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Prevalence and Clinico-Epidemiology of Familial Graves' Disease in Japan based on Nationwide Epidemiologic Survey in 2001

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Abstract. A nationwide epidemiologic survey of familial Graves' disease (GD) was conducted in 2001. "Familial GD" was defined as a patient who had at least one Graves' patient within the proband's first-degree relatives. The primary survey was performed for estimating the prevalence of patients among a random selection of 2367 departments/hospitals of internal medicine, endocrinology, thyroidology and pediatrics. Of those receiving the primary questionnaire, 1361 (57.5%) responded, and 902 familial GD patients who visited them in 2000 were reported. The total number of patients was estimated to be 2850 (95% confidence intervals: 2100–3600). Based on the nationwide survey concerning the prevalence of hyperthyroidism in 1999, 2.1–3.1% of hyperthyroidism appeared to be familial GD and the relative risk of familial GD was roughly estimated to be 19–42. Subsequently, a second survey was carried out for obtaining the clinico-epidemiologic features of those patients. Of 902 patients, 487 (54%) were reported. No significant differences between familial and non-familial GD were found in age and sex distributions, clinical features or laboratory findings. Familial GD possessed the highest association with Hashimoto's thyroiditis, approximately 8% within the first-degree relatives, suggesting a shared genetic predisposition. These findings confirm the familial clustering of GD in the Japanese population, indicating the importance of environmental factors, genetic factors or both in the development of the disease.

Key words: Graves' disease, Family clustering, Epidemiology, Japan, Relative risk

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GRAVES' disease (GD) is characterized by hyperthyroidism, goiter and ophthalmopathy, and is thought to be a complex disease caused by several genetic and environmental factors. Although numerous attempts have been made to elucidate the susceptibility genes of GD, the genetic basis still remains unclear, nor have the environmental factors of GD been precisely clarified.

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Compiling epidemiologic data concerning familial clustering of diseases is useful for performing genetic studies. The relative risk of disease in siblings, the life-time risk for siblings divided by the population frequency of the disease, is often used as an estimation of the magnitude of the genetic contribution to disease [1]. If the risk of the disease is large, a genome scan to identify the susceptibility loci affecting the disease is warranted [2]. Nonetheless, there is no nationwide study concerning the family clustering of GD in Japan. In the present study, we aimed to clarify the prevalence of Japanese familial GD and to define the clinico-epidemiologic features by a nationwide survey.

Materials and Methods

A nationwide mail survey for familial GD was conducted in 2001 in Japan. The target patients were those who visited hospitals because of the disease in 2000. According to the Nationwide Epidemiologic Survey Manual issued by the Research Committee on Epidemiology of Intractable Disease, we selected four departments for the survey target: internal medicine, endocrinology, thyroidology, and pediatrics. The hospitals studied were selected randomly from a list of all hospitals in Japan. The selection rate was decided according to the stratification classified by the number of beds in the hospitals in Japan; the more beds a hospital had, the higher the probability it had of being selected. The selection rate was 100% for hospitals with more than or equal to 500 beds and university hospitals, whereas only 5% of hospitals with less than 100 beds were selected at random. Besides the two departments, we designated a department of endocrinology or thyroidology as a special department, and all of the special departments in Japan were selected as the study department. After the selection of the study departments/hospitals, we sent a questionnaire with diagnostic guidelines [3]. The primary survey asked only the numbers of patients with GD who had at least one Graves' patients within their first-degree relatives and had visited the hospital in 2000. If a department/hospital responded that there was such a patient(s), the second mail survey questionnaire asking the detailed clinical features for each patient was sent. The present study was approved by the ethical committees of Okayama University School of Medicine and Juntendo University School of Medicine.

Considering the selection rate and the response rate to the survey, we estimated the total numbers of patients with familial GD as described previously [4, 5]. Ninety-five percent confidence intervals were calculated with an assumption of multinominal hypergeometric distribution. Age and sex distributions of the disease were estimated according to the data on the patients that were obtained by the second survey.

Results

Prevalence

For the primary survey, 2367 departments/hospitals of internal medicine, endocrinology/thyroidology and pediatrics were selected. Of them, 1361 (57.5%) responded and 902 familial GD patients were reported. The numbers of patients from department of pediatrics and other departments was 167 (18.5%) and 735 (71.5%), respectively. The total numbers of patients were estimated to be 2850 (95% confidence intervals: 2100–3600). The estimated number of patients with thyrotoxicosis who visited outpatient clinics in October of 1999 was 154,000, [6]. Since GD is the most common cause of thyrotoxicosis, ranging from 60–90% in different regions of the world including Japan [7–9] and the Japanese population in 1999 was estimated to be 126,686,000 [10], the estimated number and frequency of GD patients was 92,400–138,600 and 0.073–0.11%. Therefore, the frequency of familial GD in GD and the relative risk of familial GD were roughly calculated to be 2.1–3.1% and 19–42, respectively (Table 1).

Clinico-epidemiolgic features of familial GD

In the second survey, 487 (54%) of 902 patients were reported. Sex and age distributions are shown in Table 2. The female/male ratio was 3.2 (360/114). Patients in their twenties to fifties were the most abundant, comprising 74% of the total. As shown in Table 3, patients who had GD in parents, siblings and children were 49% (238/487), 40% (197/487) and 20% (98/487), respectively. Fourteen percent (69/487) had multiple GD members within the first-degree relatives and identical twins were found in 3% (17/487). The ratios of mother/father, sister/brother and daughter/son were 3.0 (180/60), 2.7 (152/57) and 4.6 (83/18), respectively. No significant gender-specificity was found in the disease transmission.

In the family history, HT was the most common, approximately 8% (22/282) of the patients who responded to the question whether they had HT within their

Table 1. Prevalence and relative risk of familial GD

Number of familial GD (a)	2,850
Population of Japan in 1999 (b)	126,686,000
Number of thyrotoxicosis in 1999	154,000
Estimated number of GD* (c)	92,400–138,600
Prevalence of GD (d: c/b×100)	0.073–0.11%
Frequency of familial GD in GD (e: a/c×100)	2.1–3.1%
Relative risk of familial GD (=e/d)	19–42

*: GD is estimated as 60–90% of thyrotoxicosis (Refs. 7–9)

Table 2. Age distribution of familial GD patients by sex

Age (year)	Female		Male		Unknown		Total	
	n	%	n	%	n	%	Total	%
0–9	2	1%	0	0%	0	0%	2	0%
10–19	39	11%	8	7%	2	15%	49	10%
20–29	64	18%	22	19%	0	0%	86	18%
30–39	66	18%	21	18%	3	23%	90	18%
40–49	62	17%	22	19%	2	15%	86	18%
50–59	70	19%	23	20%	3	23%	96	20%
60–69	36	10%	14	12%	3	23%	53	11%
70–79	17	5%	2	2%	0	0%	19	4%
80<	0	0%	0	0%	0	0%	0	0%
Unknown	4	1%	2	2%	0	0%	6	1%
Total	360	100%	114	100%	13	100%	487	100%

n: number

first-degree relatives (Table 4), while hypothyroidism with blocking-type TBII was seen only in 3 patients. Other autoimmune diseases were seen in 15 patients, of which type 1 diabetes was most frequent (6 cases). In complications of other diseases, approximately 10% of familial GD was associated with other autoimmune or allergic disorders (Table 5). Type 1 diabetes and atopic dermatitis were most common and each comprised 26% of the total.

Concerning clinical manifestations, goiter and orbitopathy were present in 89% (413/475) and 34% (164/479), respectively (Table 6). Of patients with orbitopathy, diplopia was found in 10% (17/156). Laboratory findings before treatment demonstrated that most patients exhibited increased serum free T4 and suppressed TSH levels, which are typical of hyperthyroidism (Table 7). The activities of TSH binding inhibitor immunoglobulins (TBII) and antimicrosomal antibody tests (MCHA) measured by the hemagglutination assay were positive in 90% and 78%, respectively. Since the number of comments concerning other clinical manifestations and laboratory findings were small, they were not shown here.

Methylmercaptoimidazole (MMI) was more widely used than propylthiouracil (PTU) (Table 8). Approximately 20% of the patients were introduced into remission by administration of anti-thyroid drugs (ATD). The side effects of ATD, of which allergy was the most frequent, were found in approximately 20% of the patients. Radioisotope therapy and surgical operation were performed in 8% and 14%, respectively.

Discussion

This is the primary national report concerning familial GD in Japan. The prevalence of familial GD was estimated as 2.1–3.1% of hyperthyroidism and the relative risk of familial GD was roughly estimated to be 19–42. In Caucasians, the relative risks of familial GD GD/hyperthyroidism of the proband's first-degree relatives were reported as 16–26 [11–14]. Since the latter included hyperthyroid disorders other than GD, one should be careful when comparing our results with theirs. Nonetheless, the Japanese relative risk estimated here was not so very different from that of the Caucasians. This finding indicates the familial clustering of GD, in particular the importance of environmental and/or genetic factors in the development of the disease. In fact, GD is thought to follow a non-Mendelian pattern of inheritance under the influence of multiple genes and variable penetrance [11, 15].

