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Skeletal Resistance to 1,25-Dihydroxyvitamin D_3 in Osteopetrotic Rats

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The osteopetrotic (*op/op*) rat mutation is a lethal mutation in which decreased osteoclast function (bone resorption) coexists with markedly elevated serum levels of 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$]. Increased circulating levels of 1,25$(OH)_2D_3$ have been reported in other osteopetrotic animal mutations and in some osteopetrotic children. This study examined the effects of 1,25$(OH)_2D_3$ infusions on serum and skeletal parameters in normal and mutant rats of *op* stock. We also examined vitamin D receptor expression and binding in bone cells from *op* normal and mutant animals. Four-week-old normal and mutant rats were infused either with propylene glycol (used as controls) or with 12.5 – 125 ng of 1,25$(OH)_2D_3$/d using osmotic minipumps implanted subcutaneously for 1 wk. Sera were analyzed for calcium, phosphorus, and 1,25$(OH)_2D_3$ levels. Histomorphometric analyses of proximal tibiae from treated normal (50 ng/d) and *op* mutant (125 ng/d) rats and their vehicle-infused controls were performed. Normal animals infused with 1,25$(OH)_2D_3$ exhibited a dose-dependent increase in serum calcium levels. Histomorphometric analyses of metaphyseal bone within the primary spongiosae region showed that 1,25$(OH)_2D_3$ increased osteoclast number with a reduction in osteoblast surface associated with a decrease in growth plate cartilage thickness. However, similar analyses on secondary spongiosae showed a decrease in osteoclast number and surface associated with an anabolic response. *Op* mutants infused with 1,25$(OH)_2D_3$ did not exhibit any change in serum calcium levels or histomorphometric parameters related to growth plate cartilage and metaphyseal bone compared with mutant controls. Vitamin D mRNA and protein levels were increased two- to threefold in *op* mutants compared to age-matched normal rats. However, binding affinity of 1,25$(OH)_2D_3$ to its receptor was similar between *op* mutant and normal animals. High dose calcitriol therapy, under the conditions and period of treatment used in this study, failed to stimulate bone turnover in *op* rats, suggesting that they are resistant to the skeletal effects of 1,25$(OH)_2D_3$. The failure of osteoclast activation in response to 1,25$(OH)_2D_3$ treatment may be associated with osteoblast incompetence in this mutation.

Key Words: Osteopetrosis; 1,25-dihydroxyvitamin D_3; skeletal resistance.

Introduction

Osteopetrosis describes a heterogeneous group of metabolic bone diseases characterized by a net increase in skeletal mass resulting from a primary reduction in osteoclast-mediated bone resorption *(1)*. Spontaneous, autosomal recessive mutations causing an osteopetrotic phenotype have been described in three animal species (mouse, rat, and rabbit) and humans *(2)*. The extent to which the skeleton is affected varies depending on the particular mutation. Decreased bone resorption results in a progressive accumulation of mineralized matrix and a reduction or absence of marrow cavity formation, thereby producing the characteristic radiographic appearance of the osteopetrotic skeleton *(3)*. Despite this common feature, there is a great deal of heterogeneity concerning the cellular components and matrices of the skeleton among the osteopetrotic mutations *(4,5)*. For example, osteoclasts are present in normal or are increased in numbers in some mutations but absent in others *(4,5)*. Ruffled borders, the ultrastructural hallmark of active osteoclasts, are absent in some mutations but present in others. It has become clear that this heterogeneity is owing to differences at the point where osteoclast development or activation is affected in each mutation *(4,5)*.

The first treatment for osteopetrosis was demonstrated in osteopetrotic (microphthalmic [*mi*]) mice in which transplantation of bone marrow from normal littermates cured the osteopetrotic hosts, resulting in complete and sustained resorption of the excess skeletal matrix *(6)*. This

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pioneering work led to the successful use of bone marrow transplantation in several osteopetrotic children *(7–10)*. However, subsequent studies have shown that some osteopetrotic animal mutations *(11–14)* and children *(15,16)* do not respond to bone marrow transplantation. This differential response to bone marrow transplantation exemplifies the pathogenetic heterogeneity among the osteopetrotic mutations.

Many osteopetrotic children either are not candidates for bone marrow transplantation or a suitable donor is not available. Therefore, alternative therapeutic strategies have been utilized. Elevated circulating levels of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] have been observed in most osteopetrotic animal mutations *(17–21)* and some osteopetrotic children *(22–24)*. This finding, coupled with the well-documented effect of $1,25(OH)_2D_3$ in stimulating osteoclast development and function *(25,26)*, led to the hypothesis that there may be a resistance to the skeletal effects of calcitriol in some forms of osteopetrosis *(19,24,27)*. High-dose calcitriol therapy has been employed with variable clinical outcomes (ranging from significant increases in bone resorption to no effect) in osteopetrotic children *(3,24,28,29)*. The effect of high-dose calcitriol treatment has also been examined in the incisors-absent (*ia*) rat *(30)* and osteosclerotic (*os*) rabbit *(27)* mutations. This treatment stimulated bone resorption in *ia* rats but was ineffective in reducing bone mass in *os* rabbits.

The osteopetrotic (*op*) mutation in the rat is a lethal mutation that was first reported in 1973 by Moutier and colleagues *(31)* as a spontaneous mutation in a nonconsanguineous *Fatty/ORL* colony. Osteoclasts in *op* mutants are either normal or reduced in number depending on age *(32)*. *Op* osteoclasts also exhibit an atypical phenotype; they are much larger than their normal counterparts, exhibit a homogeneous cytoplasm devoid of vacuoles, and have poorly developed ruffled borders *(32)*. Osteoclasts in *op* rats stain poorly, if at all, for tartrate-resistant acid phosphatase or acid ATPase, two enzymes associated with normal osteoclast function *(32,33)*. The skeletal defect in *op* mutants can be cured by transplantation of bone marrow from normal littermates *(18,34)*. In addition to defects involving osteoclasts, abnormalities in osteoblast function *(35)* and gene expression *(36)*, mineral homeostasis *(12)*, and immune function *(37)* have also been reported in *op* mutants. Serum $1,25(OH)_2D_3$ levels are markedly elevated in *op* mutants compared to normal age-matched littermates; this abnormality is present at birth and persists as the animals age *(17–19)*.

In this study, we examined the effects of high-dose calcitriol treatment on serum and skeletal parameters in *op* mutants and normal rats. We also evaluated the vitamin D receptor (VDR) expression and binding in bone from *op* and normal rats to determine whether the lack of a $1,25(OH)_2D_3$-mediated response in *op* mutants was owing to deficient expression of the VDR. The results of this study, in conjunction with those obtained from studies in other osteopetrotic animal mutations, help refine our understanding of this unique phenotypic difference between mutations so necessary for the development of effective, alternative strategies for treating this heterogeneous disorder.

