Journal of Cellular Biochemistry 53:122–134 (1993)

Murine Osteoblast Interleukin 4 Receptor Expression: Upregulation by 1,25 Dihydroxyvitamin D_3

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Abstract The immune cytokine interleukin 4 has newly recognized effects on skeletal metabolism. While the interaction of many cells ultimately determines bone mass, we have examined the possibility that the osteoblast may be an IL-4 target in bone by characterizing IL-4 receptor (IL-4R) expression by MC3T3-E1 (MC3T3) murine osteoblastic cells. Based on ^{125}I-IL-4 binding, MC3T3 cells express large numbers of IL-4 receptors (^{125}I-IL-4 Bmax = 3,000–7,500 sites/cell, ^{125}I-IL-4 K = 13–40 pM) with an affinity similar to the IL-4 receptor expressed by an IL-4–responsive T cell line. Monoclonal anti–IL-4R antibodies (M1) blocked specific MC3T3 ^{125}I-IL-4 binding and MC3T3 total cell RNA contained full-length IL-4R mRNA as detected by reverse transcription DNA amplification utilizing IL-4R primers and Northern blot analysis. Functionally, IL-4 treatment of MC3T3 cells resulted in increased cellular proliferation (10–20%) and inhibition of alkaline phosphatase levels (20–40%). While parathyroid hormone (PTH) exposure did not influence IL-4R levels, vitamin D_3 treatment augmented MC3T3 ^{125}I-IL-4 binding, in a time-dependent manner, up to threefold after a 24 h exposure with a metabolite specificity indicating the involvement of the vitamin D receptor. Equilibrium binding studies showed that the impact of 1,25 $(OH)_2$ D_3 on MC3T3 ^{125}I-IL-4 binding was due to an increased IL-4R Bmax. Cycloheximide treatment inhibited 1,25 $(OH)_2$ D_3–induced IL-4R upregulation, suggesting that protein synthesis was required. Furthermore, the steroid increased steady-state IL-4R mRNA levels in both a time- and concentration-dependent manner. The IL-4R message half-life was not altered by 1,25 $(OH)_2$ D_3, suggesting that increased IL-4R mRNA expression resulted from increased IL-4R gene transcription. Taken together, these findings raise the possibility that IL-4's influence on mineral metabolism could be mediated by osteoblasts and that the effectiveness of this cytokine may be influenced by vitamin D_3's impact on IL-4R expression.

Key words: vitamin D, cytokines, cytokine receptor, parathyroid hormone, alkaline phosphatase

Interleukin 4 (IL-4) is a 20 kDa glycoprotein produced by activated T cells [Howard et al., 1982] and mast cells [Brown et al., 1987] that impacts many biological events. Originally purified based on its capacity to induce B cell proliferation [Howard et al., 1982; Rabin et al., 1985], it is now recognized that IL-4 also influences B cell antibody isotype expression [Vitetta et al., 1985; Coffman et al., 1986], T cell activation [Kurt-Jones et al., 1987; Kupper et al., 1987], mast cell proliferation [Smith and Rennick, 1986; Mosmann et al., 1986], monocyte activation [Hart et al., 1989; Essner et al., 1989; Littman et al., 1989; Standiford et al., 1990; Abramson and Gallin, 1990; Gibbons et al., 1990], and hematopoiesis [Peschel et al., 1987, 1989; Rennick et al., 1987]. The cellular effects of IL-4 are mediated through a 140 kDa glycoprotein high-affinity plasma membrane receptor that is expressed on lymphoid cells, hemopoietic cells, fibroblasts, and stromal cells [Ohara et al., 1987; Lowenthal et al., 1988; Park et al., 1987]. The receptor has been cloned from mRNA obtained from human peripheral blood mononuclear and T cells and murine T cells [Idzerda et al., 1990; Mosley et al., 1989]. Sequence analysis of these IL-4 receptor cDNAs reveals that they are members of a cytokine receptor superfamily that includes the erythropoietin receptor, IL-6 receptor, prolactin receptor, and the IL-2 receptor beta chain, among others [Idzerda et al., 1990].

While most of the above studies have focused on IL-4's influence on immune responses and hematopoiesis, recent data suggest that this im-

Abbreviations used: interleukin, IL; IL-4 receptor, IL-4R; phosphate buffered saline, PBS; binding buffer, BB.

Received March 23, 1993; accepted June 4, 1993.

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munomodulatory cytokine could play a role in skeletal metabolism. Specifically, Watanabe et al. have documented that IL-4 is a potent inhibitor of bone resorption induced by a wide variety of osteolytic factors both in vitro [Watanabe et al., 1990] and in vivo [Watanabe et al., 1991]. Because IL-4 inhibited the resorption-inducing effects of such a diverse group of osteolytic substances, we reasoned that IL-4 impacted an essential resorptive process(es). In a recent report, we have documented that IL-4 significantly antagonizes osteoclast generation in vitro [Shioi et al., 1991] which, if true in vivo, could explain some of its actions on in vivo and in vitro bone resorption. On the other hand, in vitro osteoclast generation takes seven to ten days [Shioi et al., 1991], raising the possibility that IL-4's rapid inhibitory effect on bone-resorption (within three days) [Watanabe et al., 1991] may be mediated by cells other than osteoclasts or their precursors.

Because osteoblasts mediate the resorptive effects of a wide variety of substances [Rodan and Martin, 1981; Gallwitz et al., 1991; McSheehy and Chambers, 1986; Thomson et al., 1986, 1987], we reasoned that they were also an important IL-4 cellular target in bone. This possibility has been examined utilizing MC3T3-E1 cells, a nontransformed murine osteoblastic cell line [Sudo et al., 1983]. As will be shown, MC3T3 cells express large numbers of IL-4 receptors (IL-4Rs) as determined by ^{125}I-IL-4 binding. The MC3T3 IL-4 binding sites had a K similar to T cells and could be blocked by monoclonal anti–IL-4 receptor antibodies that recognize T cell IL-4 receptors. Furthermore, IL-4R mRNA was detectable in MC3T3 total cell RNA. MC3T3 IL-4 treatment resulted in increased proliferation (10–20%) and decreased alkaline phosphatase levels (20–40%). Lastly, we show that 1,25 $(OH)_2$ D_3, but not parathyroid hormone, increased MC3T3 IL-4R expression. These findings offer further evidence that IL-4 affects cellular activities beyond those directly related to immune responses or hematopoiesis. In bone, IL-4's influence on osteoblasts may explain IL-4's skeletal effects and, in select circumstances, serve an important antiresorptive function.

METHODS

Media, Buffers, and Chemicals

The MC3T3 culture medium was the alpha modification of Eagle's minimal essential medium supplemented with 2 mM L-glutamine, penicillin/streptomycin (100 Units/ml, 100 μg/ml), and 5% heat-inactivated fetal calf serum (HIFCS, α5-MEM). The trypsin/EDTA concentrate (Gibco/BRL, Gaithersburg, MD) was diluted in Ca^{2+}/Mg^{2+}–free PBS (TE-PBS). The binding buffer (BB) used in the IL-4R assay was RPMI 1640, HEPES 25 mM (pH 7.2), supplemented with 5% HIFCS [Kilian et al., 1986]. The alkaline phosphatase assay buffer was 100 mM 2-amino-2-methyl-1-propanol (pH 10), 1 mM $MgCl_2$ with 0.1% Triton X-100 (ICN, Irvine, CA). The buffer (Northern buffer) utilized for RNA gels was 3-(4-morpholino) propanesulfonic acid 20 mM (pH 6.8), sodium acetate 5 mM, and EDTA 1 mM. Paranitrophenylphosphate, paranitrophenol, and cycloheximide (CHX) were obtained from Sigma (St. Louis, MO).