The age and sex distribution of familial GD did not appear to be significantly different from non-familial GD: patients in their twenties to fifties were the most abundant and a female-to-male preponderance was found in the proband's first-degree relatives. In the literature, there are conflicting findings concerning the distribution of GD among the probands' female relatives. Martin and Fisher [16] found that the proband's sisters were affected more than the mothers, but Evans *et al.* [12] and Stensky *et al.* [13] found the opposite tendency and an almost equal distribution, respectively. In the present study, the ratio of affected mother/sister was 1.2 (180/152), which appeared to be almost

Table 3. Patterns of familial GD

	Pattern	n	% of total (% of subtotal)
Parents only	Mother	155	(78%)
	Father	43	(22%)
	Mother & Father	1	(1%)
	Subtotal	199	41%
Children only	One daughter	46	(78%)
	Two daughters	3	(5%)
	Son	8	(14%)
	One daughter & One son	2	(3%)
	Subtotal	59	12%
Siblings only*	One sister	118	(71%)
	Two sisters	4	(2%)
	Three sisters	2	(1%)
	One brother	31	(19%)
	Two brothers	1	(1%)
	One sister & One brother	8	(5%)
	Two sisters & One brother	1	(1%)
	One sister & Two brothers	2	(1%)
	Subtotal	167	34%
Identical twins	Sisters	13	(76%)
	Brothers	4	(24%)
	Subtotal	17	3%
Other mixed	Mother & One daughter	16	(36%)
	Father & One daughter	8	(18%)
	Mother & One brother	6	(13%)
	Father & One son	5	(11%)
	Mother & Two daughters	1	(2%)
	Father & Two daughters	1	(2%)
	Mother & Father & One daughter	1	(2%)
	Father & One daughter & One brother	1	(2%)
	One sister & One daughter	1	(2%)
	One sister & One son	1	(2%)
	One sister & One daughter & One son	1	(2%)
	One brother & One daughter	1	(2%)
	One brother & One son	1	(2%)
	Two brothers & One sister & One daughter	1	(2%)
	Subtotal	45	9%
Total		487	100%

*: identical twins were excluded.

equal. Regarding the gender-specificity of disease transmission, there are several reports that indicated gender-specific susceptibility in autoimmune thyroid diseases [17, 18]. In this study, however, it is impossible to evaluate it precisely and, at least, evidence suggesting the gender-specific susceptibility was not found. For example, the ratios of mother-children to father-children and parents-daughter to parents-son were 3.0 (180/60) and 4.6 (83/18), respectively, which were similar to the female/male ratio of the whole GD family, 3.2 (360/114).

It is well recognized that both GD and HT are frequently found in members of the same family, implying a shared genetic predisposition [19, 20]. In the

Table 4. Family history of familial GD in first-degree relatives

		Hashimoto's thyroiditis		
		n	% of total** (% of subtotal)	% of answered**
Present				
	Mother	10	(45%)	4%
	Father	2	(9%)	1%
	Sister	9	(41%)	3%
	Brother	0	(0%)	0%
	Daughter	1	(5%)	0%
	Son	0	(0%)	0%
	Subtotal	22	5%	8%
Absent		260	53%	92%
Unknown*		205	42%	
Total		487	100%	

*: includes unanswered

**: since a significant number of patients did not provide information on questions, percentages were calculated by using both the total number (% of total) and the number of patients who provided clear information (% of answered).

Table 5. Complications of autoimmune or allergic diseases in familial GD

		n	% of total** (% of subtotal)	% of answered**
Present				
	Type 1 diabetes	10	(26%)	3%
	Atopic dermatitis	10	(26%)	3%
	Asthma	5	(13%)	1%
	Rheumatoid arthritis	4	(11%)	1%
	Allergic rhinitis	4	(11%)	1%
	Allergic dermatitis	2	(5%)	0.5%
	Ulcerative colitis	2	(5%)	0.5%
	Sjögren's syndrome	2	(5%)	0.5%
	Myasthenia gravis	1	(3%)	0.3%
	Autoimmune hemolytic anemia	1	(3%)	0.3%
	Primary biliary cirrhosis	1	(3%)	0.3%
	Subtotal*	38	8%	10%
Absent		338	69%	90%
Unknown		111	23%	
Total		487	100%	

*: there were four cases who had two complications.

**: same as Table 4

present study, approximately 8% of familial GD patients had HT in their first-degree relatives, supporting the existence of shared genes. Also, GD is often associated with other autoimmune or allergic disorders, and may be part of an autoimmune polyglandular syndrome. In fact, 7–9% of familial GD were associated with such disorders as type 1 diabetes, atopic dermatitis, asthma, rheumatoid arthritis and so on.

Clinical features and laboratory findings were similar to those of non-familial GD. Most patients possessed goiter and approximately one-third had ophthalmopathy. Severe exophthalmos (≥17 mm) and diplopia, which are typical characteristics of malignant ophthalmopathy, were found in 10–25% of patients

Table 6. Clinical manifestations of familial GD

Manifestation			n	% of total* (% of subtotal)	% of answered*
Goiter					
	Present	large	26	(6%)	5%
		moderate	233	(55%)	49%
		small	154	(36%)	32%
		unknown	9	(2%)	2%
		Subtotal	422	87%	89%
	Absent		53	11%	11%
	Unknown		12	2%	
	Total		487	100%	
Orbitopathy					
	Present		164	34%	34%
	Absent		315	65%	66%
	Unknown		8	2%	
	Total		487	100%	
1) Exophthalmos					
	Present	≥17 mm	38	(28%)	25%
		<17 mm	14	(10%)	9%
		Unknown	83	(61%)	54%
		Subtotal	135	82%	88%
	Absent		18	11%	12%
	Unknown		11	7%	
	Total		164	100%	
2) Diplopia					
	Present		17	10%	11%
	Absent		139	85%	89%
	Unknown		8	5%	
	Total		164	100%	

*: same as Table 4

Table 7. Laboratory findings of familial GD before treatment

	free T4			TSH			TBII			MCHA		
	n	% of total	% of answered	n	% of total	% of answered	n	% of total	% of answered	n	% of total	% of answered
Increased	250	51%	95%	4	1%	1%	198	41%	90%	191	39%	78%
Decreased	1	0.2%	0.4%	305	63%	97%	0	0%	0%	N/A	N/A	N/A
Normal	12	2%	5%	7	1%	2%	23	5%	10%	55	11%	22%
Unknown	224	46%		171	35%		266	55%		241	49%	
Total	487	100%		487	100%		487	100%		487	100%	

N/A: not applicable
% of total and % of answered were same as Table 4

Table 8. Treatment of familial GD

			n	% of total (% of subtotal)	% of answered
Anti-thyroid drug (ATD)					
Drug	MMI		351	72%	74%
	PTU		31	6%	7%
	MMI & PTU*		90	18%	19%
	Unknown		15	3%	
	Total		487	100%	
Response	Small dose**		253	52%	62%
	Resistant**		65	13%	16%
	In remission		86	18%	21%
	Relapse		1	0.2%	0.2%
	Unknown		82	17%	
	Total		487	100%	
Side effect	Present	Urticaria	59	(71%)	13%
		Hepatopathy	17	(20%)	4%
		Arthralgia	5	(6%)	1%
		Leukopenia	3	(4%)	1%
		Agranulocytosis	1	(1%)	0.2%
		Hair loss	1	(1%)	0.2%
		Interstitial pneumonia	1	(1%)	0.2%
		Unknown	1	(1%)	0.2%
		Subtotal***	83	17%	19%
	Absent		357	73%	81%
	Unknown		47	10%	
	Total		487	100%	
Radioisotope					
Done			39	8%	
Undone/Unknown			448	92%	
Total			487	100%	
Operation					
Subtotal thyroidectomy			68	14%	92%
Total thyroidectomy			6	1%	8%
Not performed/Unknown			413	85%	
Total			487	100%	

*One of two drugs was used first, but was changed to the other due to side effects.
**: euthyroid was maintained by administration of small doses (Small dose) or large doses (Resistant) of ATD.
***: there were five cases who had two complications.
% of total and % of answered were same as Table 4.

with orbitopathy. Most patients exhibited hyperthyroidism and positive TBII activities. Approximately 75% were positive for MCHA. Concerning treatment and prognosis of familial GD, there appeared to be no distinct difference from those of non-familial GD [21]. ATD, in which MMI was dominantly used, was the treatment of choice, and radioisotope or surgery was generally not regarded as a primary therapy. The frequencies of various side effects of ATD were considered within ordinary limits [22].

In conclusion, we conducted a nationwide epidemiologic survey of familial GD and found an increased relative risk of familial GD within the proband's first-degree relatives. This finding demonstrated the famil-

ial clustering of GD in the Japanese population, with evidence for the involvement of environmental factors, genetic factors or both in the pathogenesis of the disease.

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Role of TAF_{II}-17, a VDR Binding Protein, in the Increased Osteoclast Formation in Paget's Disease

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ABSTRACT: In contrast to normal OCL precursors, pagetic OCL precursors express MVNP and form OCL at physiologic concentrations of $1,25(OH)_2D_3$, as do normal OCL precursors transfected with the *MVNP* gene. Using a GST-VDR chimeric protein, we identified TAF_{II}-17 as VDR binding protein expressed by pagetic OCL precursors and MVNP transduced normal OCL precursors. TAF_{II}-17 was in part responsible for the increased $1,25(OH)_2D_3$ responsivity of pagetic OCL precursors.

Introduction: Pagetic osteoclasts (OCLs) and their precursors express measles virus nucleocapsid protein (MVNP) and form large numbers of OCLs at low concentrations of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Similarly, normal OCL precursors transfected with MVNP also form OCLs at low concentrations of $1,25(OH)_2D_3$. These results suggest that expression of MVNP in OCL precursors enhances vitamin D receptor (VDR)-mediated gene transcription.

Materials and Methods: To determine the mechanism for the increased OCL formation capacity of pagetic OCL precursors in response to $1,25(OH)_2D_3$, lysates from pagetic and MVNP-transduced normal OCL precursors were incubated with a GST-VDR chimeric protein.

Results: A 17-kDa peptide that bound VDR was detected in MVNP-transduced cells and pagetic OCL precursors treated with $1,25(OH)_2D_3$. This peptide was identified as TAF_{II}-17, a component of the TFIID transcription complex. Expression of increased levels of TAF_{II}-17 in cells allowed TAF_{II}-17 to bind to VDR at low concentrations of $1,25(OH)_2D_3$. An antisense oligonucelotide (AS-ODN) to TAF_{II}-17 significantly decreased OCL formation in response to $1,25(OH)_2D_3$ in pagetic but not normal marrow cultures by ~40%. Transfection of TAF_{II}-17 or MVNP into NIH3T3 cells increased VDR transcriptional activity as measured by DR-3 reporter assays.

