adult animals. We find that there are no major changes in protein levels of the TR isoforms as the cells differentiate in culture.

TRs form heteromeric complexes with other members of the steroid hormone receptor superfamily, including the receptors for 9-*cis* retinoic acid (RAR) [Forman et al., 1989], all-*trans* retinoic acid (RXR) [Zhang et al., 1992], and vitamin $D_3$ (VDR) [Schrader et al., 1994]. This implies that regulatory mechanisms for thyroid hormone action in osteoblasts are coupled with those of vitamin $D_3$ and retinoids [Williams et al., 1994; Bland et al., 1997]. VDR, and multiple forms of RARs and RXRs, have each been identified in osteoblastic cells [Williams et al., 1994; Kindmark et al., 1993]. Possible differential expression of these nuclear receptors and/or differences in the TR heterodimerization pattern in femoral vs. vertebral osteoblasts may correlate with the observed skeletal site heterogeneity. It is also likely that other, perhaps unidentified, nuclear factors are involved in the differential thyroid hormone responsiveness of osteoblasts. Protein co-activators and co-repressors have been identified that interact with TRs (and other receptors of the nuclear hormone receptor superfamily) to increase or decrease levels of gene expression [Horlein et al., 1995;

Chen et al., 1996; Petty et al., 1996; Li et al., 1997; Tagami et al., 1997; Takeshita et al., 1997]. This suggests that skeletal site-specific responses to thyroid hormone may be mediated by the differential abundance of these co-regulators. Further investigation into the mechanisms mediating thyroid hormone responses at separate skeletal sites is warranted. The expression of multiple TR isoforms in differentiating osteoblast cultures from femurs vs. vertebrae underscores the ability to study thyroid hormone-regulated gene expression using this novel culture model.

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