Cytokines and Antibodies

The vitamin D_3 metabolites were the kind gift of Milan Uskokovic (Hoffmann-La Roche) and were dissolved as concentrated stock solutions in absolute ethanol and stored in the dark at −20°C. The final concentration of ethanol in all experiments was always less than 0.01%, an amount that does not affect cellular viability or ^{125}I-IL-4 binding (data not shown). The rat anti-murine monoclonal anti–IL-4 receptor antibody M1 [Beckmann et al., 1990] was obtained from Genzyme (Cambridge, MA). Recombinant interleukin 4 was expressed and purified from baculovirus transfected SF-9 (spodoptera frugiperda) cell culture supernatants as described [Ohara, 1989; Ohara et al., 1987].

Cells

The MC3T3-E1 (MC3T3) cell line was kindly provided by Dr. H. Tanaka (Okayama University, Okayama, Japan). The cells were maintained in a humidified atmosphere at 37°C in 5% CO_2 in air. For cell passage (every five to seven days), the confluent cell layers were treated with TE-PBS and, following rinsing, seeded at 2×10^4 cell/ml in T75 tissue culture flasks (15 ml total volume). To assure that the cells employed in our experiments expressed an osteoblastic phenotype, PTH induction of cAMP, monolayer staining for alkaline phosphase activity, and culture morphology were assessed (data not shown). Based on changes in cell layer morphology from a "cobblestone" to a spindled appearance together with a reduction of alkaline phosphatase activity with increasing passage number, MC3T3

cells were utilized for experiments only up to passage ten. MD10 and HT-2 cells, T cell lines that respond to IL-4, were maintained as described (Lacey et al., 1987).

^{125}I-IL-4 Binding

Murine IL-4 (10 μg) was labeled with ^{125}I ($Na^{125}I$,ICN) using enzymobeads (29) (Biorad, Richmond, CA) and the biochemical integrity verified on SDS-PAGE gels. The specific activity of the ligand ranged from 1,600–2,300 cpm/fmol. At T = 0, MC3T3 cells were cultured in 24-well plate wells at a density of 10^5 cells/well in 2 ml of α5-MEM. At this seeding density, cell confluence was reached at 48–72 h. In some experiments, the medium was replaced after 48 h with fresh medium (1 ml/well) containing different treatments and further incubated for an additional 24 h. The MC3T3 cells were then gently rinsed with cold serum-free BB (×3). In ligand binding experiments, 0.5 or 1 nM ^{125}I-IL-4 was utilized unless otherwise noted. Nonspecific binding was determined by preincubating cells with approximately 3,000 units/ml IL-4 for 1 h at 4°C prior to ^{125}I-IL-4 exposure. The final incubation volume was 0.4 ml. After an additional 1 h incubation at 4°C (2 h for Scatchard analysis), the cell layers were rinsed with cold Hank's buffered salt solution (6 × 1 ml) and then solubilized by the addition of 0.5 ml 1 N NaOH. The cell-associated radioactivity was determined in a gamma counter. Parallel culture wells were trypsinized (TE-PBS) and cell numbers established by counting with a hemocytometer. The data are reported as the mean (n = 2 or 3) and range or standard deviation, respectively, of the cpm ^{125}I-IL-4 bound/1 × 10^6 cells. Some of the data were subjected to Scatchard analysis [Scatchard, 1949]. For MD10 cells, ^{125}I-IL-4 binding was performed using a method previously described [Lacey and Erdmann, 1990] for ^{125}I-IL-1 except that ^{125}I-IL-4 and IL-4 were the ligands.

IL-4R mRNA Assays

To detect the presence of IL-4R mRNA, a cDNA amplification protocol [Kawasaki, 1989] and Northern gel analysis were utilized. Based on the murine IL-4 cDNA sequence [Mosley et al., 1989], 25 bp oligomers (5′: 409–433; 3′: 973–997) that bracket a 589 bp sequence corresponding to a portion of the IL-4R, including continuous segments of the extracellular, transmembrane, and intracellular domains, were synthesized on an Applied Biosystems oligonucleotide synthesizer, model 3801 (Foster City, CA). MC3T3 and HT-2 total cellular RNA were prepared by the method of Chirgwin [Chirgwin et al., 1979], quantified by absorbance spectroscopy (1 A_{260} = 40 μg/ml), and analyzed on a 1% Seakem agarose (FMC, Rockland, ME)/formaldehyde (0.23%) gel [Chaplin et al., 1983] in Northern buffer to assure RNA integrity. A 1 μg aliquot of MC3T3 or HT-2 total cellular RNA was reverse transcribed utilizing random hexamer primers (200 pmol), 1× DNA Taq polymerase buffer (Promega, Madison, WI), 1 mM each dATP, dCTP, dGTP, dTTP, 20 units RNasin ribonuclease inhibitor (Promega), and 200 units MMLV reverse transcriptase (Gibco/BRL) in 20 μl. Following an initial 10 min at 22°C, the reaction was adjusted to 42°C for 30 min, then heated (95°C, 10 min) and subsequently chilled to 4°C. At this time, the reagents for Taq DNA polymerase-mediated temperature-cycled DNA amplification were added. These were: 20 pmol 3′ and 5′ oligomers and 1 U Taq DNA polymerase (Promega) in 1× Taq DNA polymerase buffer (1.5 mM $MgCl_2$ final) to 50 μl. No additional dNTPs were added. The tubes were cycled in a Perkin-Elmer Cetus Thermocycler (Norwalk, CT) with an initial 3 min at 94°C for denaturation followed by 30 cycles consisting of 45 s at 94°C, 1 min at 60°C, and 2 min at 72°C. After the last cycle, the reactions were incubated for 7 min at 72°C. Some of the samples were restriction digested with Pvu II (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Aliquots were then analyzed on a 3% composite [Seakem:Nusieve (FMC), 1:2] agarose gel in an 89 mM Tris-borate, pH 8.3/25 mM EDTA buffer containing ethidium bromide (0.5 μg/ml) utilizing Hae III digested ϕX174 as a standard (Gibco/BRL). To verify that the amplified material was not the result of amplification of contaminating DNA, amplification of murine genomic DNA (1 μg) with the IL-4R primers was performed. This reaction did not yield product of the expected size (data not shown).