Conclusion: These data show that expression of the *MVNP* gene in OCL precursors results in increased levels of TAF_{II}-17. TAF_{II}-17 can bind VDR at low concentrations of $1,25(OH)_2D_3$. These results suggest that MVNP expression in Paget's OCL precursors increases expression of a component(s) of the VDR transcription complex that can increase OCL formation.

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Key words: **TAF_{II}-17, Paget's disease, 1,25-dihydroxyvitamin D_3, osteoclast formation, bone marrow cultures**

INTRODUCTION

PAGET'S DISEASE OF bone is the most exaggerated example of disordered bone remodeling with the primary cellular abnormality residing in the osteoclast (OCL). OCLs are increased in number and size and contain many more nuclei per cell compared with normal OCLs.[1] Immunocytochemical studies have shown that pagetic OCLs contain paramyxoviral-like nuclear inclusions that cross-react with antibodies to measles virus (MV), respiratory syncytial virus, and canine distemper virus nucleocapsid antigen.[2–4] In situ hybridization studies have detected expression of measles virus nucleocapsid protein (MVNP) transcripts in OCLs from patients with Paget's disease.[5] In addition, OCL precursors and circulating peripheral blood cells from patients with Paget's disease express MVNP transcripts.[6] However, the physiologic role that MV infection plays in the abnormal OCL activity in Paget's disease is unknown.

We previously reported that OCL precursors from Paget's patients formed large numbers of OCLs at low concentra-

The authors have no conflict of interest.

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tions of $1,25(OH)_2D_3$, forming OCLs in vitro at $1,25(OH)_2D_3$ concentrations that are one to two logs lower than that required for normal OCL formation.[7,8] Furthermore, we have shown that normal OCL precursors transduced with the *MVNP* gene form OCLs that were very similar to OCLs from patients with Paget's disease and formed OCLs at low concentrations of $1,25(OH)_2D_3$.[9] This increased OCL formation in response to $1,25(OH)_2D_3$ was not caused by increased numbers of vitamin D receptors (VDRs).[10] These data suggested that the enhanced capacity of MVNP transduced OCL precursors and OCL precursors from Paget's disease patients to form OCL at low $1,25(OH)_2D_3$ may result from enhanced VDR mediated transcription caused by either increased levels of one or more components of the VDR transcription complex[10] or decreased expression of a corepressor of VDR.

To test this hypothesis, we used a GST-VDR chimeric protein with lysates from Paget's bone marrow cells and MVNP-transduced normal OCL precursors to isolate and characterize VDR binding proteins for their effects on OCL formation.

MATERIALS AND METHODS

Chemicals

$1,25(OH)_2D_3$ was synthesized in Dr Ishizuka's laboratory as described previously.[11] FBS was purchased from GIBCO-BRL (Grand Island, NY, USA). All other chemicals and media were purchased from Sigma Chemical (St Louis, MO, USA), unless otherwise noted.

Subjects and cell preparation

Bone marrow cells were aspirated under 2% xylocaine anesthesia from the iliac crest of healthy normal donors or patients with Paget's disease into heparinized α-MEM containing 5% FBS, as previously described.[8] Bone marrow mononuclear cells were isolated by separation on hypaque-ficoll gradients (density, 1.077 g/ml), centrifuged at 400*g* for 30 minutes, and washed three times with α-MEM, as previously described.[8] The Institutional Review Board of the University of Pittsburgh approved these studies.

Transduction of human bone marrow cells

Human bone marrow mononuclear cells were cultivated for 2 days in α-MEM–10% FBS that contained 10 ng/ml each of interleukin-3 (IL-3), interleukin-6 (IL-6), and stem cell factor (SCF; Immunex Research and Development, Seattle, WA, USA). The bone marrow cells were then cultured for an additional 48 h at 37°C in a humidified atmosphere of 5% CO_2-air at a density of 1–2 $\times$ 10^5/ml with supernatant (10% vol/vol) containing the vector.[9] Cultures were supplemented with 4 μg/ml of polybrene, 20 ng/ml of IL-3, 50 ng/ml of IL-6, and 100 ng/ml of SCF. In preliminary experiments, we determined that this was the cytokine combination that supported the highest transduction efficiency. After 24 h, the cells were centrifuged, the spent supernatant was removed, freshly prepared viral supernatants supplemented with 4 μg/ml of polybrene and growth factors were added, and the cultures were continued for 24 h. After 48 h, cells were harvested for short-term clonogenic assays in methylcellulose as described below, and an aliquot of the cells was tested for MVNP expression by immunostaining the cells with an anti-MVNP monoclonal antibody (generously provided by Dr Don Forthal, University of California at Irvine, Irvine, CA, USA) as previously described.[2,3] As a control, some of the cells were maintained under the same culture conditions except the viral supernatant was omitted or viral supernatants containing the empty vector were added.

Granulocyte-macrophage colony-forming unit formation

Transduced marrow mononuclear cells were cultured at 5×10^5 cells/well in α-MEM containing 1.2% methylcellulose, 30% FBS, 1% deionized bovine serum albumin (BSA; Sigma), and 100 pg/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex Research and Development) with 250 μg/ml G418. Transduced cells were plated in a volume of 1.0 ml in 35-mm culture dishes (Corning; New York, NY, USA) as reported previously.[9] The dishes were incubated at 37°C in humidified atmosphere of 5% CO_2-air for 7 days. Colonies were scored after 7 days of culture using an inverted microscope, and G418-resistant colonies were individually picked, using finely drawn pipettes, and used for OCL formation assays.

Long-term cultures for osteoclast formation

G418-resistant granulocyte-macrophage colony-forming unit (CFU-GM)-derived cells (1×10^5) or human marrow mononuclear cells (1×10^5), obtained as described above, were cultured in 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in α-MEM containing 20% horse serum and various concentrations of $1,25(OH)_2D_3$ or IL-6. The cultures were fed every 3 days by replacing one-half the media, and after 14 (for CFU-GM) or 21 days (for marrow mononuclear cells) of culture, the cells were fixed with 1% formaldehyde and tested for cross-reactivity with the monoclonal antibody 23c6, which recognizes the OCL vitronectin receptor (generously provided by Michael Horton, Rayne Institute, Bone and Mineral Center, London, UK), using a Vectastatin-ABC-AP kit (Vector Laboratories, Burlingame, CA, USA). The $23c6^+$ multinucleated cells ($\geq$3 nuclei/cell) were scored using an inverted microscope. The bone resorption capacity of the multinucleated cells was assessed by measuring the number of resorption lacunae formed on dentin slices as previously described.[9]

Western blot analysis

To evaluate VDR protein levels, MVNP- or empty vector (EV)-transduced CFU-GMs were isolated as described above. After 4 days of culture with $1,25(OH)_2D_3$, cell lysates were prepared as described.[10] The protein concentration of the lysates was determined by the Bradford method, and the same amount of protein from each lysate was loaded onto 15% SDS-PAGE. Rat anti-human vitamin D receptor monoclonal antibody (Affinity BioReagents, Golden, CO, USA) was used to detect VDR. VDR content was measured by enhanced chemiluminescence (ECL) fol-

lowing the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

Binding affinity of 1,25(OH)$_2$D$_3$ to the VDR

Pooled MVNP- or EV-transduced CFU-GM (10^8 cells) and EV- or MVNP-transduced NIH3T3 cells (27 10-cm culture dishes each) were washed with PBS three times, and the cells were homogenized in phosphate buffer A (25 mM KH_2PO_4, 0.1 M KCl, 1 mM dithiothreitol) by means of 20 passes of a Potter-Elvehjem Teflon glass homogenizer kept in ice. The homogenate was centrifuged at 105,000*g* for 1 h in a Hitachi RP72 ultracentrifuge. This supernatant was used as the VDR-containing fraction. The saturation analysis of 1,25(OH)$_2$D$_3$ binding to VDR was performed as follows. Varying amounts of [^{3}H]1,25(OH)$_2$D$_3$ (specific activity 168 Ci/mmol, 3.26–182.82 fmol), with or without a 500-fold excess of unlabeled 1,25(OH)$_2$D$_3$, were dissolved in 50 μl of absolute ethanol in 12 × 75-mm polypropylene tubes (Walter Sarstedt, Numbrecht, Germany). One milliliter of the VDR-containing fraction was diluted to 1.8 mg protein/ml in phosphate buffer A, and 1 mg gelatin was added to each tube kept in an ice bath. The assay tubes were incubated in a shaking water bath for 1 h at 25°C and chilled in an ice bath. One milliliter of 40% (wt/vol) polyethylene glycol 6000 in distilled water was added to each tube, and the tubes were mixed vigorously and centrifuged at 2260*g* for 60 minutes at 4°C. After the supernatant was decanted, the bottom of the tube containing the pellet was cut off into a scintillation vial containing 10 ml of dioxane-based scintillation fluid containing 10% naphthalene and 0.5% Omnifluor (DuPont, Boston, MA, USA) in 1,4-dioxane. The radioactivity was measured in a Beckman liquid scintillation counter (Model LS6500) using an external standard. Total binding was calculated as the amount of bound [^{3}H]1,25(OH)$_2$D$_3$ in the absence of unlabeled 1,25(OH)$_2$D$_3$, and nonspecific binding was calculated as the amount of bound [^{3}H] 1,25(OH)$_2$D$_3$ in the presence of 500-fold excess of unlabeled 1,25(OH)$_2$D$_3$. Specific binding was calculated by subtracting the nonspecific binding from the total binding. The dissociation constant (K_d) was calculated by Scatchard analysis of specific binding of 1,25(OH)$_2$D$_3$.