Results

Continuous infusion of $1,25(OH)_2D_3$ (50 ng/d) into normal animals for a period 7 d averted the daily increase in body weight compared to normal propylene glycol–treated (control) rats (Fig. 1). At the lowest dose (12.5 ng/d), body weights were not significantly different from those of control rats; the intermediate dose (30 ng/d) diminished the daily weight gains, but the effect was not as dramatic as at the highest dose (data not shown). In propylene glycol–treated *op* animals, the average body weights were significantly lower than those of their propylene glycol–treated normal counterparts (Fig. 1). $1,25(OH)_2D_3$ (125 or 30 ng/d) infusion into *op* rats did not have any effect on their daily body weight gains compared with propylene glycol-treated *op* mutants (Fig. 1; data not shown for lower dose). This suggests that the *op* rats are resistant to the effect of $1,25(OH)_2D_3$ on body weight, even at very high doses. Although the growth curve for $1,25(OH)_2D_3$-treated and control *op* mutants was similar, treated *op* rats consistently had higher mean body weights compared with control *op* mutants. This was due to the fact that *op* mutants with the highest body weights were selected to receive the $1,25(OH)_2D_3$ infusions. Because *op* mutants are considerably smaller than their normal littermates, the most robust mutants were chosen for the experimental group.

Serum Biochemical Analyses

The mean serum calcium levels showed that $1,25(OH)_2D_3$ infusion into normal animals elicited a significant hypercalcemic effect at the two highest doses administered (30 and 50 ng/d, $p < 0.05$ and $p < 0.001$, respectively) when compared with untreated control rats (Fig. 2A). Serum calcium levels in untreated *op* rats were similar when compared to age-matched normal controls. Mutant *op* rats did not exhibit an increase in serum calcium levels in response to $1,25(OH)_2D_3$, including the highest dose (125 ng/d), when compared with propylene glycol–treated mutant controls (Fig. 2A). These data demonstrate that *op* mutants are resistant to the calcemic effect of $1,25(OH)_2D_3$.

Serum phosphorus levels in untreated *op* mutants were significantly decreased ($p < 0.05$) when compared to age-matched untreated normal controls (Fig. 2B). The serum phosphorus levels in $1,25(OH)_2D_3$-infused normal and mutant animals were not significantly different from those of their untreated counterparts, regardless of the dose infused (Fig. 2B).

Circulating serum $1,25(OH)_2D_3$ measurements showed that normal rats infused with 50 ng of $1,25(OH)_2D_3$/d had significantly increased levels of $1,25(OH)_2D_3$ (139 ± 28 pg/mL, $p < 0.01$) when compared with untreated normal rats (46 ± 15 pg/mL) (Fig. 3). In propylene glycol–treated *op*

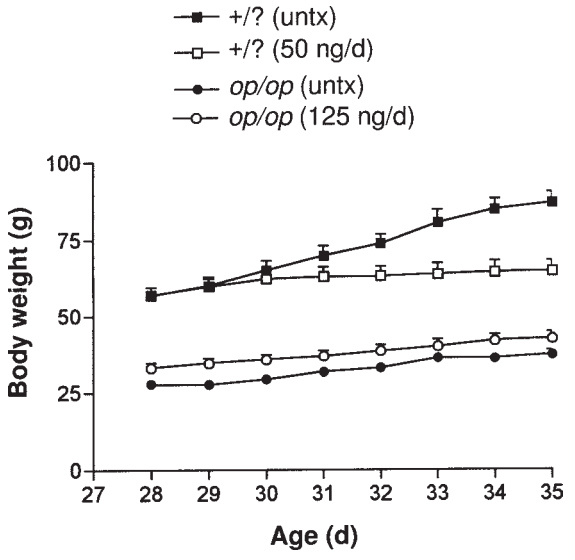

Fig. 1. Body weights of untreated (untx) and 1,25$(OH)_2D_3$-infused normal and mutant rats. Four-week-old normal (+/?) and mutant (*op/op*) rats were infused with 1,25$(OH)_2D_3$ for 7 d by subcutaneously implanted osmotic minipumps. Normal animals were infused with 50 ng/d and mutant animals were infused with 125 ng/d. Body weights were recorded daily. Data are expressed as the mean ± SEM; *n* ranges between 10 – 20 animals per group.

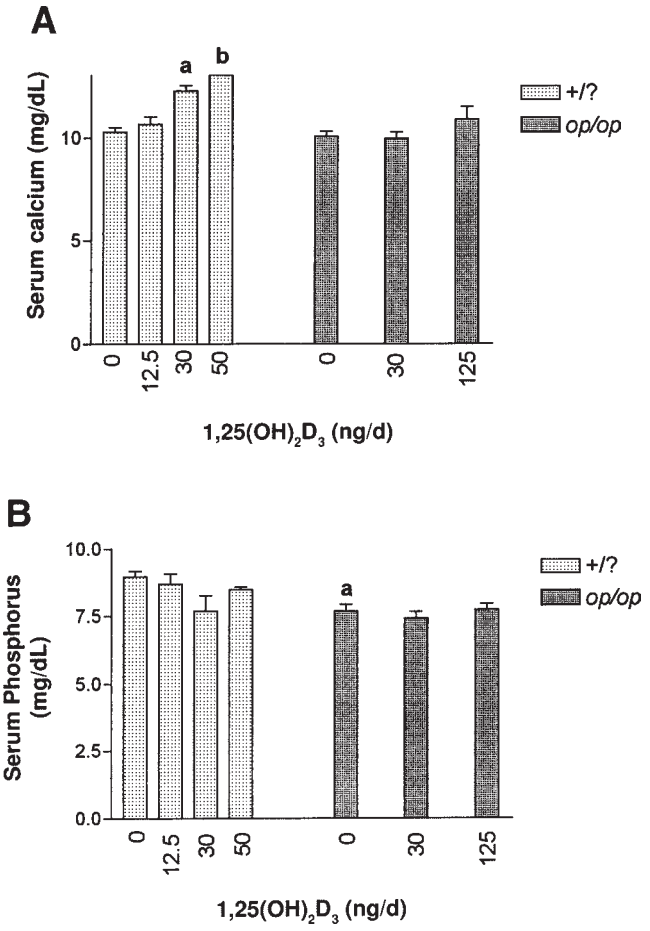

Fig. 2. Serum calcium and phosphorus concentrations in vehicle- and 1,25$(OH)_2D_3$-infused normal (+/?) and mutant (*op/op*) rats. **(A)** Normal rats were infused with 12.5, 30, or 50 ng/d; *op* mutants were infused with 30 or 125 ng/d. Controls were infused with propylene glycol. Data are expressed as the mean ± SEM for each group. There was no hypercalcemic effect in *op* rats even at the highest dose infused. [a] ($p < 0.05$) and [b] ($p < 0.001$), comparison between treated and untreated normal rats using two-factor analysis of variance (ANOVA) with Bonferroni post-test. **(B)** 1,25$(OH)_2D_3$ treatment did not alter serum phosphorus levels in normal or mutant rats. Untreated mutants were hypophosphatemic compared with untreated normal rats. Data are expressed as the mean ± SEM. [a] ($p < 0.05$), comparison between untreated normal and mutant rats using two-factor ANOVA with Bonferroni post-test.