For Northern blots, MC3T3 and HT-2 total cell RNA (15 μg) were electrophoresed in formaldehyde containing 1% agarose gels as described above. The RNA was electroblotted onto Zeta probe nylon membranes according to manufacturer's instructions (Biorad, Richmond, CA). The blots were prehybridized in cocktail containing formamide 50%, $NaHPO_4$ 0.25 M

(pH 7.2), NaCl 0.25 M, EDTA 1 mM, 2% SDS, and 0.3 mg/ml sheared herring sperm DNA for 1–2 h at 42°C. The murine IL-4R probe was generated by labeling the gel-purified DNA amplification product (same conditions as noted above) of plasmid IL-4R.5, which is PGEM 4Z (Promega, Madison, WI) containing the verified IL-4R sequence defined by the IL-4R primers utilized above, with ^{32}P-αdCTP (3,000 Ci/mmol, ICN, Irvine, CA) by random priming (Random Primed DNA Labelling Kit, Boehringer-Mannheim, Indianapolis, IN). The probe specific activity was estimated at 10^8–10^9 cpm/μg DNA. Hybridization (16–20 h, 42°C) was performed using 10^6 cpm/ml probe in the same buffer used for prehybridization. The membranes were then washed three times in 2× SSC, 0.1% SDS, 5 min/wash at room temperature, and then once in 0.2× SSC, 0.1% SDS at 56°C for one h. For autoradiography, the blots were exposed to Kodak XAR film at −80°C for two days. Some of the blots that had been hybridized with the IL-4 receptor probe were stripped with 2× SSC/0.5% SDS for 20 min at 95°C before they were reprobed with the G3PDH probe.

For IL-4R messenger RNA half-life determinations, confluent MC3T3 cultures were treated with fresh media alone or media with 1,25 $(OH)_2$ D_3 (5 nM, 3 h) prior to the addition of actinomycin D (10 μg/ml) to stop RNA synthesis. At various times following toxin exposure, total cell RNA was purified as described. Aliquots of total cell RNA (10 μg) in denaturing solution (6% formaldehyde, 10× SSC, 2 μg/ml yeast RNA) were for 10 min at 68°C, serially diluted 1:2 (×4) in the same buffer, transferred to a dot blot apparatus, and then vacuum applied to Zeta probe (Biorad, Richmond, CA) membranes. As for the Northern blots, the membranes were hybridized with the ^{32}P-IL-4R probe as described above. The dot densities on the autoradiographs were analyzed in an ELISA plate reader at 630 nm. Following normalization of the densities to the T = 0 value, the data were plotted against time. The results presented are representative of three independent experiments.

MC3T3 Proliferation Assay

MC3T3 cells (1×10^5) were plated at T = 0 into 24-well plate culture wells (1 ml) and allowed to adhere for 2–4 h. Various concentrations of IL-4 were then added (1 ml) and the cultures incubated for 72 additional h. The MC3T3 cells were released from matrix with TE-PBS and then counted using a hemocytometer. Triplicate wells were assessed.

Alkaline Phosphatase Determination

Alkaline phosphatase activity was measured by a modification of the method of Lowry [Lowry et al., 1954]. After appropriate treatment periods, the cells were washed three times with PBS. Following the addition of 1 ml of the assay buffer containing 2 mM paranitrophenylphosphate, the cultures were incubated at 37°C for 10–15 min. The reaction was stopped by the addition of 200 μl 1 N NaOH to each well. The absorbance at 410 nm was then measured using p-nitrophenol as a standard. Alkaline phosphatase activity was expressed as nmol of p-nitrophenol produced from p-nitrophenylphosphate [mean (n = 3) ± SD]/min/1 × 10^6 cells. Cells were counted in paired treated wells as described above.

RESULTS

To determine the presence of IL-4 receptors on MC3T3 cells, equilibrium binding studies were performed with ^{125}I-IL-4 to determine the affinity and number of ^{125}I-IL-4 binding sites expressed by MC3T3 cells. Concurrently, binding studies were done with MD10 cells, an IL-4 responsive murine T cell [Lacey et al., 1987], to compare osteoblast and T cell IL-4 receptors. As shown in Figure 1A and C, MC3T3 and MD10 cells express saturable ^{125}I-IL-4 binding sites. When the binding data were resolved on Scatchard plots (Fig. 1B and D for MC3T3 cells and MD10 cells, respectively), it was determined that both the osteoblasts and T cells express IL-4 receptors with nearly identical Ks (MC3T3 K = 41 pM; MD10 K = 51 pM). Further, both cells express large numbers of IL-4Rs (MC3T3 Bmax = 7,254 sites/cell; MD10 Bmax = 3,768 sites/cell).

These data suggest that osteoblast IL-4Rs are similar to those expressed by T cells. To further document this, we utilized a monoclonal antibody (M1) [Beckmann et al., 1990] raised against murine T cell IL-4Rs to block ^{125}I-IL-4 binding. As Figure 2A shows, M1 competes for ^{125}I-IL-4 binding by MC3T3 cells to the same extent as excess unlabeled IL-4 suggesting that MC3T3 cells express IL-4Rs antigenically related to those expressed by T cells. To confirm the presence of IL-4R mRNA in MC3T3 RNA, we employed both reverse transcription DNA amplification