Gene reporter assays

The promoter region of the *human 24-hydroxylase* gene (−186 to −5), which contains two vitamin D responsive elements (VDRE; a gift from Dr E Eguchi, Teijin Institute for Bio-Medical Research, Tokyo, Japan), was cloned into a luciferase reporter vector pGL3-Basic Vector (Promega, Madison, WI, USA).[12] This plasmid construct was co-transfected with the β-galactosidase expression plasmid into CFU-GM–derived cells or NIH3T3cells transduced with MVNP or EV using the DMRIE-C Reagent (Invitrogen Life Techno). Sixteen hours after transfection, vehicle (0.1% ethanol) or 1,25(OH)$_2$D$_3$ (10^{-10}–10^{-8} M) was added. Twenty-four hours later, the cells were harvested and lysed in the cell lysate solution provided with the luciferase assay kit (Promega). The luciferase activities of the cell lysates were measured with the luciferase assay kit according to the manufacturer's instructions and were standardized by comparing the galactosidase activities of the same cell lysates as determined with a β-galactosidase enzyme assay system (Promega). The DR-5 reporter gene was a generous gift from Dr MJ Tsai (Baylor College of Medicine, Houston, TX, USA). The DR-5 reporter assay was performed using the same protocol for the DR-3 gene reporter assay, except that all trans-retinoic acid (Sigma) was substituted for 1,25(OH)$_2$D$_3$.

GST-VDR affinity binding assays and protein sequencing

The GST-VDR chimeric protein was a gift from Dr S Kato (University of Tokyo, Tokyo, Japan).[13] Twenty micrograms of GST-VDR protein was incubated for 2 h at 4°C with 2 mg of lysates from cells treated with 10^{-10}–10^{-8} M 1,25(OH)$_2$D$_3$. Ten milligrams of glutathione-conjugated Sepharose 4B (Amersham) was added, the suspension was incubated for 1 h at 4°C, and the beads were collected. Protein was eluted in SDS-PAGE buffer, and the samples were resolved by SDS-PAGE (15% polyacrylamide gel; Bio-Rad, Hercules, CA, USA).

For protein sequence analysis, samples prepared as described above were transferred onto PVDF membranes (Bio-Rad). The membranes were stained with Ponceas S, and the 17-kDa band was cut out from the membrane. After being reduced and S-pyridilethylated, it was digested by Achromobacter protease I for 48 h. After sonication, the supernatant was loaded onto a reversed-phase μ-Bondasphere C8 HPLC column (Waters, Milford, MA, USA). Fractionated samples were sequenced using the 492 precise protein sequencing system (PE Applied Biosystems, Foster City, CA, USA). The amino acid sequences were subjected to analysis using Swiss protein.[14]

For Western blot analysis, the peptides that bound GST-VDR were subjected to SDS-PAGE using 15% polyacrylamide gels, and the was blot transferred onto a PVDF membrane (Millipore Corp.) After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated for 1 h with anti-rabbit-TAF$_{II}$-17 antibody[15] (generously provided by Dr RG Roeder, the Rockefeller University) at 1:3000 dilution in TBST containing 1% BSA. The blot was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO, Carpinteria, CA, USA), and the bands were visualized with an ECL system (Amersham Life Science, Arlington Heights, IL, USA).

To determine if protein levels of TAF$_{II}$-17 were increased in MVNP-transfected cells, the day 4 cell lysates described above from 1,25(OH)$_2$D$_3$ (10^{-8} M)-stimulated MVNP- or EV-transduced CFU-GMs were mixed with native sample buffer (Bio-Rad) and were loaded using 15% polyacrylamide gels (Bio-Rad) with SDS free Tris/Glysin Buffer (Bio-Rad). The blots were transferred onto PVDF membranes (Millipore Corp.). Western immunoblotting was performed using anti TAF$_{II}$-17 antibody or anti-VDR antibody as described above. The 72-kDa band that was identified with the anti-TAF$_{II}$-17 antibody was cut out and eluted from the membrane. The band was electrophoresed under denaturing conditions, and the gel was blotted with anti-VDR.

TABLE 1. SEQUENCES OF TAF_{II}-17 OLIGODEOXYNUCLEOTIDES

Oligodeoxynucleotide	*Sequence (5'–3')*
Antisense S-ODN (16-mer)	GGCCAAACTGGTTCAT
Sense S-ODN (16-mer)	ATCAAGCAGAATGGGC
MS1 S-ODN (16-mer)	GGCGAAAGTGGATCTT
MS2 S-ODN (16-mer)	GCCCATACAGGTTGAT

Underlined bases represent the four-base mismatches that differ from the antisense S-ODN.

Modified mammalian two-hybrid assays

The TAF_{II}-17 cDNA was digested by *Sma*I and *Sal*I and inserted into the pM vector (CLON-TECH; pM-TAF_{II}-17). *Eco*RI released the full-length cDNA for human VDR from the pSG5 vector and fused in-frame into the pVP16 vector, which contains the activation domain of a herpesvirus (CLON-TECH; pVP16-hVDR).[16] To examine the interaction of TAF_{II}-17 and VDR, 0.5 μg of pM-TAF_{II}-17, 0.5 μg of pVP-16-hVDR and 0.5 μg of pGVP2-GAL4BS together with 0.25 μg of plasmid containing β-galactosidase cDNA were transfected into NIH3T3 cells using Lipofectamine (GIBCO BRL). Twenty-four hours later, vehicle or 10^{-8} M $1,25(OH)_2D_3$ was added. After 24 h of incubation, the luciferase activity of the cell lysates was examined and standardized using β-gal activity.

Antisense oligodeoxynucleotide synthesis

The TAF_{II}-17 antisense phosphothioate oligodeoxynucleotide (AS-ODN) was composed of 16 bases that targeted the region that spans the translation start codon of human TAF_{II}-17 mRNA (Table 1). The sense and two four-base mismatches (MS1, MS2) of the AS-ODN were also designed as negative controls (Table 1). All these phosphothioate oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA, USA) on an automated solid-phase nucleotide synthesizer and subsequently filter-sterilized. The GC content was the same for all of these oligonucleotides

Antisense oligodeoxynucleotide uptake by osteoclast precursors

OCL precursors were incubated (1×10^5 cells) in 96-well tissue culture plates or 5×10^5 cells in 24-well tissue culture plates in the presence or absence of the antisense or either MS-1 or MS-2 in α-MEM containing 20% horse serum and Penetratin-1 (Q. Biogene) as the carrier. The effect of the AS-ODN and Penetratin-1 on MNC formation was investigated to determine the optimal transfection conditions for OCL precursors. Based on these preliminary experiments, AS-ODN in Penetratin-1 carrier was used at a concentration of 3 μM to introduce the AS-ODN, MS-1, and MS-2 into OCL precursors. The OCL precursors were cultured for 4 (for GST-VDR pull-down assays) or 14 days (for OCL formation). The media were replaced every 3 days with fresh media containing oligodeoxynucleotides.

Bone resorption assays

The CFU-GM–derived cells transduced with MVNP (10^5 cells/ well) were cultured with $1,25(OH)_2D_3$ (10^{-8} M) and/or AS-TAF_{II}-17 ODN or the MS-1 MS-ODN on mammoth dentin slices (Wako). After 2 weeks of culture, the cells were removed, the dentin slices were stained with acid hematoxylin (Sigma), and the number of resorption lacunae were counted microscopically as previously described.[9]

Generation of modified Hill equations

To determine if cells transduced with MVNP showed increased responsivity to $1,25(OH)_2D_3$ or retinoic acid compared with EV-transduced cells, a modified Hill equation was used. The relationship between $1,25(OH)_2D_3$ or retinoic acid concentrations and reporter activity or osteoclast formation by CFU-GM-EV and CFU-GM-MVNP was described by modified Hill equation:

$$E = \frac{E_{max} \times C^H}{C^H_{50\%} \times C^H}$$

In this equation, the effect (E) is the change in DR-3 or DR-5 reporter activity or OCL formation, E_{max} is the maximum effect possible, which was fixed at 100%; C is the concentration of $1,25(OH)_2D_3$ or retinoic acid; $C_{50\%}$ is the concentration of $1,25(OH)_2D_3$ or retinoic acid associated with 50% E_{max}; and H is the Hill constant, an indication of the sigmoidicity of the curve.

Statistical analysis

The results of culture assays are reported as the mean ± SEM for typical experiments. Significance was evaluated by a two-sided nonpaired Student's t-test, with differences of $p < 0.05$ considered significant. A similar pattern of results was seen in experiments using bone marrow cells from 10 separate normal marrow donors transduced with EV or MVNP and 5 Paget's patients.

RESULTS

Expression of the VDR

To determine the mechanism responsible for the increased OCL formation by OCL precursors from pagetic patients to low levels of $1,25(OH)_2D_3$, we tested if MVNP- or EV-transduced normal OCL precursors (CFU-GM) expressed increased amounts of VDR protein or bound increased amounts of $1,25(OH)_2D_3$/mg protein. We previously had reported using competitive PCR that VDR mRNA levels did not differ in Paget's or normal marrow.[10] The amounts of VDR (Fig. 1) and $1,25(OH)_2D_3$ specifically binding to VDR was similar in EV- or MVNP-transduced CFU-GM (3.25 versus 2.97 fmols/mg protein). The amounts of the 80-kDa VDR band, whose occurrence has been reported by others,[17] also did not differ between EV and MVNP transduced CFU-GM.

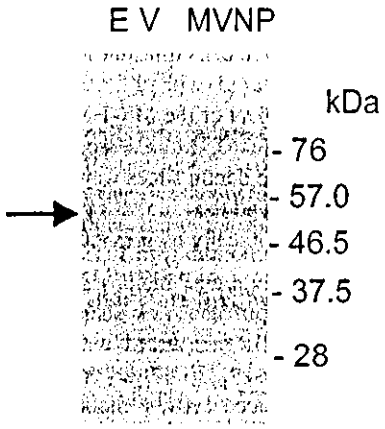


FIG. 1. Expression of the VDR in MVNP- and EV-transduced OCL precursors. The cell lysates (20 μg) were obtained from MVNP- or EV-transduced CFU-GMs cultured for 4 days in α-MEM–10% FBS and separated on 15% SDS-PAGE gels. VDR protein (50 kDa) was detected by immunoblot analysis using the rat monoclonal antibody 9A7.