controls, 1,25$(OH)_2D_3$ levels were significantly higher (138 ± 32 pg/mL) when compared with normal rats, and these baseline levels were similar to those observed in 1,25$(OH)_2D_3$-treated (50 ng/d) normal rats (Fig. 3). When *op* mutants were treated with 1,25$(OH)_2D_3$ (125 ng/d), serum levels increased significantly (369 ± 111 pg/mL, $p < 0.01$) compared with their mutant controls.

Bone Histological and Histomorphometric Analyses

Histological examination and histomorphometric analysis of proximal tibial growth plate cartilage revealed that in normal rats, 1,25$(OH)_2D_3$ treatment (50 ng/d) caused a 35–40% reduction in resting zone/proliferative zone (RZ/PZ), hypertrophic cell zone (HCZ), and total growth plate thickness (GPTh) when compared with propylene glycol–treated control rats (Fig. 4, Table 1). Control *op* mutants exhibited a significant decrease in growth plate thickness including both the RZ/PZ and the HCZ when compared with normal controls (Fig. 4, Table 1). However, even when the highest dose (125 ng/d) of 1,25$(OH)_2D_3$ was infused into *op* rats, no differences in zonal thickness (RZ/PZ and HCZ) or GPTH were observed compared with vehicle-infused mutants (Fig. 4, Table 1).

Qualitative and quantitative evaluations of proximal tibial metaphyses in normal rats revealed metaphyseal site differences in several static bone parameters following 1,25$(OH)_2D_3$ infusion (Fig. 5, Table 2). The principal effects on primary spongiosae were to increase significantly the numbers of osteoclasts and the fractional surface of bone covered by osteoclasts. This activity appeared to be at the expense of osteoblasts because their number and fractional surfaces were decreased compared with untreated normal controls.

Although osteoclast activation seemed to be the principal 1,25$(OH)_2D_3$-mediated effect in the primary spongiosae region, increased osteoblastic and reduced osteoclastic activity was observed in the region containing secondary spongiosae. Bone volume and trabecular numbers were significantly increased and trabecular separation was decreased in treated normal rats. The number of osteoblasts/millimeter of bone perimeter and fractional surface were slightly, though not significantly, increased. 1,25$(OH)_2D_3$ infusion may have impaired matrix mineralization because osteoid volume, osteoid surface, and osteoid thickness

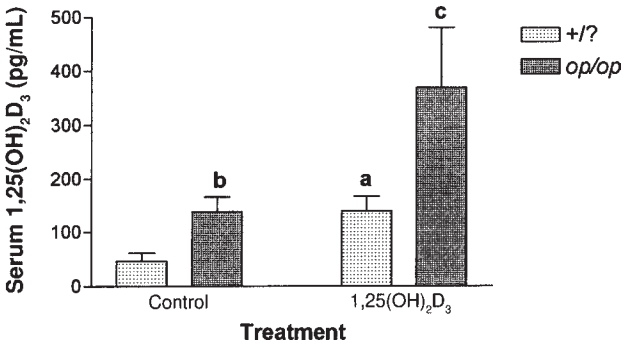

Fig. 3. Serum levels of $1,25(OH)_2D_3$ in untreated normal and $1,25(OH)_2D_3$-infused normal (+/?) and mutant (*op*/*op*) rats. Untreated *op* mutants have markedly elevated serum levels of $1,25(OH)_2D_3$ compared with untreated normal rats. $1,25(OH)_2D_3$ treatment caused hypervitaminosis in both normal and mutant animals when compared to their untreated littermates. Data are expressed as the mean ± SEM. [a] ($p < 0.05$), comparison between untreated normal and mutant rats; [b] ($p < 0.01$), comparison between treated and untreated normal rats; [c] ($p < 0.01$), comparison between treated and untreated mutant rats using two-factor ANOVA with Bonferroni post-test.

parameters were markedly increased. This anabolic response and the hyperosteoidosis may have inhibited osteoclastic activity, as evidenced by a fivefold decrease in osteoclast number and fractional surface. Under normal, unstimulated conditions, this metaphyseal region is an active area of resorption, as suggested by the increased number of osteoclasts and fractional surface compared to the primary spongiosae region. In addition, between these anatomic sites, bone volume decreased 63% in untreated normal rats compared to 42% in $1,25(OH)_2D_3$-infused normal controls.

These findings of increased bone cell activity and bone turnover in normal rats contrast sharply with the lack of effect of $1,25(OH)_2D_3$ to alter static parameters of bone in *op* rats (Fig. 5, Table 3) and support our hypothesis that this mutation is resistant to this hormone. No differences were observed between treated and untreated mutant animals within an equivalent area of the metaphysis corresponding to the primary spongiosae in normal rats. By definition, all of the trabeculae in *op* mutants represent primary spongiosae containing central cores of calcified cartilage surrounded by bone matrix and intertrabecular spaces devoid of organized hemopoietic tissue. Because there are no secondary spongiosae in *op* mutants, quantitation of this tissue was not possible. Furthermore, there were no noticeable cellular or bone changes in the area of the metaphysis that corresponded to the secondary spongiosae in normal rats.

VDR Expression and Binding Analyses

Steady-state VDR mRNA levels were evaluated in calvaria from *op* rats and their normal counterparts at 4 and 6 wk of age by the RNase protection assay (RPA). VDR transcript levels were increased in *op* rats when compared with age-matched normal littermates (Fig. 6A). The relative amounts of VDR mRNA determined by scanning densitometry of protected RNA fragments showed that the mutant/normal (M/N) ratio was 2.75 and 2.25 at 4 and 6 wk of age, respectively (Fig. 6B). Nonlinear regression analysis of saturation binding assays was performed to estimate receptor number (N_{max}) and ligand-binding affinity (K_d). The number of VDRs was significantly increased in *op* mutants compared with normal littermates at 4 (twofold) and 6 (threefold) wk of age ($p < 0.001$) (Fig. 7).

The ligand-binding affinities of the VDR were similar in *op* mutant and normal rats (Table 4). This indicates that the inability of $1,25(OH)_2D_3$ to elicit a functional response in *op* bone is not related to VDR numbers or ligand-binding affinity.