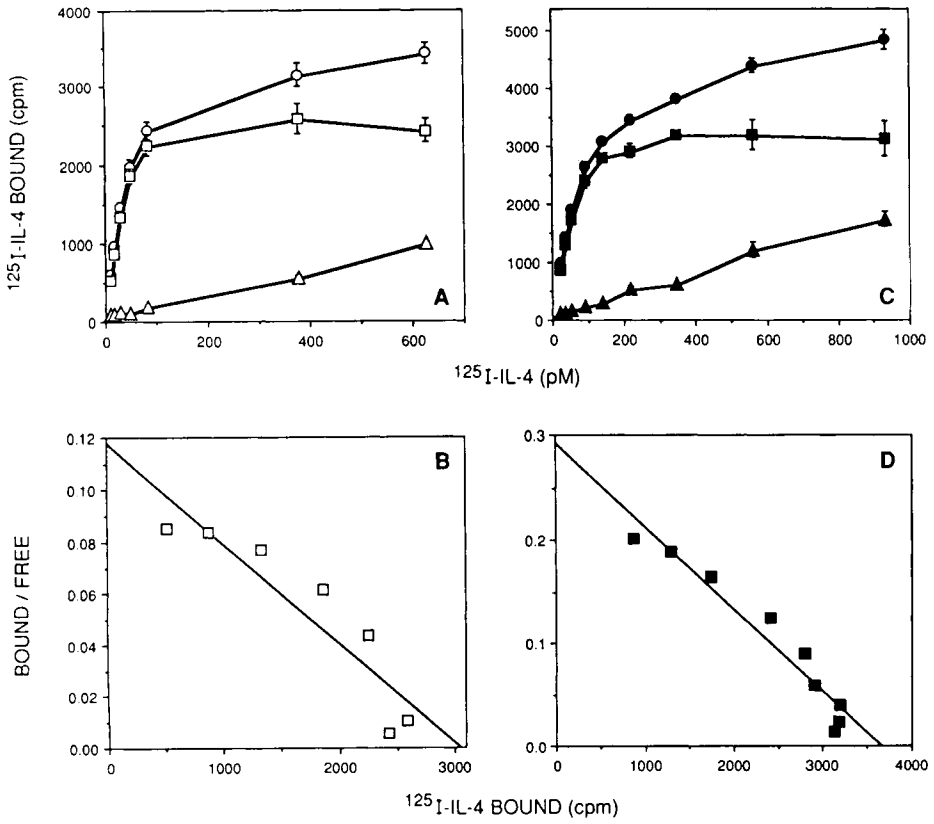

Fig. 1. MC3T3 cells express saturable, high-affinity ^{125}I-IL-4 binding sites. Confluent, adherent MC3T3 cultures (1.5×10^5 cells/well) were employed in ^{125}I-IL-4 equilibrium binding studies as described (see Methods) using various concentrations of ^{125}I-IL-4. For a T cell control, MD10 cells (2.4×10^6 cells/ml, 0.15 ml) were utilized in a suspension binding assay (see Methods). In **A** and **C**, the binding curves of the total (○, ●), nonspecific (△, ▲), and specific (□, ■) ^{125}I-IL-4 bound for MC3T3 (open) and MD10 (solid) cells are shown and represent the mean (n = 3) and SD. In **B** (MC3T3) and **D** (MD10), Scatchard analysis of the specific ^{125}I-IL-4 bound is shown.

and Northern blot protocols. Utilizing 5′ and 3′ primers specific for a murine IL-4R sequence [Mosley et al., 1989] we amplified DNA with a mobility on 3% agarose gels (Fig. 2B) near the predicted size (589 bp) from total cell RNA obtained from both HT-2 (lane b, IL-4R expressing T cell line [Ohara and Paul, 1987; Lowenthal et al., 1988]) and MC3T3 cells (lane d). Pvu II (hexameric restriction site) restriction digests of DNA amplified from both HT-2 (lane c) and MC3T3 (lane e) RNA resulted in restriction fragments near the size predicted (198 bp, 391 bp) by the IL-4R sequence. Autoradiograms (Fig. 2C) of Northern blots of MC3T3 and HT-2 RNA hybridized with a ^{32}P-IL-4R DNA probe revealed the presence of RNA species migrating slightly faster than 28s RNA. These findings are consistent with the presence of full-length IL-4R transcripts based on comparison to autoradiograms presented in earlier reports [Mosley et al., 1989; Idzerda et al., 1990]. Additionally, HT-2 cells appear to contain more IL-4R mRNA than MC3T3 cells.

To explore IL-4's impact on MC3T3 activities, we examined IL-4's effects on proliferation and alkaline phosphatase levels. As shown in Figure 3A, IL-4 enhances MC3T3 cellular proliferation after a 72 h exposure approximately 10–20% with a stimulatory effect observed at 25 U/ml. To determine whether IL-4 affected alkaline phosphatase expression, confluent monolayers of MC3T3 cells were treated for 48 h with various concentrations of IL-4. As shown in Figure 3B, we found that IL-4 inhibited alkaline phosphatase levels with the maximal inhibition (50%) observed at 100 U/ml IL-4. Together, these findings underscore IL-4's capacity to modulate osteoblast function.

The in vitro and in vivo resorption-inhibiting effects of IL-4 together with the presence of

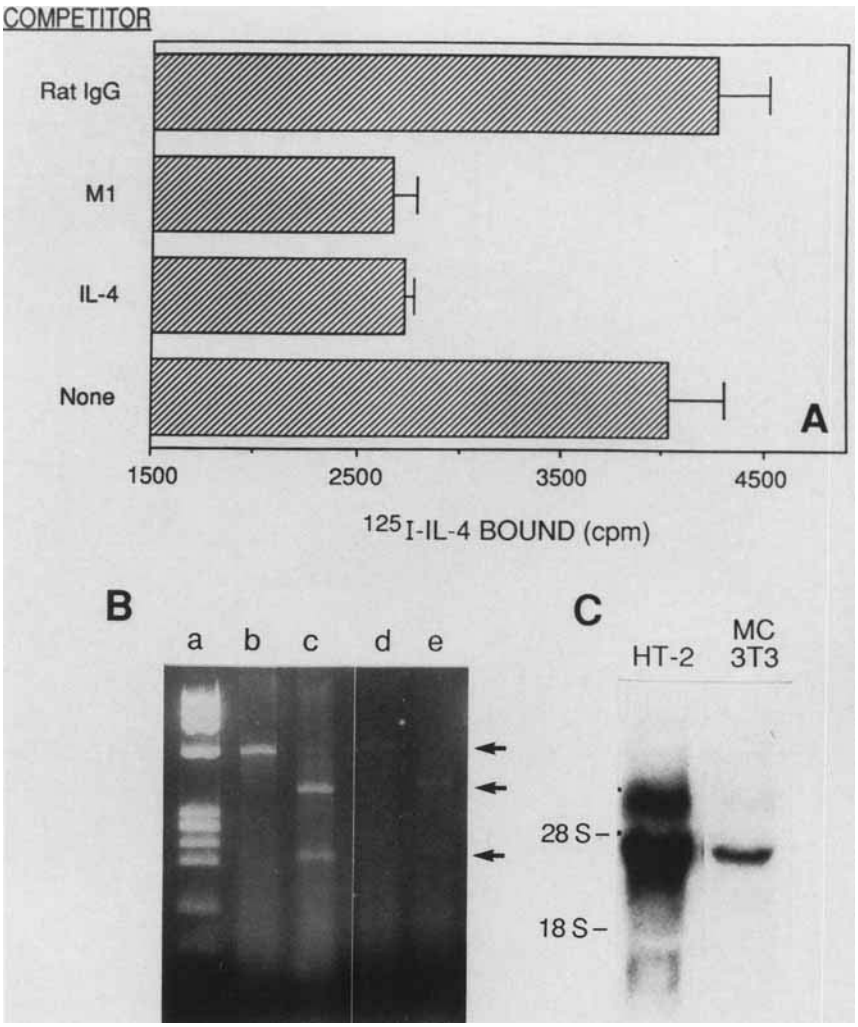

Fig. 2. MC3T3 cells express IL-4R antigenically and molecularly identical to T cells. **A,** Confluent cultures of MC3T3 cells were exposed to Rat IgG (7.5 μg/ml), M1 (7.5 μg/ml), IL-4 (3,000 U/ml), or buffer alone for 1 h prior to ^{125}I-IL-4 (0.5 nM) binding. The results represent the mean (n = 3) and SD of the ^{125}I-IL-4 (cpm) bound/1 × 10^6 cells. **B,** To detect sequence specific IL-4R mRNA, total cell RNA from HT-2 and MC3T3 cells was reverse transcribed with random hexamers and then amplified utilizing IL-4R specific primers, Taq DNA polymerase, and a thermocycling device. Aliquots of these reactions and Pvu II restriction digests thereof were separated on 3% agarose gels as described as follows: lane a, Hae III ϕX174 restriction fragments; lane b, HT-2 RNA; lane c, Pvu II digest of b; lane d, MC3T3 RNA; lane e, Pvu II digest of d. Arrows denote the position of cut and uncut amplified DNA. **C,** To confirm the presence of full-length IL-4R transcripts, HT-2 and MC3T3 total cell RNA (15 μg) was electrophoresed in 1% agarose gels, electroblotted onto nylon, and probed with a ^{32}P-IL-4R cDNA probe. The migration distance of the 28s and 18s RNAs are marked adjacent to the autoradiogram.