DR-3, DR-5 reporter activity in MVNP-transduced normal CFU-GM–derived cells

To determine whether VDR-mediated gene transcription was increased in MVNP-transduced cells and whether it was relatively specific to VDR, a luciferase reporter vector containing a DR-3 (VDR) or DR-5 (retinoic acid receptor [RAR]) response element was inserted into EV- or MVNP-transduced early osteoclast precursors, CFU-GMs. VDR-mediated gene transcription was significantly increased in CFU-GM cells transduced with the MVNP cDNA compared with EV-transduced cells. This increase was detectable at concentrations of $1,25(OH)_2D_3$ that were one log less than that required increasing DR-3 reporter activity in EV-transduced CFU-GM cells (Fig. 2A). Using a modified Hill equation to compare the dose response curves, the concentration of $1,25(OH)_2D_3$ that induced a 50% of maximal response ($C_{50\%}$) for the $1,25(OH)_2D_3$ dose response curve ($0–10^{-8}$ M) for MVNP-transduced cells differed significantly from EV-transduced cells (0.47×10^{-9} versus 2.0×10^{-9} M, $p < 0.05$). In contrast, transduction of the DR-5 reporter construct into CFU-GM cells transduced with the MVNP or EV construct showed that, although basal transcription was increased in CFU-GM cells transduced with the MVNP gene, the $C_{50\%}$ for the retinoic acid dose–response curve ($0–10^{-8}$ M retinoic acid) for MVNP-transduced cells did not differ significantly from that of the EV-transduced cells ($0.7 \pm 1.25 \times 10^{-9}$ versus $1.5 \pm 0.72 \times 10^{-9}$ M, respectively; Fig. 2B). As shown in Figs. 2C and 2D, NIH3T3 cells transfected with MVNP also showed enhanced VDR-mediated transcriptional activity in response to $1,25(OH)_2D_3$ in analogous fashion to OCL precursors expressing MVNP. The transfection efficiency for the reporters in NIH3T3 cells, (85% versus 15%), which are adherent, was much greater compared with CFU-GMs, which are nonadherent hematopoietic cells, as determined by co-transfection of the *β-galactosidase* gene. NIH3T3 cells normally express VDR.

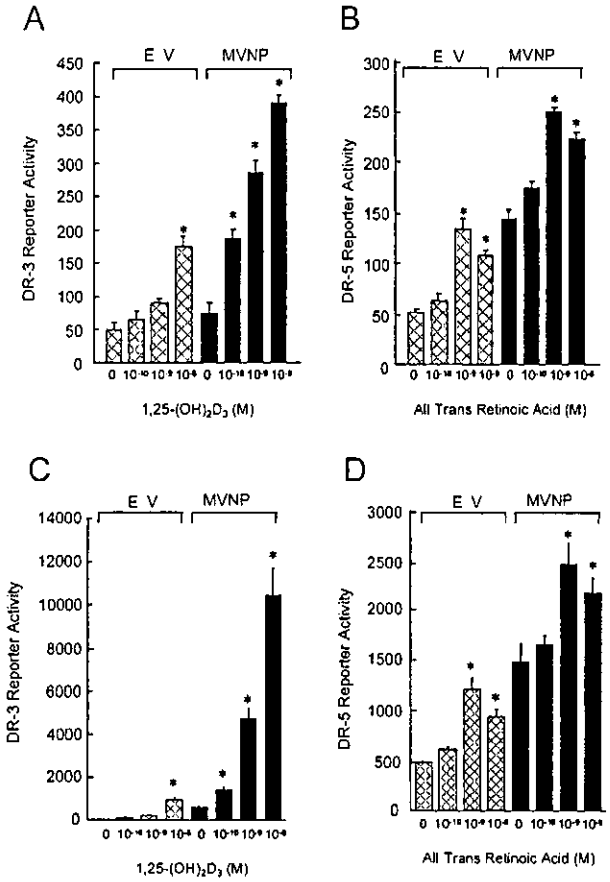


FIG. 2. Effects of transducing MVNP or EV into CFU-GM or NIH3T3 cells expressing a VDR (DR-3) or RAR (DR-5) reporter construct. The reporter plasmid containing the DR-3 or DR-5 promoter and β-gal expression vector were introduced into CFU-GM (CFU-GM-EV and CFU-GM-MVNP) cells or NIH3T3 (NIH3T3-EV and NIH3T3-MVNP) cells. Different concentrations of (A) $1,25(OH)_2D_3$ or (B) all trans-retinoic acid were added 24 h after the transfection of CFU-GM. The same concentrations of (C) $1,25(OH)_2D_3$ or (D) all trans-retinoic acid were added to NIH3T3 cells. Results are expressed as the mean ± SE. $^*p < 0.01$ compared with cells treated with media alone. Similar results were seen in three independent experiments.

Binding affinity of VDR in EV- and MVNP-transduced NIH3T3 cells

A potential mechanism that could account for increased VDR-mediated transcription would be increased affinity of VDR for its ligand. To test this possibility, NIH3T3 cells were used as a surrogate for OCL precursors and were transfected with MVNP or EV, and the VDR binding affinity was determined. It would have been preferable to perform these experiments with MVNP- or EV-transduced OCL precursors, but it is impossible to obtain sufficient numbers of cells required for this assay from human bone marrow cells. As noted above, NIH3T3 cells transfected with MVNP display enhanced VDR-mediated transcription in analogous fashion to OCL precursors expressing MVNP (Figs. 2C and 2D). Scatchard analysis showed that the affinity constants (K_d) and B_{max} for $1,25(OH)_2D_3$ binding VDR in EV- or MVNP-transduced NIH-3T3 cells were

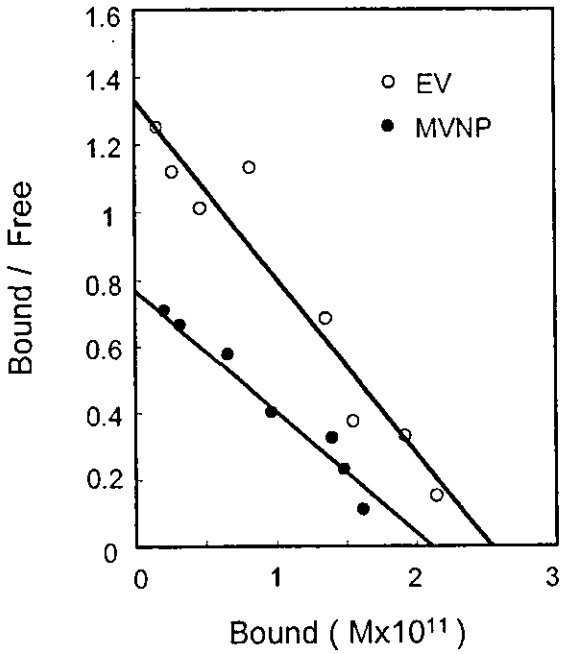


FIG. 3. Scatchard analysis of $1,25(OH)_2D_3$ receptor binding in EV- or MVNP-transduced NIH3T3 cells. Scatchard analysis was performed as described in the Materials and Methods section. Only one class of $1,25(OH)_2D_3$ receptor was detected. Similar results were seen in two independent experiments.

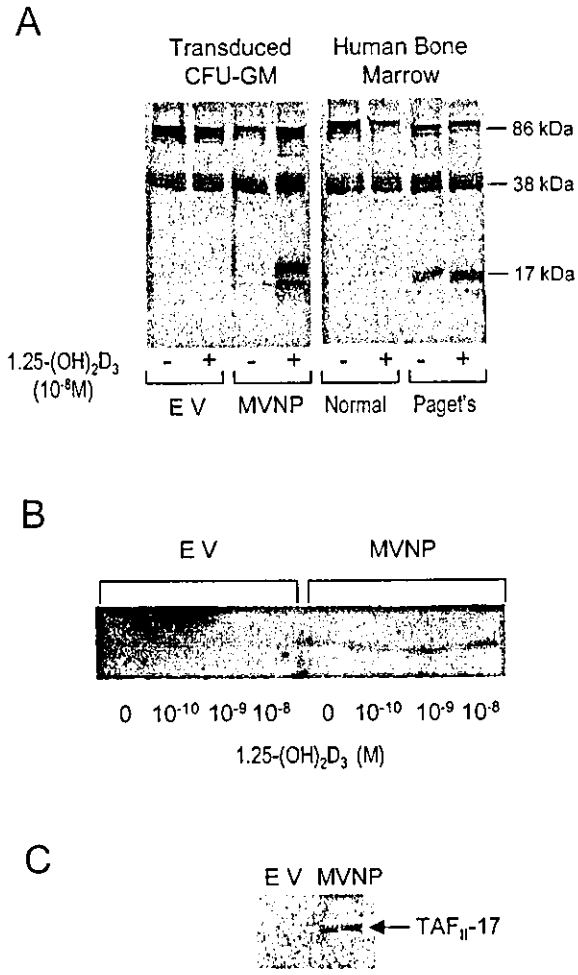


FIG. 4. Binding of a GST-VDR chimeric protein to a 17-kDa protein expressed at high levels in Paget's marrow cells and MVNP-transduced cells. (A) GST-VDR (20 μg) was incubated for 2 h at 4°C with 2–4 mg of cell lysates from cells treated with 10^{-8} M $1,25(OH)_2D_3$ for 48 h. Two micrograms of immobilized Sepharose 4B was incubated with labeled proteins for 1 h at 4°C. Samples were resolved by SDS-PAGE and detected by Coomassie blue staining. Similar results were seen in three independent experiments. (B) Western blot analysis showing that the 17-kDa band is TAF_{II}-17. GST-VDR (20 μg) was incubated for 2 h at 4°C with 24 mg cell lysates from cells treated with 10^{-10}–10^{-8} M $1,25(OH)_2D_3$ for 4 days. Two micrograms of immobilized sepharose 4B was incubated with labeled proteins for 1 h at 4°C. Similar results were seen in four independent experiments. (C) Western blot analysis of total cell lysates from MVNP- or EV-transfected CFU-GMs. TAF_{II}-17 protein levels were detected as described above. Similar results were seen in two independent experiments.