Discussion

Previous studies have identified abnormalities of the vitamin D–endocrine system in various osteopetrotic mutations including significantly elevated serum levels of $1,25(OH)_2D_3$ *(4,14,17,19,21–24)*. This hormone, the most active metabolite of vitamin D_3, is at least 1000 times more potent in stimulating bone resorption than any of the other vitamin D_3 metabolites *(38,39)*. Despite its well-documented effects on stimulating osteoclast development and the activity of preexisting osteoclasts *(25,32,40)*, bone resorption is decreased in all osteopetrotic mutations *(20)*. Based on the possibility that there may be a resistance to calcitriol in these mutations, high-dose treatment has been demonstrated to overcome this resistance and stimulate osteoclast development and/or activity in some mutations *(24,28,30)*.

In the present study, we examined the effects of $1,25(OH)_2D_3$ infusion on serum parameters and skeletal histomorphometric indices in *op* and normal rats. We have previously shown that continuous infusion of $1,25(OH)_2D_3$ via subcutaneously implanted osmotic minipumps is a reliable method to achieve steady-state elevations of circulating $1,25(OH)_2D_3$ over extended periods *(27)*. The doses infused into normal and mutant rats in the present study were different. In normal rats, the highest dose used (50 ng/d) caused a threefold elevation in serum $1,25(OH)_2D_3$ levels, elicited a calcemic response, and stimulated bone turnover. This dose prevented the daily body weight gains observed in normal controls during the infusion period, but it did not have a lethal (toxic) effect on the animals. However, lower doses (12.5 and 30 ng/d) had either no effect or an intermediary effect on these same parameters (data not shown). In *op* mutants, the highest dose infused (125 ng/d) caused a similar increase (2.7-fold) in the already high baseline circulating levels of $1,25(OH)_2D_3$. However, none of the doses infused in *op* mutants had any effect on serum calcium levels or static histomorphometric parameters. These data demonstrate that *op* mutants are resistant to the effects of $1,25(OH)_2D_3$ on calcium mobilization and bone resorption.

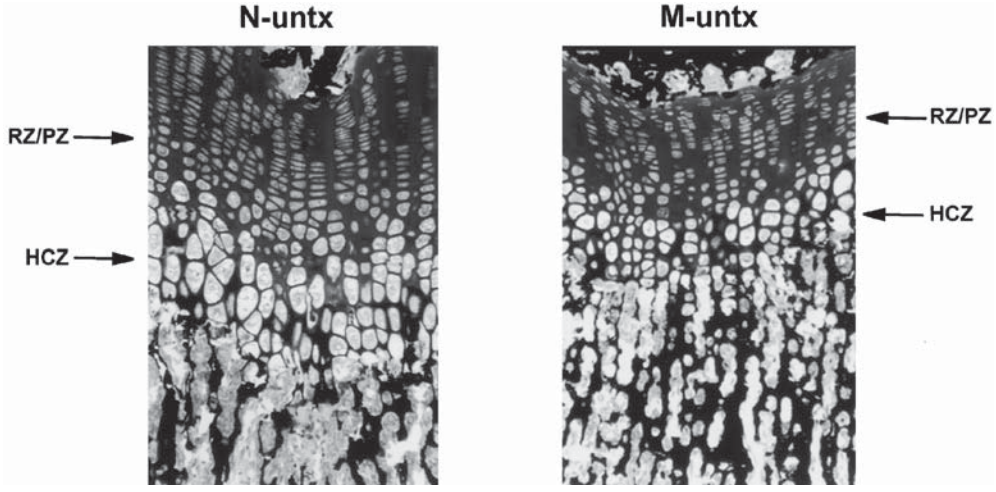

Fig. 4. Photomicrographs of proximal tibial growth plate cartilage in normal untreated (N-untx), and *op*/*op* mutant untreated (M-untx) rats. RZ/PZ, Resting Zone/Proliferative Zone; HCZ, Hypertrophic Cell Zone.

Table 1
Effects of 1,25$(OH)_2D_3$ Infusion on Tibial Growth Plate Cartilage[a]

Phenotype	*n*	Tx group (ng/d)	RZ/PZ (μm)	HCZ (μm)	GPTh (μm)
+/?	7	0	267.8 ± 10.5	162.6 ± 7.9	430.4 ± 16.3
+/?	8	50	174.1 ± 8.3[b]	97.4 ± 3.1[b]	271.5±10.1[b]
op/*op*	8	0	187.9 ± 12.5[c]	107.0 ± 7.8[c]	294.9 ± 18.6[c]
op/*op*	9	125	208.5 ± 14.0	107.7 ± 4.8	316.3 ± 13.2

[a]All values represent means ± SEM. Tx, Treated group; RZ/PZ, resting zone/proliferative zone; HCZ; hypertrophic cell zone; GPTh; total growth plate thickness; n, number of animals used. [b]$p < 0.001$ represents the comparison between normal untreated and normal treated rats.
[c]$p < 0.001$ represents the comparison between untreated normal and untreated mutant rats using two-factor ANOVA with Fisher post-test.

The histomorphometric results indicate different functional effects of 1,25$(OH)_2D_3$ on normal metaphyseal bone. Within the primary spongiosae region, 1,25$(OH)_2D_3$ acts to increase osteoclasts and is largely without effect in stimulating osteoblastic parameters. In the secondary spongiosae region, however, 1,25$(OH)_2D_3$ acts bifunctionally to decrease osteoclast numbers and fractional surface as well as to stimulate an anabolic response. Whether these responses are direct or elicited by the activities of skeletal growth factors released during osteoclastic bone resorption is unclear. That elevated 1,25$(OH)_2D_3$ impairs matrix mineralization was evident by the finding of excess trabecular osteoid in the present study.

Previous in vivo studies examining the effects of 1,25$(OH)_2D_3$ on mineralization and bone formation have yielded conflicting results.(*26,41–44*). Continuous infusions of physiological doses of calcitriol in normal young mice promoted the calcification of bone matrix but inhibited the formation of new bone *(26,41)*. However, another study in which rats were chronically treated with calcitriol demonstrated an anabolic effect; increased bone formation and decreased mineralization *(42)*. Other studies have confirmed hyperosteoidosis associated with increased osteoblast synthetic activity as a result of calcitriol treatment in rats *(43,44)*. The normal rats infused with 50 ng/d in this study also exhibited a significant increase in osteoid volume. Perhaps these discrepancies in prior studies can be explained, at least in part, by the differential response within primary vs secondary spongiosae.

