1,25-Dihydroxyvitamin D_3 induction of the tissue-type plasminogen activator gene is mediated through its multihormone-responsive enhancer

Pascal Merchiers[a], Frank Bulens[a], Ingrid Stockmans[b], Astrid De Vriese[a], Renilde Convents[b], Roger Bouillon[b], Désiré Collen[a], Alexandra Belayew[a,*], Geert Carmeliet[b]

[a] *Center for Molecular and Vascular Biology, University of Leuven, Campus Gasthuisberg, O and N, Herestraat 49, B-3000 Leuven, Belgium*
[b] *Laboratory for Experimental Medicine and Endocrinology, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium*

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Abstract Tissue-type plasminogen activator (t-PA) is a positive modulator of the plasminogen-plasmin system, which is involved in bone remodeling. In the present study, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] was found to stimulate *t-PA* gene expression in ROS17/2.8 osteosarcoma cells. Transient transfection analysis and in vitro DNA binding studies identified two vitamin D-responsive elements (*VDRE*) in the human *t-PA* enhancer. The first *VDRE* (bp −7175 to −7146) comprised an inverted palindrome separated by 9 bp (*IP9*) overlapping a palindrome separated by 3 bp. The second *VDRE* (bp −7315 to −7302) is an *IP2* element overlapping the previously identified retinoic acid-responsive element. 1,25$(OH)_2D_3$ treatment of primary osteoblasts derived from t-PAlacZ transgenic mice containing 9 kb of 5′ sequence of the human *t-PA* gene increased the number of lacZ-positive cells, fitting with the probability model of enhancer function.

Key words: 1,25-Dihydroxy vitamin D_3; Tissue-type plasminogen activator; Enhancer; Vitamin D-responsive element; Probability model

1. Introduction

The plasminogen–plasmin proteolytic cascade is involved in different biological processes like fibrinolysis [1], ovulation [2], neuronal long-term potentiation [3], neuronal excitotoxin-induced cell death [4] and bone remodeling [5]. The main components of the system are urokinase- and tissue-type plasminogen activator (t-PA) which convert the inactive plasminogen to the active serine protease plasmin. This proteolytic cascade is controlled by plasminogen activator inhibitor 1 and α_2-antiplasmin, which inhibit either t-PA or u-PA and plasmin respectively [6].

t-PA is produced by osteoblasts and osteoclasts, cells involved in the process of bone remodeling. Plasmin activity is dispensable for bone resorption in explanted mouse fetal metatarsal bones [7], suggesting that the t-PA-plasmin cascade is rather involved in the activation of latent collagenase, regulating the processes of extracellular matrix degradation, and activation of latent transforming growth factor β or latent insulin-like growth factor leading to activation of osteoblasts [5]. t-PA binding to an osteoclast or osteoblast cell membrane receptor might also generate an intracellular signal triggering cell motility, as was shown for urokinase-type plasminogen activator binding to its receptor [8].

Expression of t-PA is subject to regulation by osteotrophic hormones, like parathyroid hormone [9]. In addition, stimulation of rat UMR106, human SAOS-2 osteosarcoma cells or primary osteoblasts derived from rat calvariae with 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) increased plasminogen activator activity, as was determined by the release of radiolabeled fibrin fragments in the presence of these cells or by fibrin overlay gel experiments [10,11]. However, the role of 1,25$(OH)_2D_3$ in *t-PA* gene expression has not been investigated in detail.

The effects of 1,25$(OH)_2D_3$ on gene transcription are primarily mediated by the vitamin D receptor (VDR), which belongs to the superfamily of the steroid hormone receptors [12]. Binding of 1,25$(OH)_2D_3$ to VDR induces formation of homodimers or heterodimers with retinoid X receptors (RXR) and subsequently binding to vitamin D-responsive elements (*VDRE*) of target genes. The consensus binding site for such a VDR/RXR heterodimer is a direct repeat of a hexanucleotide sequence separated by 3 bp (*DR3*; GGGTCA gng GGGGCA), although other types of binding elements, like inverted palindromes, have also been reported [12]. The DNA-bound VDR/RXR complex mediates transcriptional activation by several mechanisms that, as for other nuclear receptors, are becoming unraveled: interactions with other transcription factors and co-activators loosen the chromatin structure and attract the RNA polymerase II pre-initiation complex [13–16].