2.70×10^{-11} M and 14.0 fmol/mg protein versus 1.90×10^{-11} M and 11.7 fmol/mg protein, respectively, and were not significantly different (Fig. 3). Only one class of VDR was detected.

Binding of the GST-VDR chimeric protein to a 17-kDa peptide in Paget's marrow cells and MVNP-transduced normal CFU-GM-derived cells: We used a GST-VDR chimeric protein with lysates from MVNP-transduced normal OCL precursors (CFU-GM–derived cells) and marrow cells from involved bones from patients of Paget's disease to try to isolate potential VDR binding proteins. As shown in Fig. 4A, a 17-kDa peptide was detected that bound VDR and was expressed at high levels in MVNP-transduced cells treated with $1,25(OH)_2D_3$, but was expressed in EV-transduced cells. Bone marrow cells derived from patients with Paget's disease also expressed high levels of this peptide in the presence or absence of added $1,25(OH)_2D_3$ (Fig. 4A). Furthermore, NIH3T3 cells that were transduced with the MVNP construct also expressed a similar 17-kDa VDR binding protein when treated with $1,25(OH)_2D_3$ (Fig. 4A). This 17-kDa band was not present in EV-transduced NIH3T3 cells (Fig. 4A). However, a faint 15-kDa band was present in EV-transduced cells that also co-migrated with the 17-kDa band in MVNP-transduced cells. At present, we have not been able to determine the identity of this band.

Microsequence analysis of the 17-kDa band in two independent experiments identified the protein (N-terminal; PEPASXPPQG; TAF_{II}-17; 20–29; fragment 21): (K)ACTTXAH; TAF_{II}-20 (140; 141–147). The 17-kDa band in patients with Paget's disease and MVNP-transduced CFU-GM samples was identical to TAF_{II}-17, which is a component of the TFIID transcription complex.[15,18]

To determine if the increased levels of TAF_{II}-17 were present in the cells rather than TAF_{II}-17 having increased interaction with VDR, we measured the relative levels of TAF_{II}-17 in EV- and MVNP-transfected CFU-GMs by Western blot analysis as shown in Fig. 4C. MVNP-transduced cells expressed higher levels of TAF_{II}-17. To determine if the increased levels of TAF_{II}-17 could form complexes with VDR at low concentrations of

$1,25(OH)_2D_3$, CFU-GM that had been transfected with the MVNP cDNA or EV were treated with 10^{-10}–10^{-8} M $1,25(OH)_2D_3$ and the lysates used for GST-VDR pull-down experiments. The identity of the 17-kDa band in these experiments as TAF_{II}-17 was confirmed by Western blot analysis (Fig. 4B). TAF_{II}-17 could complex to VDR in MVNP-transduced cells at very low concentrations of $1,25(OH)_2D_3$ (10^{-10} M) and could complex to VDR even when the cells were only exposed to the very low concentrations of $1,25(OH)_2D_3$ present in the sera used in the media.

Interaction of $1,25(OH)_2D_3$ and VDR with TAF_{II}-17

To further examine protein–protein interactions between VDR and TAF_{II}-17, the mammalian two-hybrid system was used. An expression vector in which human TAF_{II}-17 was fused to GAL4DBD (pM-TAF_{II}-17) was used as the bait construct and the human VDR (pVP16-hVDR) was used as the prey vector (Fig. 5A). VDR mediated gene transcription was induced when 10^{-8} M $1,25(OH)_2D_3$ was added with the VDR and TAF_{II}-17 constructs (Fig. 5B), confirming that VDR interacted with TAF_{II}-17 or a complex containing TAF_{II}-17.

To determine if TAF_{II}-17 could directly bind VDR, cell lysates from MVNP transduced CFU-GM were fractionated on native gels and blotted with either anti-VDR or anti-TAF_{II}-17. As shown in Fig. 5C, a 72-kDa complex that contained TAF_{II}-17 (MW = 17 kDa) and VDR (MW = 55 kDa) was detected. To confirm that VDR was present in the 72-kDa complex, the band was cut out, eluted from the membrane, run under denaturing conditions, and blotted with anti-VDR. Figure 5D show that VDR was part of the 72-kDa complex.

Effects of anti-sense TAF_{II}-17-ODN on OCL formation: To determine the potential physiologic relevance of increased TAF_{II}-17 on OCL formation, the effects of the TAF_{II}-17 AS-ODN on OCL formation by Paget's OCL precursors and MVNP-transduced CFU-GM were investigated. Treatment of Paget's OCL precursors with TAF_{II}-17-antisense ODN markedly decreased the protein expression levels of TAF_{II}-17 compared with MS-ODN–treated osteoclast precursors as assessed by GST-VDR pull-down assays. As shown in Fig. 6, treatment with either MS-ODN did not alter the protein levels of TAF_{II}-17 (100 versus 69 densitometry units). When Paget's patient derived or MVNP-transduced CFU-GMs were cultured for 14 days in the presence or absence of 3 μM TAF_{II}-17 AS-ODN and treated with 10^{-11}–10^{-7} M $1,25(OH)_2D_3$, OCL formation was inhibited (37 ± 6% Paget's patients and 64 ± 4% MVNP-transduced cells; Fig. 7A). In contrast, 3 μM of TAF_{II}-17 AS-ODN did not block OCL formation in cultures of EV transduced CFU-GM (Fig. 7B). Importantly, TAF_{II}-17 AS-ODN did not affect OCL formation induced by IL-6 in Paget's patients and normal donors. (Fig. 7C). Treatment with either MS-ODN did not significantly decrease OCL formation.

To confirm that antisense TAF_{II}-17-ODN affected OCL formation, bone resorption assays were performed with MVNP transduced CFU-GM treated with TAF_{II}-17-AS-ODN. Treatment with 3 and 10 μM of TAF_{II}-17-AS-ODN markedly decreased pit formation compared with MS-ODN– and $1,25(OH)_2D_3$-treated cultures (Fig. 7D). The number of pits formed on dentin slices in cultures containing $1,25(OH)_2D_3$ or $1,25(OH)_2D_3$ and 3 μM of MS-ODN were 132 ± 12 versus 129 ± 20, respectively. Rare resorption pits were detected in cultures treated with 3 and 10 μM of TAF_{II}-17-AS-ODN and low concentrations of $1,25(OH)_2D_3$ (10^{-9} M).

Effects of transfection of TAF_{II}-17 on VDR-mediated gene transcription: NIH3T3 cells were stably transfected with TAF_{II}-17 or EV and tested for DR3 reporter activity. Transfection of TAF_{II}-17 increased VDR mediated transcription in response to low levels of $1,25(OH)_2D_3$ in an analogous fashion as MVNP (Figs. 2C and 8). In contrast, transfection of DR-5 reporter construct into these NIH3T3 cells showed that the retinoic acid dose–response curve was similar in EV- and MVNP-transfected cells, although basal transcription was increased (Fig. 8).

DISCUSSION

We have previously reported that OCL precursors from patients with Paget's disease contain MVNP and form OCL at $1,25(OH)_2D_3$ concentrations that are one to two logs less than required by normal OCL precursors.[8–10] We have further shown that MVNP-transduced normal OCL precursors also form OCL that share many of the features of pagetic OCLs and form OCLs at low concentrations of $1,25(OH)_2D_3$.[9] Our hypothesis to explain this phenomenon was that VDR-mediated transcription was enhanced because of increased levels of VDR coactivators or decreased levels of VDR corepressors. In support of this hypothesis, as shown in Fig. 2A, MVNP-transduced OCL precursors showed increased DR-3 reporter activity at $1,25(OH)_2D_3$ concentrations that were one to two logs less than that required for the EV-transduced cells. This increase transcription activity seemed to be relatively specific for VDR because, although basal transcription was increased in MVNP- or EV-transduced cells transfected with a DR-5 reporter construct, the $C_{50\%}$ for the retinoic acid dose–response curves of MVNP-transduced cells did not differ from that of the EV-transduced cells (Fig. 2B).

Several mechanisms may be responsible for the increased VDR-mediated transcriptional activity induced by MVNP and the increased OCL formation by pagetic and MVNP-transduced normal OCL precursors treated with low levels of $1,25(OH)_2D_3$. One possibility is that VDR numbers are increased by expression of MVNP in OCL precursors. However, as shown in Fig. 1, VDR protein levels were similar in EV- or MVNP-transduced OCL precursors. These data are consistent with our previous report using competitive RT-PCR, which showed similar levels of VDR mRNA in pagetic and normal OCL precursors.[10]

A second possibility is that, in cells expressing MVNP, the affinity of VDR for $1,25(OH)_2D_3$ is increased. As shown in Fig. 2C, although NIH3T3 cells transfected with MVNP show increased VDR-mediated transcription in response to low levels of $1,25(OH)_2D_3$ in an analogous fashion to OCL precursors expressing MVNP, Scatchard analysis showed that the affinity (K_d) of $1,25(OH)_2D_3$ for VDR and VDR