Histomorphometric analysis of the proximal tibial growth plate from normal animals infused with 1,25$(OH)_2D_3$ (50 ng/d) showed that the RZ/PZ, the HCZ, and GPTh were significantly reduced compared with control rats. The effect on the RZ/PZ is consistent with another study demonstrating that 1,25$(OH)_2D_3$ inhibits the proliferation of chondrocytes in primary culture *(45)*. The reduction in thickness of the RZ/PZ may also be caused by 1,25$(OH)_2D_3$ in promoting chondrocyte maturation toward the hypertrophic phenotype, as demonstrated in previous studies *(46,47)*. The decrease in thickness of the HCZ may be owing to an accelerated rate of terminal differentiation of the hypertrophic chondrocytes and/or the induction of apoptosis *(48,49)* among these cells. Compared with the vehicle-treated normal rats, the vehicle-treated mutant controls had a significant reduction in growth plate thickness (all zones); calcitriol-treatment of *op* mutants failed to elicit any response in the growth plate. Although the elevated baseline levels of 1,25$(OH)_2D_3$ could

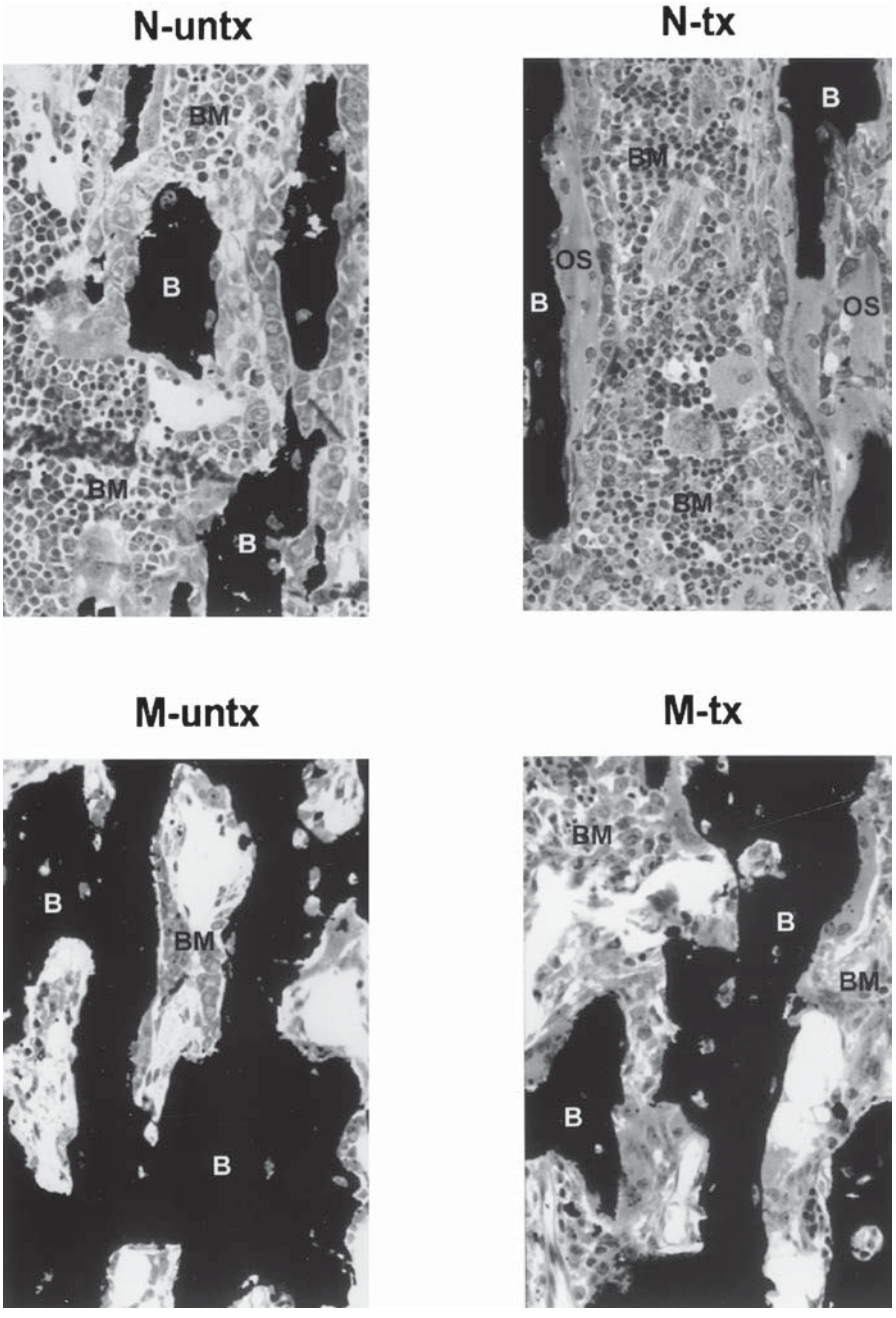

Fig. 5. Photomicrographs of proximal tibial metaphyses of secondary spongiosae from normal untreated (N-untx), normal 1,25$(OH)_2$D-treated (N-tx) (50 ng/d), mutant untreated (M-untx), and mutant 1,25$(OH)_2$D-treated (M-tx) (125 ng/d) rats. Compared with normal controls, normal rats treated with 1,25$(OH)_2D_3$ showed an increase in osteoid volume and trabecular thickness. No significant differences were noted between control and 1,25$(OH)_2D_3$-treated mutant rats. OS, Osteoid; B, Bone; BM, Bone Marrow.

account for the reduced growth plate thickness in control *op* mutants, our results suggest that *op* chondrocytes are also unresponsive to increased circulating levels of 1,25$(OH)_2D_3$ induced by exogenous administration of the hormone.

The *op* rat mutation is a lethal mutation characterized by severe skeletal sclerosis that persists with age *(50)*. Among the osteopetrotic animal mutations that have been treated with 1,25$(OH)_2D_3$, the *op* mutation is the only one that has demonstrated a complete lack of response to 1,25$(OH)_2D_3$ treatment. The failure to stimulate bone resorption and mobilize calcium with high-dose calcitriol therapy in this mutation requires further explanation. It is unlikely that circulating levels were not high enough to overcome vitamin D–dependent forms of resistance. At the highest dose (125 ng/d), serum levels of 1,25$(OH)_2D_3$ were similar to or in excess of those levels that stimulated bone resorption in *ia* rats *(30)* and vitamin D–responsive osteopetrotic children *(24,28)*. In a follow-up experiment, higher doses (up to 600 ng/d) of 1,25$(OH)_2D_3$ were infused in *op* mutants for 7 d, yet even at these doses there was no calcemic or

Table 2
Static Histomorphometric Parameters of Bone in Normal Rats Infused with $1,25(OH)_2 D_3$[a]