t-PA is produced not only in osteoblasts and osteoclasts, but also in endothelial and neuronal cells where its expression is regulated by several hormones. The upstream region of the human *t-PA* gene contains an enhancer at −7.2 kb, which mediates the response to retinoic acid (RA) and to all classical steroid hormones, except estrogens [17,18]. These responses are mediated through a perfect *DR5* element (bp −7319; *t-PADR5*) and a hormone-responsive unit comprising four glucocorticoid-responsive elements (*GRE*; bp −7501, bp −7703, bp −7942 and bp −7960), respectively. In contrast, the *t-PA* proximal promoter, which is primarily regulated through a cAMP-responsive element and two Sp1 binding sites [19,20], contains no hormone-responsive elements.

*Corresponding author. Fax: (32) (16) 34 59 90.
E-mail: alexandra.belayew@med.kuleuven.ac.be

Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CAT, chloramphenicol acetyltransferase; DR*n*, direct repeat separated by *n* nucleotides; EMSA, electrophoretic mobility shift assay; IP*n*, inverted palindrome separated by *n* nucleotides; NF-I, nuclear factor-I; oc, osteocalcin; Pal*n*, palindromic element separated by *n* nucleotides; RA, retinoic acid; RAR, RA receptor; RARE, RA-responsive element; RXR, retinoid X receptor; TK, thymidine kinase; t-PA, tissue-type plasminogen activator; VDR, vitamin D receptor; VDRE, vitamin D-responsive element

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In this study we show that $1,25(OH)_2D_3$ stimulates *t-PA* gene expression in ROS 17/2.8 rat osteoblast cells and in primary osteoblasts from murine calvariae. The human *t-PA* enhancer, located at −7.2 kb, is involved in the $1,25(OH)_2D_3$ induction through two non-classical *VDRE*s.

2. Materials and methods

2.1. Reagents

ROS 17/2.8 osteoblast cells were obtained from Dr. G. Rodan (Merck Research Laboratories, West Point, PA, USA). The expression vector encoding the human retinoid X receptor α (*pRSh-RXRα*) and the human vitamin D receptor (*phVDR*) were gifts from Dr. R.M. Evans (The Salk Institute for Biological Studies, La Jolla, CA, USA) and Dr. M. Haussler (University of Arizona, Tucson, AZ, USA), the bacterially expressed and partially purified myc-hRXRα, myc-hVDR and hRARβ proteins from Dr. M. Baes (Laboratory for Clinical Chemistry, University of Leuven, Leuven, Belgium), $1,25(OH)_2D_3$ from Dr. J.P. van de Velde (Solvay, Weesp, The Netherlands). t-PA-lacZ transgenic mice were obtained from Dr. W.D. Schleuning (Research Laboratories, Schering AG Berlin, Germany). Trizol, Dulbecco's modified Eagle's F12 medium (MEM), αMEM and all medium supplements were purchased from Life Technologies, Inc. (Ghent, Belgium), tissue culture recipients from Corning, Inc. (New York, NY, USA) and Becton Dickinson (Franklin Lakes, NJ, USA), chloramphenicol from Sigma (St. Louis, MO, USA), DNA purification columns from Qiagen (Chatsworth, CA, USA), acetyl-CoA and [^{3}H]acetyl-CoA from ICN Biomedicals (Costa Mesa, CA, USA), Lipoluma from Lumac-LSC (Olen, Belgium). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA), 5-Br-4-Cl-3-indolyl-β-D-galactopyranoside (X-gal) from Immunosource (Zoersel-Halle, Belgium), Galacto Light system from Tropix, Inc. (Bedford, MA, USA), poly(dIdC)·poly(dIdC) from Pharmacia (Roosendaal, The Netherlands).

2.2. Cell culture

Rat osteoblast ROS 17/2.8 cells were maintained in supplemented DMEM, containing L-alanyl-glutamine (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum. For transfection, ROS 17/2.8 cells were seeded at a density of 2–4×10^4 cells/cm^2 and grown overnight at 37°C in a humidified 95% air, 5% CO_2 atmosphere in medium with 2% charcoal-treated heat-inactivated fetal calf serum. $1,25(OH)_2D_3$ was dissolved in ethanol and stored at −20°C. The appropriate concentration was added to the medium in a volume corresponding to 0.1% of the culture medium. Control medium contained an equal amount of excipient.