osteoblast IL-4Rs suggest that this cytokine could function physiologically as a local negative regulatory factor in bone. One mechanism by which this effect could be potentiated is through osteoblast IL-4R modulation. This prospect was examined by assessing ^{125}I-IL-4 binding in MC3T3 cells stimulated for 24 h with various concentrations of either 1,25 $(OH)_2$ D_3 or parathyroid hormone. Figure 4A shows that 1,25 $(OH)_2$ D_3 (50 and 5 nM) increased MC3T3 ^{125}I-IL-4 binding over twofold when compared to control cells. In contrast, parathyroid hormone exhibited minimal effects on ^{125}I-IL-4 binding at all concentrations tested. To assess the possible involvement of the nuclear 1,25 $(OH)_2$ D_3 receptor, the effect of 25 (OH) D_3 on MC3T3 IL-4R expression was examined. As Figure 4B shows, both vitamin D_3 metabolites augmented ^{125}I-IL-4 binding after a 24 h treatment with 1,25 $(OH)_2$ D_3 being active at concentrations two log orders below 25 (OH) D_3. These findings are consistent with an effect mediated by the 1,25

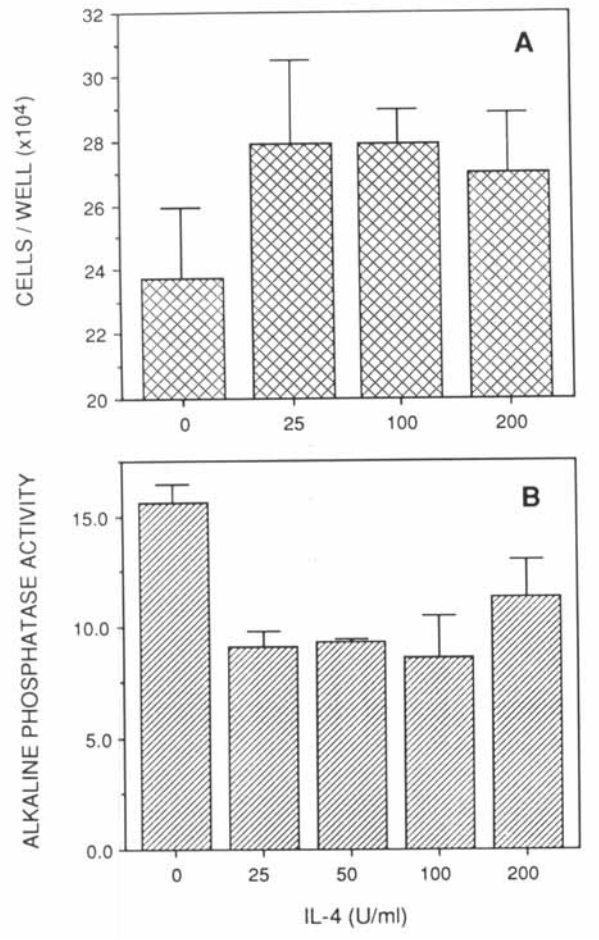

Fig. 3. IL-4 treatment stimulates MC3T3 growth but inhibits alkaline phosphatase expression. **A,** MC3T3 cells were plated in a 24-well plate at T = 0 and cultured for 72 h in the presence of various IL-4 levels. The cells were then trypsinized and counted. The data represent the mean (n = 3) and SD of the cell#/well. **B,** MC3T3 cells were grown to confluence and the media exchanged with fresh media containing various IL-4 concentrations. After an additional 48 h, alkaline phosphatase activity was assessed as described and is expressed as nmol of p-nitrophenol produced from p-nitrophenylphosphate [mean (n = 3) ± SD]/min/1 × 10^6 cells.

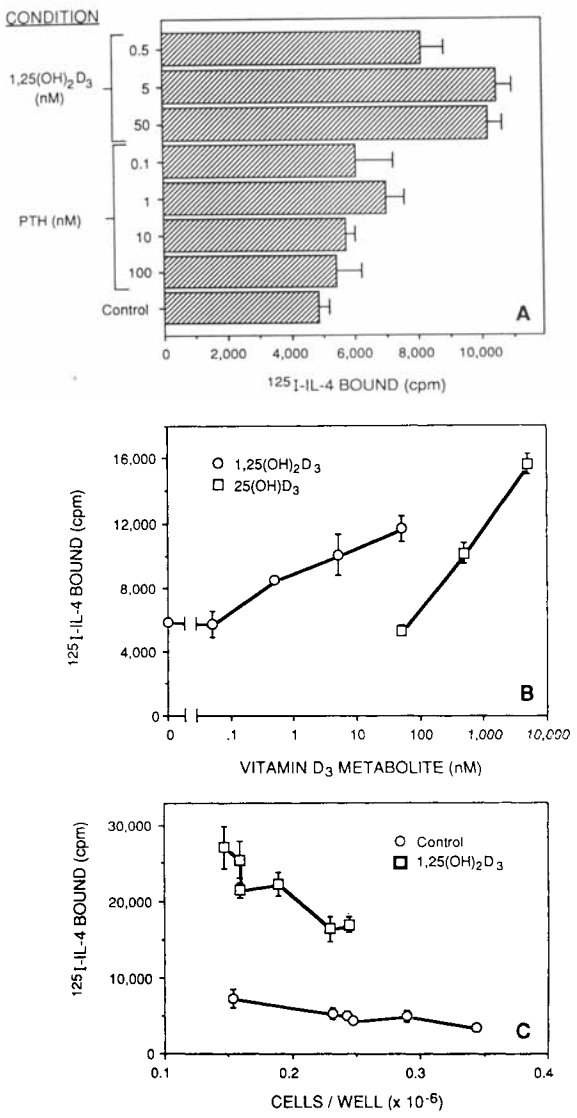

Fig. 4. Parathyroid hormone and 1,25 $(OH)_2$ D_3 effects on MC3T3 IL-4R expression. **A,** MC3T3 cultures (48 h after initiation) were exposed to various concentrations of either 1,25 $(OH)_2$ D_3 or bovine PTH (1–34) for 24 h. The cell layers were then utilized in ^{125}I-IL-4 binding experiments as described. **B,** To further explore the vitamin D_3 effect, cells cultured as in A were exposed to various levels of either 1,25 $(OH)_2$ D_3 or 25 (OH) D_3 for 24 h prior to ^{125}I-IL-4 binding. **C,** To exclude the possibility that cell density effects were mediating 1,25 $(OH)_2$ D_3 effects on IL-4R expression, MC3T3 cultures were initiated at various densities and allowed to grow for 48 h. Paired groups of wells were then exposed to 5 nM 1,25 $(OH)_2$ D_3 or media alone for an additional 24 h, after which ^{125}I-IL-4 binding was performed as described. The data in all experiments in this figure represent the mean (n = 3) and SD of the specific ^{125}I-IL-4 bound/1 × 10^6 cells.