Metaphyseal site	Tx group (ng/d)	BV/TV (%)	OV/BV (%)	Tb.Th (μm)	Tb.Sp (μm)	Tb.N	N.Ob/B.Pm	N.Oc/B.Pm	Ob.S/BS (%)	Oc.S/BS (%)	OS/BS (%)	O.Th (μm)
Primary	0	36.3 ± 2.1	0.02 ± 0.02	15.1 ± 1.4	26.3 ± 0.3	24.2 ± 0.9	24.7 ± 2.1	1.4 ± 0.1	35.3 ± 2.3	4.8 ± 0.5	0.1 ± 0.1	0.4 ± 0.4
spongiosae	50	34.7 ± 1.9	0.2 ± 0.1	17.2 ± 0.6	33.1 ± 2.9	20.3 ± 1.5	20.2 ± 0.6	2.5 ± 0.3^{b}	28.9 ± 1.2^{b}	9.9 ± 1.0^{c}	0.6 ± 0.3	2.2 ± 0.7
Secondary	0	13.3 ± 0.5*A*	0.2 ± 0.06^{B}	26.3 ± 1.2^{C}	171.9 ± 9.9^{A}	5.1 ± 0.3^{A}	12.8 ± 1.6^{C}	2.5 ± 0.4^{B}	18.0 ± 2.1^{C}	10.3 ± 2.3	1.5 ± 0.3^{C}	1.8 ± 0.2^{B}
spongiosae	50	$20.1 \pm 2.1^{b,D}$	$13.1 \pm 3.8^{b,C}$	27.3 ± 0.7^{A}	$114.9 \pm 16.3^{b,C}$	$7.4 \pm 0.8^{b,A}$	15.3 ± 0.8^{C}	$0.5 \pm 0.07^{d,D}$	22.6 ± 1.3^{C}	$1.8 \pm 0.3^{c,A}$	$23.7 \pm 5.4^{c,C}$	$7.1 \pm 0.6^{d,D}$

[a]All values represent means ± SEM from at least four rats/treatment group. [b–d] ($p < 0.005$, $p < 0.01$, $p < 0.001$, respectively) represent the comparison between normal untreated and normal treated rats at respective metaphyseal sites. [A–D] ($p < 0.0001$, $p < 0.05$, $p < 0.001$, $p < 0.001$, respectively) represent the comparison among similar treatment groups and metaphyseal sites. Data were analyzed using two-factor ANOVA with Fisher post-test. Tx group, treated group; BV/TV, bone volume as a fraction of total tissue volume; OV/BV, osteoid volume as a fraction of bone volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N., trabecular number; N.Ob/B.Pm, number of osteoblasts/mm bone perimeter; N.Oc/B.Pm, number of osteoclasts/mm bone perimeter; Ob.S/BS, osteoblast surface as a fraction of bone surface; Oc.S/BS, osteoclast surface as a fraction of bone surface; OS/BS, osteoid surface as a fraction of bone surface; O.Th, osteoid thickness.

Table 3
Static Histomorphometric Parameters of Bone in *op*/*op* Rats Infused with $1,25(OH)_2D_3$[a]

Tx group (ng/d)	BV/TV (%)	OV/BV (%)	Tb.Th (μm)	Tb.Sp (μm)	Tb.N	N.Ob/B.Pm	N.Oc/B.Pm	Ob.S/BS (%)	Oc.S/BS (%)	OS/BS (%)	O.Th (μm)
0	58.8 ± 2.5	0.3 ± 0.1	21.5 ± 1.8	15.0 ± 1.0	27.6 ± 1.5	19.7 ± 1.0	1.3 ± 0.3	32.9 ± 1.5	9.0 ± 2.6	1.2 ± 0.4	1.8 ± 0.6
125	59.3 ± 4.8	0.2 ± 0.04	20.8 ± 1.5	14.3 ± 1.9	28.5 ± 0.5	15.6 ± 1.9	1.7 ± 0.1	25.3 ± 2.8	11.0 ± 1.9	0.7 ± 0.2	2.5 ± 0.4

[a]All values represent means ± SEM from at least four rats/treatment group. Tx group, treated group; BV/TV, bone volume as a fraction of total tissue volume; OV/BV, osteoid volume as a fraction of bone volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb. N., trabecular number; N.Ob/B.Pm, number of osteoblasts/mm bone perimeter; N.Oc/B.Pm, number of osteoclasts/mm bone perimeter; Ob.S/BS, osteoblast surface as a fraction of bone surface; Oc.S/BS, osteoclast surface as a fraction of bone surface; OS/BS, osteoid surface as a fraction of bone surface; O.Th, osteoid thickness.

skeletal response (data not shown). These data suggest that there is a complete inability to override the resistance despite the amount of exogenous $1,25(OH)_2D_3$ administered.

Resistance to $1,25(OH)_2D_3$ can be caused by a structural defect of the VDR, some of which lead to a decrease in the number of functional receptors or to defective ligand-binding affinity *(49,51,52)*. We examined VDR mRNA levels and nuclear receptor numbers and affinity to determine whether the skeletal resistance in the *op* rat may be caused by a decrease in the VDR. Since it is known that osteoblasts possess receptors for $1,25(OH)_2D_3$, we harvested osteoblasts from the endocranial surface of calvaria for the receptor saturation binding assays. The results showed that both VDR transcript and nuclear receptor levels were increased (two- to threefold) in *op* mutant vs normal bone. Furthermore, ligand-binding affinity was similar. Although upregulation of skeletal VDR in *op* mutants may be caused by abnormal levels of circulating factors known to regulate VDR expression, such as elevated $1,25(OH)_2D_3$ and parathyroid hormone levels, our results suggest that a decrease in VDR numbers or defective ligand-binding affinity are not responsible for the skeletal resistance in this mutation.

It is possible that a defect could reside in the DNA-binding domain of the VDR. We also examined the expression of several vitamin-responsive osteoblast-related gene products (including type I collagen, alkaline phosphatase, and osteocalcin) in $1,25(OH)_2D_3$-treated *op* rats and found that the mRNA levels of these genes were responsive to the increased circulating levels of $1,25(OH)_2D_3$ in *op* rats. These data suggest that the VDR is functional in *op* osteoblasts.

It is generally believed that the stimulatory effect of $1,25(OH)_2D_3$ on osteoclastic bone resorption is mediated by osteoblasts that produce paracrine factor(s) that can directly interact with and upregulate osteoclast function. Studies by Sundquist et al. *(53)* showed that osteoblasts from another osteopetrotic mutation (toothless [*tl*]) were incapable of regulating osteoclast function in a coculture system using $1,25(OH)_2D_3$ as a stimulator for osteoclast formation. These studies concluded that *tl* osteoblasts were defective in their ability to stimulate osteoclast activity, a possibility that might also explain the inability of a high dose of $1,25(OH)_2D_3$ to stimulate bone resorption in the *op* mutation. Coculture studies are currently under way to examine this interaction using osteoblasts and osteoclasts obtained from *op* and normal rats.