2.3. Northern blot and t-PA-related antigen level determination

Total RNA was isolated from the cells using Trizol reagent and purified according to the manufacturer's instructions. For each condition, 20 μg of total RNA was separated by electrophoresis and transferred as previously described [21]. Northern blots were probed using standard hybridization techniques with a 0.8 kb *mtPA* cDNA fragment [22] that had been ^{32}P-random labeled, and reprobed with an 18S RNA probe. Bands from autoradiographs were quantified on the PhosphorImager (Molecular Dynamics) and the values for the t-PA transcript were normalized to the corresponding values of the 18S RNA. Rat t-PA-related antigen levels were determined in the conditioned medium by specific enzyme-linked immunosorbent assays (ELISA) as described [23].

2.4. Isolation, cell culture and analysis of primary murine osteoblasts

Transgenic mice contained the *lacZ* gene fused to either the complete 9 kb of the upstream region of the human *t-PA* gene (bp −9578 to +197; lines 9kbht-PAlacZ #5706 and 9kbht-PAlacZ #6582) or to 3 kb of the latter region, with an internal deletion from −0.5 kb to −1.4 kb (line 2.1kbht-PAlacZ #7656) [24]. Genotyping was performed using PCR [25]. t-PAlacZ transgenic males were mated with Swiss wild type females. Primary osteoblasts were prepared by six sequential digestions of individual calvariae from newborn mice in phosphate buffer saline containing collagenase (2 mg/ml). Cells isolated from the third digest on were pooled, plated out in four wells of a 12 well plate and cultured in αMEM medium with 10% heat-inactivated fetal calf serum at 37°C in a humidified 95% air, 5% CO_2 atmosphere. The osteoblasts were grown to confluence and were then treated with either vehicle or 100 nM $1,25(OH)_2D_3$. After 48 h the conditioned medium was collected, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min at 4°C and stained with X-gal (1 mg/ml) for 48 h at 30°C or the β-galactosidase production was quantified in a Berthold luminometer with the Galacto Light kit. t-PA-related antigen levels were determined in the conditioned medium by ELISA as described [26]. β-Galactosidase production and t-PA-related antigen levels were corrected for protein concentration of the cell lysate.

2.5. Reporter constructs

Isolation of *t-PA* upstream sequences and their incorporation in chloramphenicol acetyltransferase (CAT) reporter plasmids have been described previously [17]. All genomic sequences analyzed in this study are numbered relative to the transcription start site [27]. *t-PA9578-CAT* and *t-PA632-CAT* contain the human *t-PA* sequence from bp −9578 to +197 and from bp −632 to +197 respectively. *t-PA2.4-632-CAT* has in addition the *t-PA* enhancer (*t-PA2.4*; bp −9578 to −7144) in front of the *t-PA* promoter. *t-PA2.4-TK-CAT* and *t-PA1.9-TK-CAT* have respectively the enhancer *t-PA2.4* and the 3′ deletion of the enhancer *t-PA1.9* (bp −9578 to −7650) in front of the *TK* promoter. Single mutations in the reporter constructs were introduced with a PCR-based strategy, after which the double mutation was cloned with standard techniques. All mutations were sequenced with the ABI 310 sequencer (Perkin Elmer). Sequences of the oligonucleotides used in the mutagenesis are listed in Table 1.

2.6. Transfection analysis

For transient transfection of ROS 17/2.8 cells, the calcium phosphate co-precipitation method was applied to a six well dish using a DNA mixture of 20–60 μg of reporter plasmid with 1.4 μg of the indicated nuclear receptor expression plasmid. Cells were treated for 48 h with 10 nM $1,25(OH)_2D_3$. Cell extracts were prepared by three freeze-thaw cycles in 100 mM Tris-HCl pH 7.8, 5 mM EDTA. CAT activity was determined by the liquid scintillation method [28]. All data shown represent values obtained from at least two independent experiments, each performed in triplicate ($n=6$ or 9) and for which at least two different plasmid preparations were used.