$(OH)_2$ D_3 receptor [Haussler, 1986]. To exclude the possibility that the 1,25 $(OH)_2$ D_3 effect could be attributable to growth inhibition, MC3T3 cells were seeded at varying densities at T = 0 and cultured for 48 h. The cell layers were then treated with 5 nM 1,25 $(OH)_2$ D_3 for 24 additional hours followed by ^{125}I-IL-4 binding. The data were normalized to cell number and then plotted against cell density. At all cell densities examined, 1,25 $(OH)_2$ D_3 enhanced ^{125}I-IL-4 binding (Fig. 4C), suggesting that the vitamin D_3 effect was not indirectly mediated by density effects. The vitamin D_3 growth inhibitory effect was apparent in this experiment because the range of cell densities in the 1.25 $(OH)_2$ D_3 treated group was lower than control treated cells (D3, 0.148–0.245 × 10^6 cells/well; control, 0.154–0.345 × 10^6 cells/well).

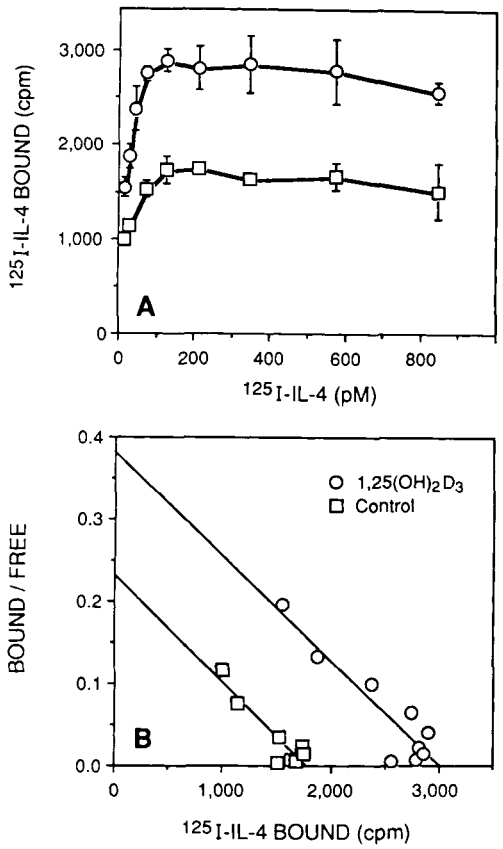

Fig. 5. 1,25 $(OH)_2$ D_3 increases the MC3T3 ^{125}I-IL-4 binding site number with no change in affinity. Confluent MC3T3 cultures were treated with 5 nM 1,25 $(OH)_2$ D_3 or media alone for 24 h, after which ^{125}I-IL-4 equilibrium binding was performed as in Figure 1. **A,** Binding curves of specific ^{125}I-IL-4 binding. **B,** Scatchard analysis of the data in A.

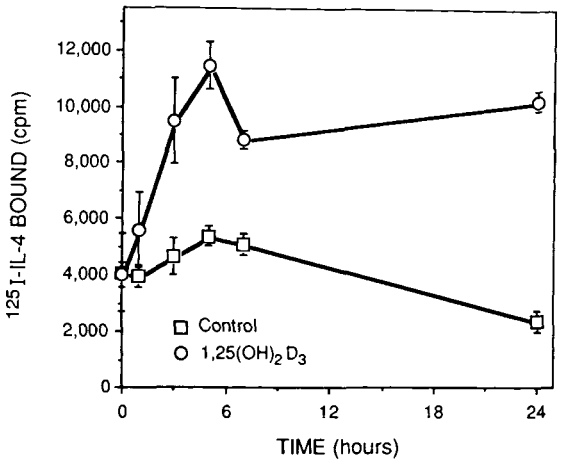

Fig. 6. 1,25 $(OH)_2$ D_3 enhancement of MC3T3 ^{125}I-IL-4 binding is time dependent. Confluent cultures of MC3T3 cells were treated with 5 nM 1,25 $(OH)_2$ D_3 or media alone for various time periods, after which ^{125}I-IL-4 binding was performed as described. The data represent the mean (n = 3) and SD of the specific ^{125}I-IL-4 bound/1 × 10^6 cells.

TABLE I. Cycloheximide Effects on MC3T3 IL-4R Expression

Condition[a]	Inhibitor[b]	Recovery[c]	^{125}I-IL-4 Bound[d]
Control	none	–	2,277 ± 1,158[e]
	none	+	2,588 ± 815
	CHX	–	1,214 ± 256
	CHX	+	4,261 ± 702
1,25-$(OH)_2D_3$ (5 nM)	none	–	6,147 ± 1,038
	none	+	6,374 ± 403
	CHX	–	2,568 ± 132
	CHX	+	6,984 ± 643

[a]MC3T3 cells were cultured as described for 48 h, after which time the media was changed and 1,25-$(OH)_2$ D_3 added.
[b]One hour prior to the addition of 1,25-$(OH)_2D_3$, CHX (200 nM) was added.
[c]To document the reversibility of the inhibitor effects, paired wells were rinsed (×3) and the media replaced with fresh media with or without 1,25-$(OH)_2D_3$ and incubated for an additional 24 h.
[d]^{125}I-IL-4 binding was determined as described (see Methods) after a 24 h treatment with agonist in the presence and absence of the respective inhibitor. For recovery cultures, ^{125}I-IL-4 binding was performed after a total of 48 h of treatment.
[e]The data are reported as the mean (n = 2) ± the range/2 of the net cpm ^{125}I-IL-4 bound/1 × 10^6 cells.

To further characterize the effect of 1,25 $(OH)_2$ D_3 on MC3T3 IL-4R expression, equilibrium binding studies were performed. As Figure 5A shows, both control and 1,25 $(OH)_2$ D_3 treated cells express saturable ^{125}I-IL-4 binding sites. Resolution of the data on Scatchard plots (Fig. 5B) and normalization to cell number reveal that the principal effect of 1,25 $(OH)_2$ D_3 treatment was to increase Bmax almost threefold (control Bmax = 2,351 sites/cell; D_3 Bmax = 6,831 sites/cell) with no change in the IL-4R K (control K = 13 pM; D_3 K = 13 pM).

The final series of experiments explored aspects of the mechanism operative in 1,25 $(OH)_2$ D_3 IL-4R upregulation. Figure 6 shows the results of a 1,25 $(OH)_2$ D_3 time-course experiment where ^{125}I-IL-4 binding was performed at various times after steroid exposure. Note that 1,25 $(OH)_2$ D_3 enhancement of IL-4R expression is detectable after a 3 h exposure and essentially plateaus at 5 h, where it is almost threefold above control cells.

The role of protein synthesis in 1,25 $(OH)_2$ D_3–induced IL-4R upregulation was then explored. As shown in Table I, 1,25 $(OH)_2$ D_3 treatment increased ^{125}I-IL-4 binding after a 24

h exposure by almost threefold when compared to control. In the presence of CHX, cells treated with media alone experienced a decline in ^{125}I-IL-4 binding. After CHX removal, control cells exhibited a rebound in ^{125}I-IL-4 binding after a 24 h incubation. Importantly, the increase induced by 1,25 $(OH)_2$ D_3 was largely prevented by CHX treatment. This CHX effect was not attributable to toxic effects as the viability of control and inhibitor treated cells exceeded 95% as assessed by trypan blue exclusion. Furthermore, inhibitor treated cells that were rinsed and restimulated recovered to show enhanced ^{125}I-IL-4 binding. The reason for the increased ^{125}I-IL-4 binding seen in cells recovering from CHX in the presence of control media is not understood but was never elevated to the extent that the CHX + 1,25 $(OH)_2$ D_3 recovery condition exhibited. These data imply that CHX's inhibition of 1,25 $(OH)_2$ D_3–induced IL-4R upregulation was reversible and that most of the 1,25 $(OH)_2$ D_3–driven IL-4R increase required protein synthesis to occur.