Materials and Methods

Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. $1,25(OH)_2D_3$ was a gift from Dr. Milan N. Uskokovic (Hoffman La-Roche, Nutley, NJ). DDK-Plast methylmethacrylate resin was purchased from Delaware Diamond Knives (Wilmington, DE), and the calcium diagnostic kit was purchased from Sigma.

Animals

Animals used in this study were the offspring of mating pairs of heterozygous (+/*op*) breeders. Phenotypically, mutant (*op*/*op*) rats were identified on or about the tenth day after birth by the failure of the incisors to erupt. Normal littermates (+/*op*, +/+), used as controls, were indistinguishable phenotypically, except by breeding. Normal animals were fed ad libitum using standard laboratory chow (Rodent Laboratory Chow 5001; Ralston-Purina, St. Louis, MO) containing 1.0% calcium, 0.6% phosphorus, and 4.5 IU/g of vitamin D_3. Since osteopetrotic mutants have an unerupted dentition, laboratory chow was provided in a pulverized and hydrated form. Animals were bred and maintained in a pathogen-free barrier core facility at Temple University Medical School according to the principles in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (1985) *(54)* and guidelines established by the Institutional Animal Care and Use Committee of Temple University.

$1,25(OH)_2D_3$ Infusion

Four-week-old normal and mutant rats were infused with propylene glycol (vehicle controls), or with different concentrations of $1,25(OH)_2D_3$ ranging between 12.5 and 125 ng/d for 7 d. $1,25(OH)_2D_3$ was delivered in propylene glycol via subcutaneously implanted osmotic minipumps (Model 2001; Alza, Palo Alta, CA) allowing continuous infusion as described previously *(27)*. The doses used in

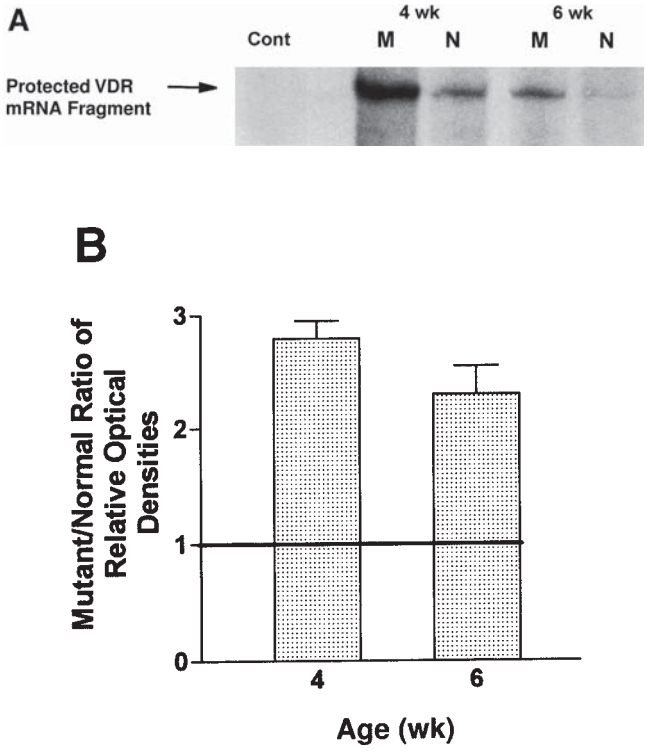

Fig. 6. Ribonuclease protection assay RPA of skeletal VDR mRNA levels in *op* rats and their normal littermate controls. **(A)** Representative autoradiograph from an RPA using total RNA obtained from pooled osteopetrotic (M) or normal (N) calvaria at 4 and 6 wk of age. The protected VDR mRNA fragment (0.5 kb) was not present in controls (Cont). **(B)** Mutant/Normal ratio of skeletal VDR levels. Relative amounts of VDR mRNA were determined by scanning densitometry of protected RNA fragments. Data from pooled mutant (M) and normal (N) sample pairs were used to calculate individual M/N ratios. Data are expressed as the mean M/N ± SEM with a minimum of six individual ratios at each age.

this study ranged from 1 to 10 times the dose known to stimulate bone resorption in normal mice *(32)*. The doses of $1,25(OH)_2D_3$ infused into normal rats were selected to prevent any lethal effect on these animals. However, in *op* rats, the highest dose (125 ng/d) was chosen to elevate the already high baseline levels of serum $1,25(OH)_2D_3$ proportional to the effect of the highest dose (50 ng/d) used in normal rats. Body weights were recorded daily to monitor the effects of treatment on general physical condition.

Tissue Preparation and Histomorphometric Analyses

At necropsy, proximal tibiae from normal and mutant rats treated with 50 ng/d and 125 ng/d, respectively, and those from their control counterparts were removed, fixed in 10% neutral buffered formalin, dehydrated, and embedded undecalcified in DDK-Plast methylmethacrylate resin. Four-micrometer-thick frontal sections were stained with von Kossa/tetrachrome. Proximal tibial growth plate cartilage was measured for GPTh, RZ/PZ thickness, and HCZ thickness at ×30 magnification using the OsteoMeasure histomorphometry system (Osteometrics, Atlanta, GA) as

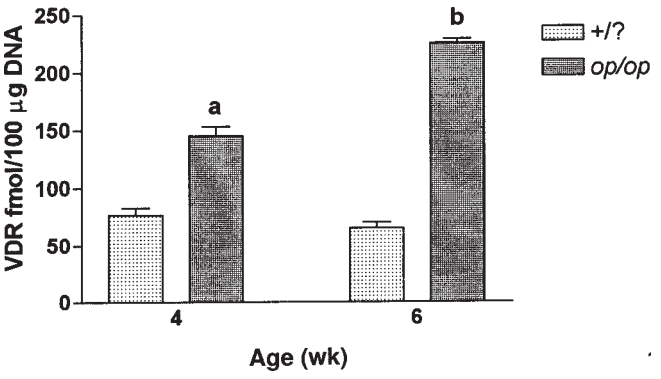

Fig. 7. Chromatin-associated, unoccupied 1,25-dihydroxyvitamin D_3 receptors in osteopetrotic (*op*) and normal bone (calvaria). Data are expressed as the mean ± SEM. Data were analyzed using two factor ANOVA with Bonferroni post-test. [a] ($p < 0.05$) and [b]($p < 0.01$), comparison between mutant and normal littermates.

described previously *(55,56)*. Static bone histomorphometric parameters such as bone volume as a fraction of total volume, osteoid volume as a fraction of bone volume, trabecular thickness, trabecular separation, trabecular number, osteoblast number per millimeter of bone perimeter, osteoclast number per millimeter of bone perimeter, osteoclast surface as a fraction of bone surface, osteoblast surface as a fraction of bone surface, osteoid surface as a fraction of bone surface, and osteoid thickness were measured using the same system. Measurements were made from two different regions of proximal tibial metaphyses according to the definition of Kimmel and Jee *(57)*. Primary spongiosae, trabeculae lined by osteoblasts, and osteoclasts separated by osteoprogenitor cells were examined in a 1.6 × 0.5 mm area just beneath the growth plate. Secondary spongiosae, identified by a preponderance of marrow cells within the intertrabecular spaces, were examined in a 1.6 × 1.5 mm area immediately subjacent to the primary region.