2.7. Electrophoretic mobility shift assay

The recombinant proteins myc-hVDR, hRAR and myc-hRXR were overexpressed in BL21 *Escherichia coli* cells and partially purified by ammonium sulfate precipitation as described previously [29]. Sequences of the oligonucleotides are listed in Table 1. 20 pmol DNA oligonucleotides were labeled by T4 polynucleotide kinase in the presence of [γ^{32}P]ATP and purified via polyacrylamide gel electrophoresis. 20 ng of purified myc-hRXRα and myc-hVDR or hRARβ were pre-incubated for 15 min at room temperature before addition to the mixture (10 mM Tris pH 8, 1 mM dithiothreitol, 500 μM EDTA, 80 mM KCl, 0.1% Triton X-100, 1 μg bovine serum albumin, 0.5 μg poly(dIdC), 12% glycerol) containing 20 000 cpm of the labeled oligonucleotide and incubated for 25 min at room temperature. Incubation with the antibody directed against the myc tag was performed for 15 min between the pre-incubation and the final protein-DNA incubation. The samples were loaded on a 4% polyacrylamide gel and separated by electrophoresis at 4°C for 90 min in 0.5×Tris-borate buffer. Gels were dried and the bands were visualized by autoradiography at −80°C for 12–48 h. Signals were quantified using the PhosphorImager (Molecular Dynamics).

3. Results

3.1. $1,25(OH)_2D_3$ induces t-PA gene expression in ROS 17/2.8 osteoblast cells

Secretion of t-PA-related antigen by ROS 17/2.8 cells was measured following treatment with $1,25(OH)_2D_3$. A time-dependent increase was observed for several doses with the highest induction of t-PA-related antigen secretion after 48 h (Fig. 1A). Regulation of *t-PA* gene expression by $1,25(OH)_2D_3$ in ROS 17/2.8 cells was also evaluated at the mRNA level. A comparable time-dependent induction of message level was

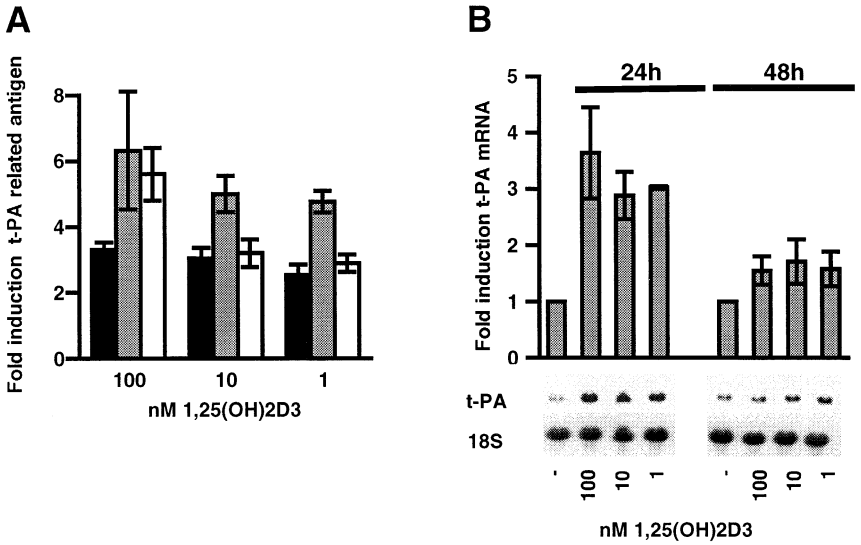

Fig. 1. $1,25(OH)_2D_3$ induction of t-PA-related antigen secretion and t-PA mRNA levels in ROS 17/2.8 cells. A: ROS 17/2.8 cells were treated with indicated concentrations of $1,25(OH)_2D_3$. Samples of the conditioned medium were taken after 24 h (■), 48 h ([NOCHAR:z.sqne]) and 72 h (□). Data are expressed as fold induction versus untreated cells (vehicle) and represent the mean ± S.E.M. ($n = 3$). B: Total RNA of ROS 17/2.8 cells treated with indicated concentrations of $1,25(OH)_2D_3$ for 24 h or 48 h was subjected to a Northern blot analysis. RNA blots were hybridized with a *t-PA* cDNA probe and rehybridized with an 18S rRNA probe. Quantified signals were normalized to the 18S rRNA signal and are presented as fold induction compared to untreated cells. Given values represent the mean ± S.E.M. of three independent experiments and a representative blot is shown.

observed that preceded the alterations in antigen level by 24 h: maximal increase was detected after 24 h (Fig. 1B).