Because 1,25 $(OH)_2$ D_3–induced IL-4R upregulation involved protein synthesis, we next queried whether IL-4 mRNA levels were also impacted by the steroid in time-course experiments. As shown in Figure 7, 1,25 $(OH)_2$ D_3 treated MC3T3 cells exhibited increased levels of IL-4R mRNA as early as 4 h after steroid exposure. Further, this 1,25 $(OH)_2$ D_3–induced increase in steady-state IL-4R mRNA levels was maintained throughout the 24 h treatment period.

Based on the time-course experiments, we then assessed the concentration dependence of the steroid on IL-4R mRNA levels. As shown in Figure 7, a 3 h 1,25 $(OH)_2$ D_3 treatment resulted in elevated IL-4R mRNA levels in the presence of both 50 and 5 nM of the steroid. Lesser concentrations of the steroid were ineffective. The increased level of steady-state IL-4R mRNA induced by 1,25 $(OH)_2$ D_3 reflected the steroid's impact on gene transcription, message stability, or both. To determine whether 1,25 $(OH)_2$ D_3 treatment altered message stability, IL-4R mRNA half-life experiments were performed. As shown in Figure 9, the half-lives of IL-4R mRNA in both 1,25 $(OH)_2$ D_3 and control treated cells were similar (approximately 60 min), indicating that the steroid did not enhance IL-4R mRNA stability. These data suggest that 1,25 $(OH)_2$ D_3, directly or indirectly, increased IL-4R gene transcription in MC3T3 cells.

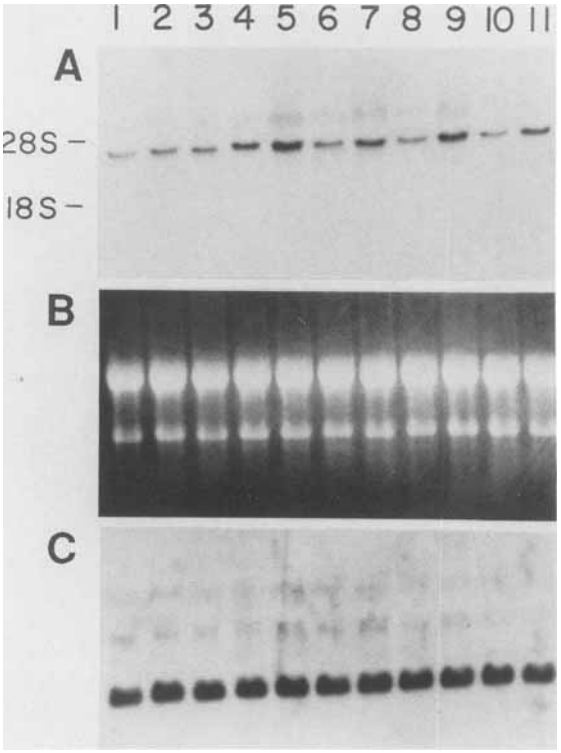

Fig. 7. 1,25 $(OH)_2$ D_3 increases IL-4R steady-state mRNA levels in a time-dependent manner. Confluent cultures of MC3T3 cells were treated with media supplemented with and without 5 nM 1,25 $(OH)_2$ D_3 for various times up to 24 h. Total cell RNA was purified from paired steroid and control treated cells, electrophoresed, blotted, and assessed for IL-4R and G3PDH mRNA as described (see Methods). Lane 1 is the time = 0 control and the additional lanes represent samples following 1 (lanes 2, 3), 4 (lanes 4, 5), 6 (lanes 6, 7), 9 (lanes 8, 9), and 24 (lanes 10, 11) h of treatment. Control and 1,25 $(OH)_2$ D_3 samples are in the even and odd numbered lanes (except for lane 1), respectively. **A** and **C** are the autoradiographs of the blots probed with the ^{32}P-IL-4R and ^{32}P-G3PDH probes, respectively. **B** is a photograph of the ethidium bromide stained gel.

DISCUSSION

This is the first report documenting IL-4 receptor expression by osteoblasts. The IL-4 receptor expressed by MC3T3 cells is identical to that expressed by T cells based on ligand binding (Fig. 1), M1 antibody blocking of ^{125}I-IL-4 binding (Fig. 2A), and the presence of IL-4R mRNA (Fig. 2B,C). The size of the IL-4 mRNA (Fig. 2C) and the ability to amplify an intracellular fragment (Fig. 2B) suggest that the osteoblasts predominantly express a full-length IL-4R and not an alternatively spliced form, which has been detected in a cDNA library derived from a murine T cell line [Mosley et al., 1989].

While the function of the intracellular portion of the IL-4R is unknown, it is reasonable to

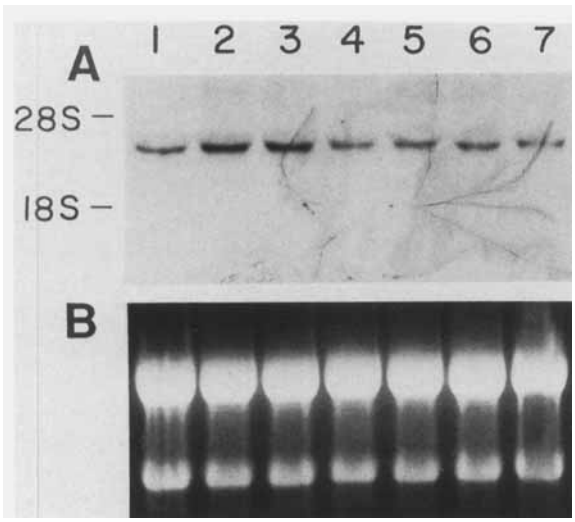

Fig. 8. 1,25 $(OH)_2$ D_3 augments IL-4R steady-state mRNA levels in a concentration-dependent manner. Confluent MC3T3 cells were treated with various amounts of 1,25 $(OH)_2$ D_3 for 3 h. Northern blots were prepared from total cell RNA and were hybridized with the ^{32}P-IL-4R probe as described. Lanes 1 and 7 are the T = 0 and 3 h controls, respectively. Lanes 2 through 6 are the 50, 5, 0.5, 0.05, and 0.005 nM 1,25 $(OH)_2$ D_3 treatment groups, respectively. **A** and **B** are the autoradiograph and ethidium bromide stained gel, respectively.