Serum Calcium, Phosphorus, and 1,25(OH)$_2$D$_3$ Measurements

Treated and control normal and mutant animals were anesthetized (25 µg/kg Nembutal) and blood was collected by cardiac puncture. All serum determinations were performed on samples isolated from individual animals. Serum calcium levels were measured using a commercial diagnostic kit (Sigma, MO) according to the manufacturer's protocol. Serum phosphorus levels were determined using a standard colorimetric assay as previously described *(57)*. The intra- and interassay coefficients of variation (CVs) were 3 and 7%, respectively, for calcium and 5 and 8%, respectively, for phosphorus. Serum $1,25(OH)_2D_3$ was extracted and assayed using the calf thymus receptor assay as described by Reinhardt et al. *(59)*. Briefly, a minimum of 0.5 mL of serum was required for each sample assayed and all volumes were brought up to 1 mL with isotonic saline before extraction. The use of $1,25(OH)_2[^3H]D_3$ at a specific

Table 4
Ligand Binding Affinity (K_d) of VDR
in Normal and Mutant Bone Cells

Age (wk)	Normal (+/?)	Mutant (*op*/*op*)
2	0.11 ± 0.007	0.08 ± 0.007
4	0.14 ± 0.012	0.09 ± 0.006
6	0.11 ± 0.009	0.09 ± 0.006

All values represent means ± SEM. K_d in n*M*.

activity of 176 Ci/mmol provided a limit of detection of 2 pg. The intra- and interassay CVs were 8 and 12%, respectively.

RNA Isolation

Calvaria from normal and *op* mutant rats were excised, freed of soft tissue, flash-frozen in liquid nitrogen, and stored at –80°C. Total RNA was isolated using a modification of the guanidine isothiocyanate method as described by Thiede et al. *(60)*. Briefly, calvariae were rapidly pulverized in a Bessman tissue pulverizer (Fisher, Itasca, IL) precooled in a bath of dry ice/ethanol. Bone powder was homogenized in an RNA extraction buffer consisting of 5 *M* guanidine isothiocyanate, 72 m*M* β-mercaptoethanol, and 0.5% Sarkosyl. Homogenates were layered over a cesium chloride cushion and centrifuged at 100,000g_{av} overnight at 20°C. RNA was recovered as a translucent pellet after centrifugation. A minimum of six calvariae were pooled for each phenotype at each age examined, and three separate pooled samples were assayed at each age for both phenotypes.

RPA and Probe Preparation

VDR cDNA (kindly provided by Dr. H. DeLuca, University of Wisconsin) was ligated into a bluescript SK^+ vector. The vector was then transcribed with T_7DNA-dependent RNA polymerase using a T_7 MAXIscript in vitro transcription kit (Ambion, Austin, TX) in the presence of 10 m*M* ATP, CTP, and GTP; 0.25 m*M* cold UTP; and α-^{32}P-UTP (800 Ci/mmol), (New England Nuclear, Chadds Ford, PA). The remaining DNA template was digested with RNase-free DNase I. Ten micrograms of total RNA and purified probe were concentrated, resuspended in hybridization buffer (RPA II kit; Ambion, Austin, TX), and incubated overnight at 45°C. Yeast RNA and rat liver served as internal controls to check for erroneous protection of the probe from digestion by RNases. Samples were separated on 8 *M* urea/5% polyacrylamide gels, and the resulting bands were quantitated using a Fujix BAS 2000 Bio-Imaging system.

Receptor Saturation Assay and DNA Assay

All steps of the receptor assay were performed at 4°C unless otherwise stated. Cells were gently scraped from the endocranial surface of calvariae into a low-ionic strength homogenization buffer according to the procedure of Walters et al. *(61)*. For each sample, cells from a minimum of six age- and phenotype-matched calvaria were pooled in approx 4 mL of TEDMo buffer (10 m*M* Tris base, 1.5 m*M* EDTA, 1.0 m*M* dithiothreitol, 10 m*M* sodium molybdate, pH 7.4) and various protease inhibitors including 500 kallikrein-inhibiting U/mL of Traysol (aprotinin; Miles, Kankakee, IL), 300 μ*M* phenylmethylsulfonylfluoride (Sigma), and 20 μg/mL of soybean trypsin inhibitor (Sigma). After homogenization and centrifugation, pellets were washed three times in a TED/Triton buffer, and the final pellet of nuclear chromatin was resuspended in 4 mL of the homogenization buffer (minus soybean trypsin inhibitor). For the competitive binding assay, 200-μL aliquots of each sample were incubated overnight in six different concentrations (0.03 – 1.0 n*M*) of radiolabeled steroid (1α-25-dihydroxy[23,24 *(n)* ^{3}H]-cholecalciferol; 80–120 Ci/mmol), (Amersham, Arlington Heights, IL) in the presence or absence of 1000-fold excess of unlabeled $1,25(OH)_2D_3$. Following incubation, 150 μL of a 50% hydroxyapatite (HAP) solution were added to each tube, and HAP-receptor-ligand complexes were washed three times with TED/Triton to remove all nonspecifically bound tracer molecules. Receptor-ligand complexes were then dissociated from HAP by the addition of 1 mL of 100% ethanol and subsequent heating at 30°C for 30 min. The radioactivity contained in the supernatant was counted in a liquid scintillation counter. Total and nonspecific binding were determined by the amount of radioactivity contained in the tubes incubated in the absence and presence of the unlabeled steroid. Specific binding was calculated by subtracting nonspecific binding from total binding. These data were then analyzed by Scatchard (linear regression) analysis to yield estimates of receptor number (N_{max}) and affinity (K_d). DNA determinations were performed on each sample using the diphenylamine reaction and used to express N_{max} as femtomoles of receptor per 100 microgram of DNA.

Statistical Analyses

All data were expressed as the mean ± SEM. For comparisons between two group means in which the response was affected by a single variable, an unpaired t-test was performed to evaluate statistical significance. For multiple comparisons in which a response was affected by two different categorical variables, two-factor ANOVA was used followed by a Bonferroni or Fisher post-test to compare selected pairs of group means. Statistical significance was defined as any effect with a probability of <0.05.

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