3.2. *1,25(OH)$_2$D$_3$ induces LacZ expression in primary osteoblasts isolated from t-PAlacZ transgenic mice*

Primary osteoblasts were isolated from individual calvariae of newborn mice from two transgenic lines containing the *lacZ* gene fused to 9 kb (bp −9578 to +197) of the human *t-PA* gene upstream region (respectively 9kbht-PAlacZ #5706 and 9kbht-PAlacZ #6582). Treatment of transgenic osteoblasts for 48 h with 100 nM $1,25(OH)_2D_3$ increased the number of cells expressing the *lacZ* gene compared to untreated cells as observed by light microscopy (2.2 ± 0.22- and 2.0 ± 0.25-fold increase for the 9kbht-PAlacZ #6582 and #5706 cells respectively; Fig. 2A,B). Accordingly, β-galactosidase activity in the treated cells was increased 1.9 ± 0.12- and 1.7 ± 0.2-fold compared to untreated cells respectively (Fig. 2C: $P < 0.05$). In contrast, osteoblasts derived from a transgenic line containing the *lacZ* gene fused to 3 kb of the upstream region of the human *t-PA* gene, with an internal deletion from −0.5 kb to −1.4 kb (2.1kbht-PAlacZ #7656), did not express the *lacZ* gene either in the untreated cells or in the $1,25(OH)_2D_3$-stimulated cells (Fig. 2A). Wild type cells did not show any detectable β-galactosidase expression.

Secreted murine t-PA-related antigen was also significantly induced 2.0 ± 0.3-fold in the conditioned medium of treated primary osteoblasts compared to untreated cells (8.9 ng/ml ± 1.35 and 4.5 ng/ml ± 1.35 respectively; $P < 0.01$), indicating that $1,25(OH)_2D_3$ acts in a similar way on the transgene and on the endogenous *t-PA* gene (Fig. 2D).

In aggregate, these results indicate that the upstream region of the human *t-PA* gene (bp −9578 to +197) mediated $1,25(OH)_2D_3$ induction in primary mouse osteoblasts.

3.3. *Delineation in the t-PA enhancer of the region mediating the 1,25(OH)$_2$D$_3$ response*

The human *t-PA* enhancer, located 7.2 kb upstream of the transcription start site, mediates the response to RA and to all classical steroid hormones except estrogens [17,18].

Table 1
Oligonucleotides

Name	Sequence
VDRE 1 wt	TCACAGTTCACTGCAACCTCAAATTTTCAGGTTCAAGG
VDRE 1 mut1	TAACAATTGACTGCAACCTCAAATTTTCAGGTTCAAGG
VDRE 1 mut4	TCACAGTTCACTGCAACCTCAAATTTTCATACATTGG
VDRE 1 mut6	TCACAATTGACTGCAACCTCAAATTTTCAGGTTCAAGG
VDRE 1 mut7	TCACAGTTCACTGATTTACCAAATTTTCAGGTTCAAGG
VDRE 1 mut8	TCACAATTGACTGATTTACCAAATTTTCAGGTTCAAGG
VDRE 1 mut9	TCACAGTTCACTGATTTACCAAATTTTCATACATTGG
t-PA DR5	GATCCACTCTGGGGTCACCCTGGGGTCAGAAGGAA
t-PA DR5 mod	GATCCACTCTGCACTCACCCTGGGGTCAGAAGGAA
t-PA DR5 mut	GATCCACTCTGGGTACCCCTGGGGATTGAAGGAA
oc VDRE	ACTGTGCACTGGGTGAATGAGGACATGACTCA
t-PA NFI	GAATAGGGCTTTGGCCGCTCTCCCAAAGGAGCCCG

Sequences of the oligonucleotides used in the competition analysis or to introduce site-specific mutations in the *t-PA2.0-TK-CAT* vector. Putative *VDRE* half sites are double underlined in the wild type sequences. Introduced mutations are underlined.