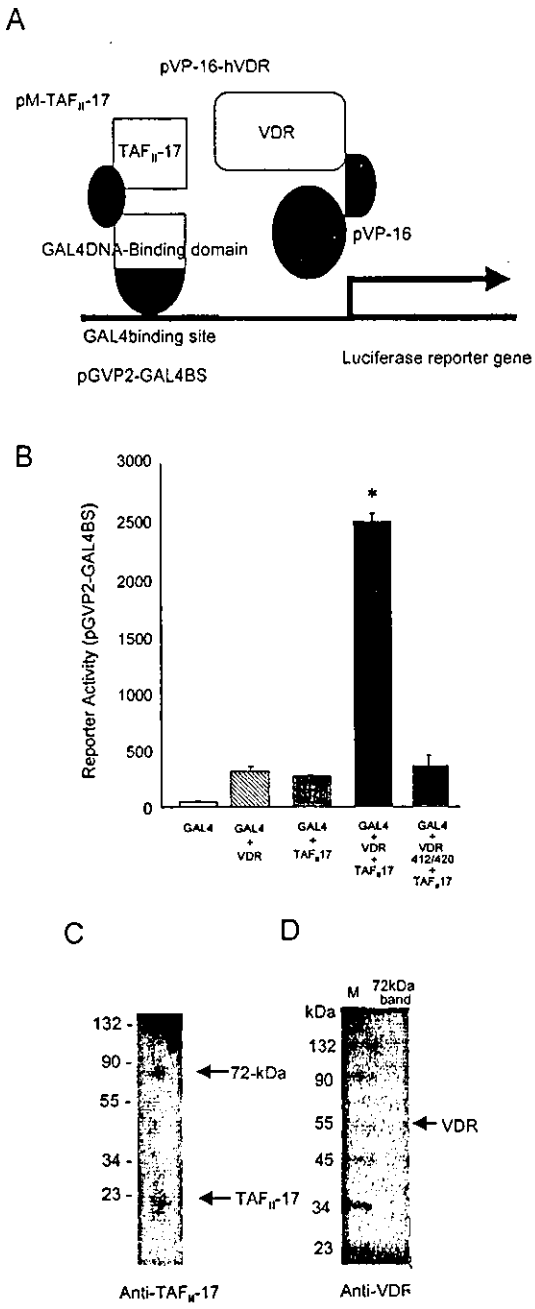


FIG. 5. Interaction between VDR and TAF$_{II}$-17. (A) Modified mammalian two-hybrid assay was used to examine the interaction between VDR and TAF$_{II}$-17. The TAF$_{II}$-17 cDNA was inserted at the *Eco*RI site of the pMvector, which contains a GAL4 DNA binding domain (pM-TAF$_{II}$-17). (B) The interaction of VDR and TAF$_{II}$-17 was examined by measuring the reporter activity in NIH3T3 cells transduced with pM-TAF$_{II}$-17, pVP16-hVDR, and the reporter plasmid containing the GAL4 DNA-binding sites (pGVP2-GAL4BS). 1,25(OH)$_2$D$_3$ (10^{-8} M) was added after transfection. Results are expressed as the mean ± SE. *$p < 0.01$ compared with cells treated with pGVP2-GAL4BS, pGVP2-GAL4BS + pVP16-hVDR, or pGVP2-GAL4BS + pM-TAF$_{II}$-17 transfected groups. Similar results were seen in three independent experiments. (C) Western immunoblotting to determine if TAF$_{II}$-17 directly binds VDR. Day 4 cell lysates from 10^{-8} M 1,25(OH)$_2$D$_3$-stimulated MVNP-transduced CFU-GMs were mixed with native sample buffer and were loaded onto 15% polyacrylamide gels in non–SDS-containing Tris/Glysine Buffer (Bio-Rad). A 17-kDa TAF$_{II}$-17 band and a 72-kDa band, which contained TAF$_{II}$-17, were detected with the anti-TAF$_{II}$-17 antibody. (D) Western analysis of the 72-kDa band from C. The 72-kDa band was eluted and run on SDS gels, and the gels were transferred to membranes and blotted without VDR. Similar results were seen in two independent experiments.

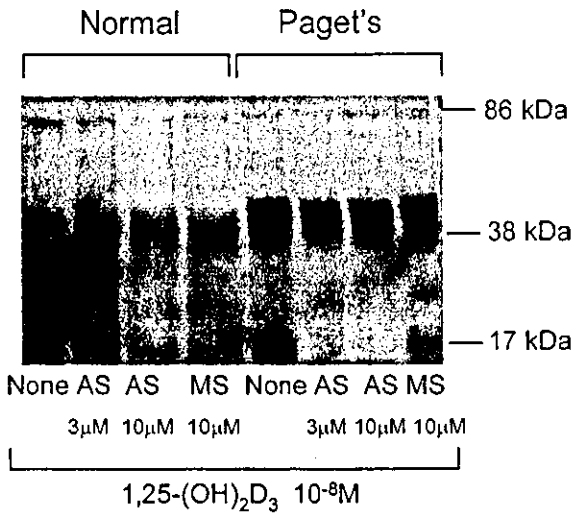
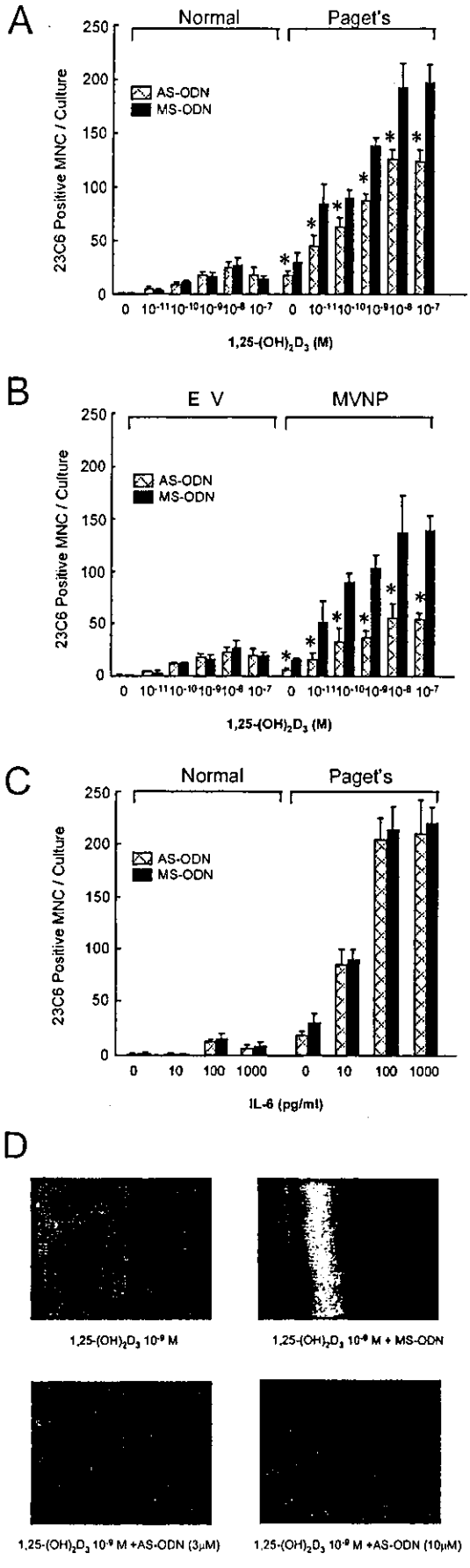


FIG. 6. TAF$_{II}$-17 antisense oligonucleotide decreases the amount of TAF$_{II}$-17 in Paget's OCL precursors. GST-VDR (20 μg) was incubated for 2 h at 4°C with 2 mg cell lysates from cells treated with 10^{-8} M 1,25(OH)$_2$D$_3$ for 4 days. Two micrograms of immobilized sepharose 4B was incubated with reaction mixture for 1 h at 4°C. Samples were resolved by SDS-PAGE and detected by silver staining. Similar results were seen in three independent experiments.

receptor numbers were similar in EV- or MVNP-transfected NIH3T3 cells (Fig. 3).

A third possibility is that MVNP induces expression of a component of the VDR transcription complex or decreases the levels of a VDR corepressor. Experiments using a GST-VDR chimeric protein with lysates from marrow cells from Paget's disease patients or MVNP-transduced normal OCL precursors did not show that any VDR binding protein was decreased in pagetic or MVNP-transduced cells compared with EV-transfected cells. These results suggest that a corepressor of VDR was not decreased by MVNP, although more extensive experiments are required to confirm this preliminary observation. Instead, these experiments showed that TAF$_{II}$-17 levels were increased in MVNP-transduced CFU-GM and pagetic cells and bound VDR. Interestingly, increased amounts of TAF$_{II}$-17 were detected in GST-VDR pull-down experiments from cells treated with increasing concentrations of 1,25(OH)$_2$D$_3$, using other MVNP-transduced CFU-GM or pagetic marrow cells. TAF$_{II}$-17 was only detected in GST-VDR pull-down experiments with lysates from pagetic cells cultured in the absence of 1,25(OH)$_2$D$_3$. This difference most likely reflects the prolonged exposure of pagetic cells to low concentrations of 1,25(OH)$_2$D$_3$ in vivo before isolation and testing (Fig. 4A). Consistent with this possibility, when MVNP-transduced CFU-GMs were exposed to low levels of 1,25(OH)$_2$D$_3$ for 4 rather than 2 days, as shown in Fig. 4B, increased levels



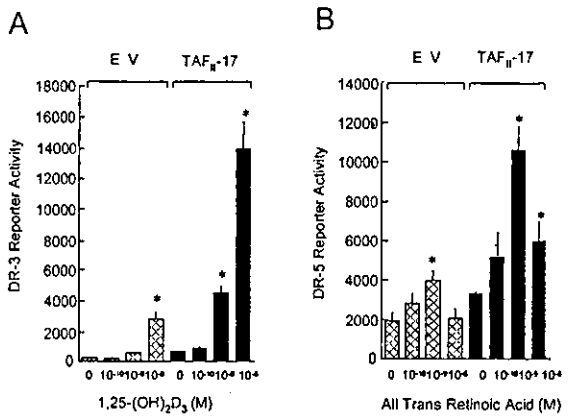


FIG. 8. Effects of transfecting TAF_{II}-17 into NIH3T3 cells expressing a VDR (DR3) or retinoic acid receptor (DR5) reporter constructs. The reporter plasmid was transfected into NIH3T3 (NIH3T3-EV and NIH3T3-TAF_{II}-17) cells. Different concentrations of (A) $1,25(OH)_2D_3$ or (B) all trans-retinoic acid were added to the cultures for 24 h after the transfection. Results are expressed as the mean ± SE. $*p < 0.01$ compared with cells treated with media alone. Similar results were seen in three independent experiments.

of TAF_{II}-17 could be detected in the absence of added $1,25(OH)_2D_3$.