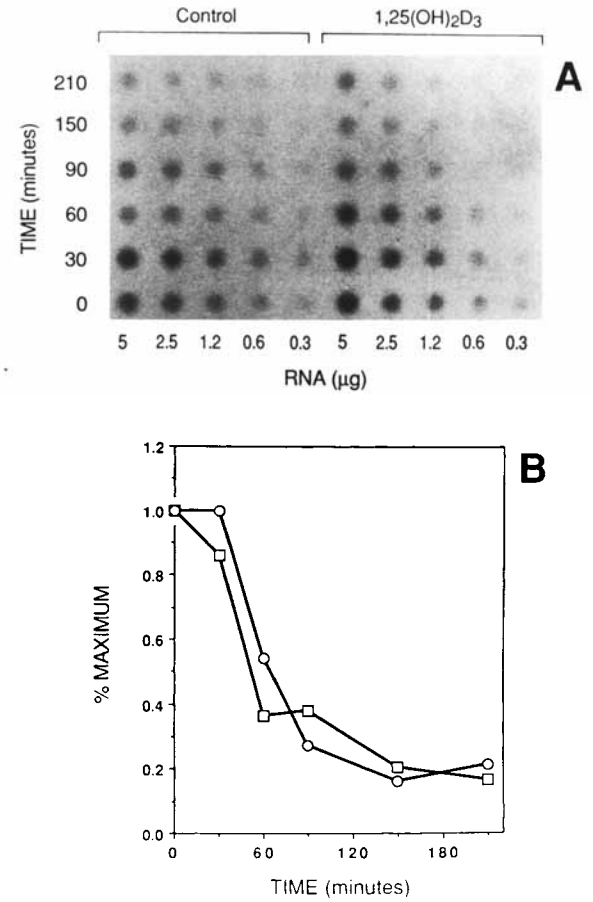

Fig. 9. 1,25 $(OH)_2$ D_3 does not alter IL-4R mRNA stability. Confluent MC3T3 monolayers were treated with media (□) or media with 5 nM 1,25 $(OH)_2$ D_3 (○) for 3 h, at which time 10 µg/ml actinomycin D was added. Total cell RNA was purified from paired plates at various times following actinomycin D addition. Dot blots were prepared with dilutions of the purified RNA and the membranes hybridized with the ^{32}P-IL-4R probe as described. Dot densities on the autoradiograph (developed after a four day exposure at −80°C) were determined and normalized as described. The values are plotted against the time of toxin exposure.

assume that it is required for biological activity. During this study we examined the IL-4 effect on cellular proliferation (Fig. 3A) and alkaline phosphatase (Fig. 3B) expression. Both activities were influenced by IL-4 levels that we observed to affect T cell proliferation [Lacey and Erdmann, 1990; Shioi et al., 1991] and inhibit in vitro osteoclast formation [Shioi et al., 1991]. This level of IL-4 has also been reported to inhibit in vitro and in vivo bone resorption [Watanabe et al., 1990, 1991]. Whether these IL-4–affected osteoblast activities are related to IL-4's inhibition of bone resorption is unknown, but they underscore the potential for yet another immunomodulatory cytokine to influence osteoblast activities.

Reasoning that osteotropic hormones may influence IL-4R expression as a means to potentially blunt their own proresorptive actions, the regulation of MC3T3 IL-4R by parathyroid hormone and 1,25 $(OH)_2$ D_3 was investigated. While parathyroid hormone did not alter IL-4R expression, vitamin D_3 increased MC3T3 IL-4R in a dose- (Fig. 4A,B), metabolite- (Fig. 4B), and time-dependent (Fig. 6) manner via a mechanism requiring protein synthesis (Table I). Furthermore, the steroid increased IL-4R mRNA levels in both a time- (Fig. 7) and concentration-dependent (Fig. 8) manner. Because 1,25 $(OH)_2$ D_3 did not alter the IL-4R mRNA half-life (Fig. 9), we conclude that increased IL-4R gene transcription likely plays a role in this effect. Additional evidence suggesting that vitamin D may affect IL-4R gene regulation comes from sequence analysis of a recently published murine IL-4R genomic clone [Wrighton et al., 1992]. In this report, approximately 1 kb of 5′ untranslated genomic sequence is presented that appears structurally consistent with a promoter. In this region, directly 5′ to an SP-1 element, is the motif GGGTGA arranged as a direct repeat separated by nine nucleotides. This sequence is similar to other described vitamin D receptor binding sequences [Carlberg et al., 1993] and

may function as a vitamin D response element in the murine IL-4R promoter. Obviously, further studies are needed to test this hypothesis.

Despite its experimental effects on in vitro and in vivo bone resorption, it is unclear whether IL-4 participates either physiologically or pathologically *in bone remodeling*. We have not detected IL-4 mRNA in MC3T3 total cell RNA utilizing reverse transcription/DNA amplification with murine IL-4 primers making it unlikely that IL-4 is an osteoblastic autocrine factor (data not shown). Other IL-4 producing cells in the marrow compartment, such as mast cells, basophils, and/or T cells, may provide IL-4 locally. Mast cells are increased in hyperparathyroidism [reviewed in McKenna and Frame, 1985] and postmenopausal osteoporosis [Fallon et al., 1983], suggesting that mast cells play a role in the pathogenesis of these remodeling disorders. While heparin, prostaglandins, histamine, and kininogenase activity have been invoked as mast cell mediators involved in these diseases [Lidor et al., 1990], it is possible that IL-4 also plays a role. A previous report [Levi-Schaffer and Bar-Shavit, 1990] documenting the capacity of osteoblastic cells to maintain mast cell viability and function *in vitro* provides experimental evidence that osteoblasts and mast cells could interact in vivo with IL-4, possibly functioning as a local paracrine mediator. Furthermore, the skeletal lesions of systemic mastocytosis, which are associated with markedly increased numbers of marrow mast cells [Fallon et al., 1981], may reflect the impact of mast cell–produced IL-4 on the local osteoblast population. Regarding T cells, inflammatory conditions associated with T cell activation could result in the production of cytokines known to influence resorptive activity including interferon γ [Gowen and Mundy, 1986], tumor necrosis factor β (lymphotoxin) [Bertolini et al., 1986], and interleukin 6 [Ishimi et al., 1990], amongst others. Interleukin 4 can now be added to this list.

The extent to which IL-4's inhibition of agonist-induced bone resorption is mediated by the osteoblast is not understood. As noted previously, the significant time required to generate osteoclasts in vitro [Shioi et al., 1991] coupled with IL-4's rapid effects in vitro and in vivo suggests that cells other than osteoclast precursors mediate IL-4's immediate inhibitory effects on bone resorption. The osteoblast represents a logical target cell because it modulates the effects of many resorption-inducing cytokines. In addition to secreting proteases that may prepare the bone surface for osteoclast attachment [Partridge et al., 1987; Chambers et al., 1985], osteoblasts release factors that serve to activate osteoclasts [Thomson et al., 1986, 1987; McSheehy and Chambers, 1986; Gallwitz et al., 1991]. Interruption of either activity could impede resorptive activity. Alternatively, IL-4–activated osteoblasts may secrete factors that suppress osteoclast function. In any event, the observation that osteoblasts express IL-4Rs and respond to this cytokine suggests that IL-4 effects on the skeleton could be mediated, to a significant extent, by osteoblastic cells.

ACKNOWLEDGMENTS

Portions of this work were supported by a grant from the National Institutes of Health (AI26814).

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