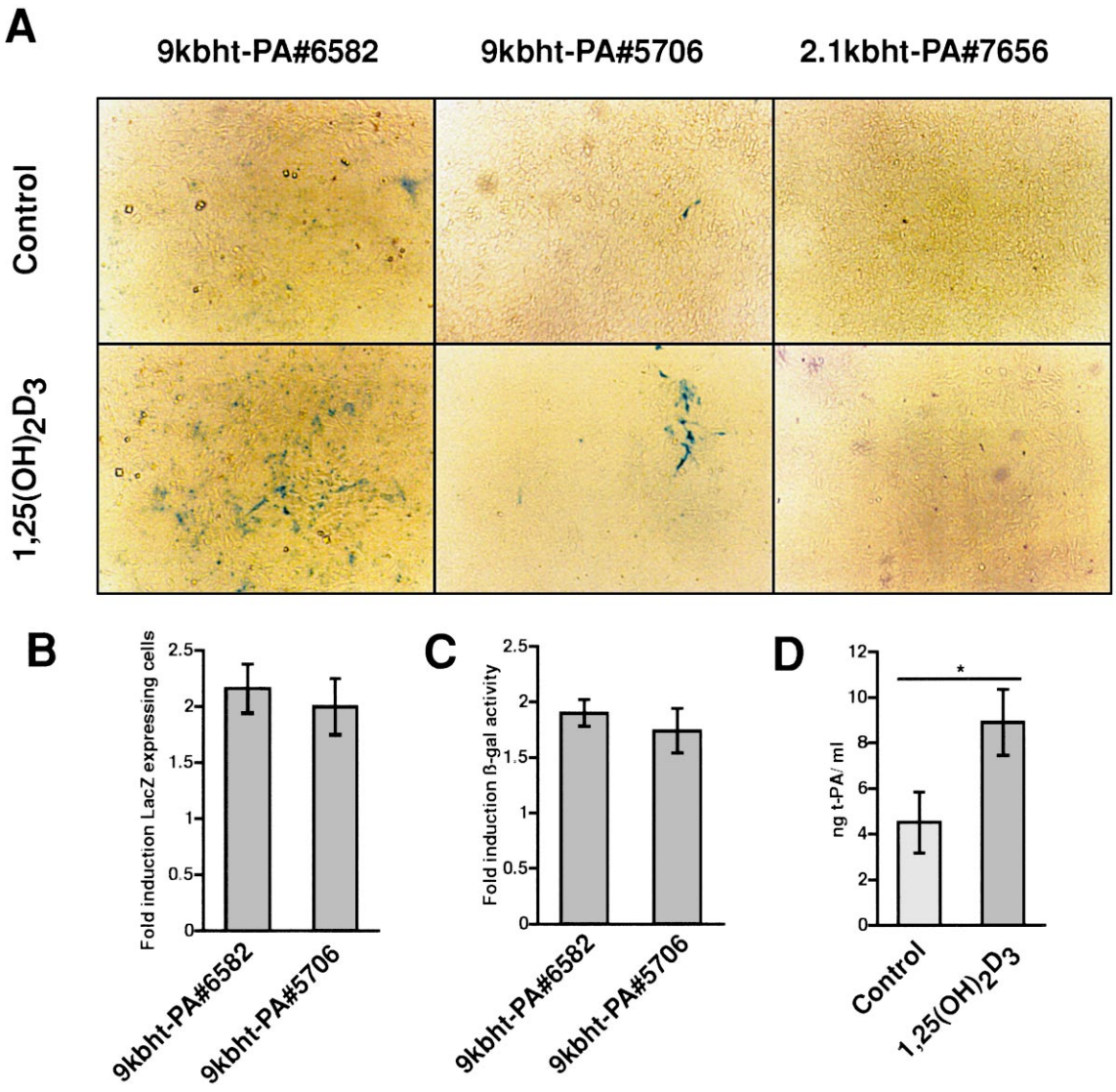

Fig. 2. $1,25(OH)_2D_3$ treatment of primary t-PAlacZ osteoblasts. Primary osteoblasts were isolated from newborn t-PAlacZ transgenic mice as described in Section 2 and allowed to grow to confluence before treatment for 48 h with 100 nM $1,25(OH)_2D_3$. A: Cells were fixed and stained with X-gal for 48 h. Representative pictures of untreated and $1,25(OH)_2D_3$-treated cells derived from mice of the 9kbht-PAlacZ #5706, 9kbht-PAlacZ #6582 and 2.1kbht-PAlacZ #7656 lines are shown. B: Number of stained cells was determined in a bright field microscopic analysis with the aid of a gridded ocular. Fold induction of the number of stained cells treated with $1,25(OH)_2D_3$ compared to control cells for the 9kbht-PAlacZ #5706 and 9kbht-PAlacZ #6582 lines is presented. C: Cells were harvested and β-galactosidase activity was measured and corrected for protein concentration. Fold induction of β-galactosidase activity of $1,25(OH)_2D_3$-treated cells versus control cells for the 9kbht-PAlacZ #5706 and 9kbht-PAlacZ #6582 lines is shown. D: ELISA for murine t-PA-related antigen was performed on the conditioned medium of the primary osteoblasts and statistical significance (*) was at the $P < 0.01$ level (Student's *t*-test).