Mammalian two hybrid assays showed that TAF_{II}-17 interacted with VDR (Fig. 5B). Furthermore, Western blot analysis of lysates from MVNP-transduced CFU-GMs (Figs. 5C and 5D) showed the direct association of TAF_{II}-17 and VDR in cells. However, the mechanism for how TAF_{II}-17 increases VDR-mediated transcription is still unclear. Possibly, TAF_{II}-17 may act as a coactivator for VDR-mediated gene transcription or the increased amounts of TAF_{II}-17 permit formation of the VDR transcription complex at low concentrations of $1,25(OH)_2D_3$ bound to VDR. Consistent with the latter hypothesis, high concentrations of TAF_{II}-17 in MVNP-transduced OCL precursors allowed the transcription complex to form at low concentrations of $1,25(OH)_2D_3$ bound to VDR (Fig. 4B). In support of this finding, overexpression of TAF_{II}-17 in NIH3T3 cells increased VDR-mediated gene transcription (Fig. 8).

FIG. 7. Effects of TAF_{II}-17 antisense oligonucleotide (AS-ODN) or missense oligonucleotide (MS-ODN) on OCL formation by CFU-GM-derived cells from Paget's and normal marrow mononuclear cells, or normal CFU-GM-derived cells transduced with MVNP or EV. CFU-GM-derived cells (10^5 cells/well) from normal or Paget's marrow (A) or CFU-GM-derived cells transfected with MVNP or EV (B) were cultured for OCL cell formation in the presence of 10^{-11} M to 10^{-7} M $1,25(OH)_2D_3$. Alternatively, CFU-GM-derived cells from normal or Paget's marrow mononuclear cells were also cultured with 10–1000 pg/ml of IL-6 (C). Three micromolar AS-TAF_{II}-17 ODN or MS-AS ODN were added to these cultures. After 14 days of culture, the cells were fixed and stained with the 23C6 monoclonal antibody, which identifies OCLs. The results are expressed as mean ± SE for quadruplicate cultures from a typical experiment, $p < 0.001$ compared with results of cultures treated with the same concentration of MS-ODN. A similar pattern of results was seen in five independent experiments. In addition, the CFU-GM-derived cells transduced with MVNP (10^5 cells/well) were cultured for OCL formation with $1,25(OH)_2D_3$ (10^{-8} M) with AS-TAF_{II}-17 ODN or MS-ODN on the mammoth dentin slices (D). After 2 weeks of culture, the dentin slices were stained with acid hematoxylin. Magnification, ×100.

Further support for a role of TAF_{II}-17 in the enhanced OCL formation by pagetic OCL precursors in response to low levels of $1,25(OH)_2D_3$ are our experiments using TAF_{II}-17 AS-ODN. When MVNP-transfected OCL precursors were treated with an AS-ODN to TAF_{II}-17, the TAF_{II}-17 AS-ODN decreased the amount of TAF_{II}-17 bound to VDR in Paget's patient–derived OCL precursors and MVNP-transduced CFU-GMs and significantly reduced OCL formation in response to $1,25(OH)_2D_3$. Decreased OCL formation was not observed in normal CFU-GM– or EV-transduced CFU-GM cultures treated with TAF_{II}-17 AS-ODN, consistent with the very low levels of TAF_{II}-17 bound to VDR in normal OCL precursors. This effect seems to be relatively specific for VDR-mediated OCL formation by pagetic or MVNP-transduced cells, because OCL formation by IL-6 was not affected by blocking TAF_{II}-17 expression, and the antisense construct had no effect on the expression of IL-6 in these cultures (Fig. 7C). In addition, other components of the VDR transcription complex may also be increased in pagetic cells and MVNP-transduced CFU-GMs to $1,25(OH)_2D_3$ and play a role in the increased OCL formation.

It is currently unknown if the transcriptional effects of TAF_{II}-17 are specific for VDR-mediated transcription because other TFIID subunits can also bind the thyroid hormone receptor.[19,20] However, our experiments using DR3- and DR5-reported constructs in NIH3T3 cells transfected with TAF_{II}-17 suggest that the effects of TAF_{II}-17 seem to be relative-specific for VDR-mediated TAFIID gene transcription. The TFIID transcription factor complex is composed of a TATA binding protein and at least four TAF_{II} subunits. Until recently, TFIID was thought to be responsible only for promoter recognition and directing RNA polymerase II to core promoters in response to activators of transcription.[21,22] This function of TFIID was thought to be universal rather than gene-specific because TFIID is expressed in all tissues. Recent reports have shown that TFIID components can be cell type specific and regulate specific genes. For example, TAF_{II}-105 is highly expressed in testes and ovary and regulates transcription of specific genes in granulosa cells.[23] Furthermore, mice lacking TAF_{II}-105 are sterile.[23] These data show that different components of the TFIID complex can mediate the transcription of specific genes that can have dramatic effects on the function of specific cells.

It is still unknown if our in vitro observations that pagetic OCL precursors form OCLs at low levels of $1,25(OH)_2D_3$ also occurs in Paget's patients. Currently, there are no animal models of Paget's disease available to rigorously test these findings in vivo. To do this, a VDR antagonist or a $1,25(OH)_2D_3$ antagonist would have to be tested in patients with Paget's disease and normals to determine the effects of these agents on bone turnover and bone resorption. However, our results suggest that enhanced TAF_{II}-17 expression plays an important role in OCL formation by pagetic OCL precursors.

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Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching

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Vitamin D receptor (VDR) is essential for ligand-induced gene repression of 25(OH)D_3 1α-hydroxylase (1α(OH)ase) in mammalian kidney, while this gene expression is activated by protein kinase A (PKA) signaling downstream of the parathyroid hormone action. The mapped negative vitamin D response element (1αnVDRE) in the human 1α(OH)ase gene promoter (around 530 bp) was distinct from those of the reported DR3-like nVDREs, composed of two E-box-like motifs. Unlike the reported nVDREs, no direct binding of VDR/RXR heterodimer to 1αnVDRE was detected. A bHLH-type factor, designated VDIR, was identified as a direct sequence-specific activator of 1αnVDRE. The transactivation function of VDIR was further potentiated by activated-PKA signaling through phosphorylation of serine residues in the transactivation domains, with the recruitment of a p300 histone acetyltransferase co-activator. The ligand-dependent association of VDR/RXR heterodimer with VDIR bound to 1αnVDRE caused the dissociation of p300 co-activators from VDIR, and the association of HDAC co-repressor complex components resulting in ligand-induced transrepression. Thus, the present study deciphers a novel mechanism of ligand-induced transrepression by nuclear receptor via co-regulator switching.

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Introduction

Members of the nuclear receptor (NR) superfamily act as ligand-inducible transcription factors. Fat-soluble NR ligands, such as the steroid/thyroid hormones vitamin A and vitamin D, are believed to exert their biological actions through both positive and negative transcriptional control of specific sets of target genes (Mangelsdorf *et al*, 1995; Chambon, 1996). NR proteins can be divided into several functional domains, with the central highly conserved DNA-binding C domain (DBD) and the less-conserved ligand-binding E domain (LBD) at the C-terminal end present in all members of the NR superfamily. Both the N-terminal A/B and C-terminal E domains are responsible for ligand-inducible NR transactivation functions (Tora *et al*, 1989). While autonomous transactivation function 1 (AF-1) in the A/B domain is constitutively active, it is suppressed by the presence of an unliganded LBD domain. In contrast, AF-2 in the LBD domain is dependent on ligand binding (Tora *et al*, 1989; Beato *et al*, 1995).

In the promoters of target genes transactivated by liganded NRs, homo- or heterodimers of NRs recognize and directly bind to their cognate hormone-responsive elements (HREs) through chromatin remodeling, presumably by ATP-dependent chromatin remodeling complexes (Belandia and Parker, 2003; Kitagawa *et al*, 2003). Liganded NRs bound to their cognate HREs induce the recruitment of a number of histone acetyltransferase (HAT) and non-HAT co-activators to activate transcription (McKenna and O'Malley, 2002). The HAT co-activator complexes CBP/p160 (Onate *et al*, 1995; Kamei *et al*, 1996; Spencer *et al*, 1997) and TRRAP/GCN5 (Yanagisawa *et al*, 2002), and the non-HAT DRIP/TRAP complexes (Fondell *et al*, 1996; Rachez *et al*, 1999) are thought to act as common co-activator complexes for NRs as well as for other classes of DNA-binding activators. In the absence of ligand, NRs bound to HREs appear to be transcriptionally silent due to association with histone deacetylase (HDAC) co-repressor complexes, which are thought to contain NCoR/SMRT, Sin3A and HDACs, along with other components (Chen and Evans, 1995; Heinzel *et al*, 1997; Glass and Rosenfeld, 2000). Thus, ligand binding leads to structural alterations and the switching of NR function from transcriptional inactivation by co-repressors to transcriptional activation via the recruitment of co-activators (Shiau *et al*, 1998).

In sharp contrast to the molecular basis of NR-mediated gene activation, little is known about ligand-induced gene repression at the molecular level. To address this issue, we characterized a negative VDRE (1αnVDRE) in the promoter of the human 25(OH)D_3 1α-hydroxylase (1α(OH)ase) gene (CYP27B1), which is negatively controlled by 1α,25(OH)D_3-bound receptors (VDR) in cultured kidney cells and in the kidneys of intact animals (Murayama *et al*, 1999). 1α(OH)ase is a key enzyme in vitamin D biosynthesis, hydroxylating 25(OH)$_2D_3$ to the active form of vitamin D, 1α,25(OH)$_2D_3$ (Takeyama *et al*, 1997; Panda *et al*, 2001). Expression of the 1α(OH)ase gene is positively and negatively regulated by multiple hormonal factors. 1α,25(OH)$_2D_3$ negatively regulates 1α(OH)ase gene expression through VDR binding to the promoter, while protein kinase A (PKA) signaling downstream of activated parathyroid hormone/parathyroid hormone-related protein (PTH/PTHrP) receptor complexes is thought to be involved in PTH/PTHrP-induced gene induction (Henry, 1985; Brenza *et al*, 1998). 1αnVDRE has been previously mapped to around −500 bp in the human

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