$1,25(OH)_2D_3$ responsiveness of reporter constructs containing this enhancer was evaluated by transient transfection of ROS 17/2.8. Since it was observed that the endogenous VDR expression levels decreased in time, co-transfection of expression vectors for RXR and VDR was performed.

Transient transfection of ROS 17/2.8 cells with *t-PA9578-CAT* yielded only a moderate 1.4 ± 0.1-fold induction by 10 nM $1,25(OH)_2D_3$. This is similar to the weak response to RA or glucocorticoids that is observed after transient transfection of HT1080 fibrosarcoma cells with *t-PA9578-CAT* [17,18]. Since the responsive elements mediating the latter inductions had been successfully identified using a reporter construct containing the t-PA enhancer in front of the *t-PA* or *TK* promoter, we followed the same approach for the delineation of the *VDRE*s. After treatment of the ROS 17/2.8 cells with 10 nM $1,25(OH)_2D_3$ a 3.6 ± 0.2-fold induction compared to the basal level was observed for a construct linking the *t-PA* enhancer (*t-PA2.4*) to the *t-PA* promoter (*t-PA2.4-632-CAT*; Fig. 3). Since no response was found for the *t-PA* proximal promoter alone (*t-PA632-CAT*), the enhancer was linked to a heterologous promoter (*t-PA2.4-TK-CAT*) resulting in an even stronger induction (5.8 ± 0.4-fold; Fig. 3).

Evaluation of a 3′ deletion mutant of the t-PA enhancer (*t-PA1.9-TK-CAT*) revealed an almost complete suppression of the $1,25(OH)_2D_3$ induction, indicating the presence of essential responsive elements between bp −7650 and −7144 (*t-PA0.5*).

In conclusion, the enhancer located 7.2 kb upstream of the

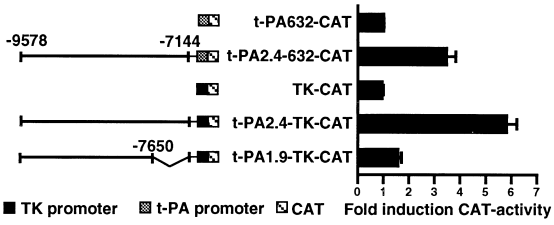

Fig. 3. Delineation of a $1,25(OH)_2D_3$-responsive region in the upstream sequences of the human *t-PA* gene. ROS 17/2.8 cells were transiently co-transfected with expression vectors for VDR and RXR and with CAT reporter constructs containing the TK or *t-PA* promoter (*TK-CAT*; *t-PA632-CAT*) alone or fused to the *t-PA* enhancer (*t-PA2.4-632-CAT*; *t-PA2.4-TK-CAT*) or to a 3′ deletion mutant *t-PA1.9* (*t-PA1.9-TK-CAT*). After treatment for 48 h with 10 nM $1,25(OH)_2D_3$, cells were harvested and CAT activity was determined. The results are the mean ± S.E.M. and are presented as the fold induction of CAT activity of treated versus control cells.

t-PA gene is able to mediate a response to $1,25(OH)_2D_3$, through *cis*-elements situated at its 3′ end.

3.4. Interaction of VDR/RXR with sequences in t-PA0.5

Nucleotide sequence analysis of *t-PA0.5* (bp −7650 and −7144) identified putative nuclear receptor binding sites. Interestingly, a half site identical to the mouse osteopontin (*mop*) *VDRE* was present at bp −7153. This sequence constitutes the 3′ half site of a putative inverted palindromic element separated by nine nucleotides (*IP9*), while the 5′ half site of this *IP9* forms a palindromic element separated by three nucleotides (*Pal3*) in combination with a more upstream half site (Fig. 4A). Interaction of bacterially expressed myc-hVDR/myc-hRXR heterodimers with an oligonucleotide comprising this sequence (*VDRE 1*: bp −7175 to −7146) was investigated by electrophoretic mobility shift assay (EMSA; Fig. 4B). Compared to *oc VDRE*, binding of myc-hVDR or myc-hVDR/myc-hRXR to *VDRE 1* resulted in a weak complex

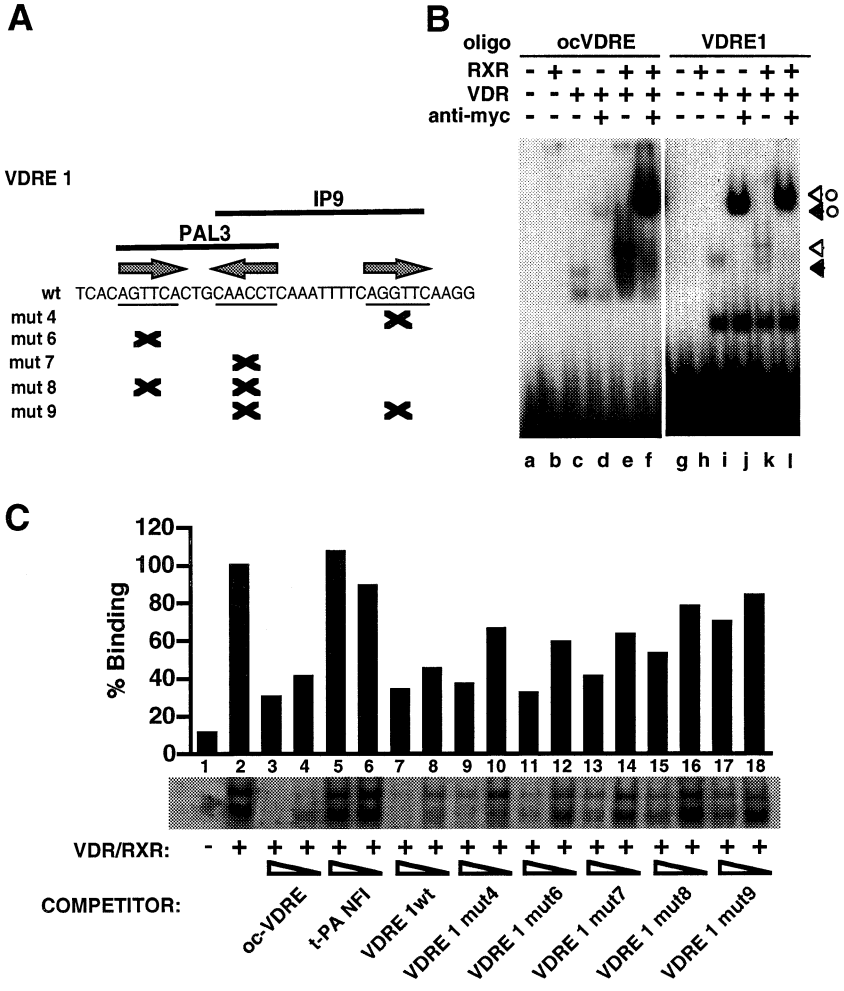

Fig. 4. Identification of the sites mediating VDR/RXR binding to *VDRE 1*. A: Sequence of *VDRE 1* (bp −7175 to −7146) and its putative half sites (arrows). Mutated half sites are indicated with a bold cross. Putative binding elements are indicated at the top of the sequence. B: EMSA was performed with the ^{32}P-labeled oligonucleotide *VDRE 1* or the control *oc VDRE* incubated with bacterially expressed myc-hRXR, myc-hVDR or myc-hVDR/myc-hRXR proteins as described in Section 2. Because of differences in binding affinity the gel for *VDRE 1* was exposed longer. Myc-hVDR and myc-hVDR/myc-hRXR complexes are indicated with a black and white arrow respectively. The supershifted complexes with antibody directed against the myc tag are indicated with a circle. C: EMSA was performed with ^{32}P-labeled *oc VDRE* oligonucleotide, myc-hVDR/myc-hRXR proteins and *VDRE 1* competitor oligonucleotides as described in Section 2. Complexes formed with VDR homodimers (lower complex) and VDR/RXR heterodimers were quantified and are shown below the graph. The relative intensities of the quantified signals are presented as percent binding compared to binding in the absence of competition. Competitor oligonucleotides are indicated below the corresponding lanes and were added in 100-fold and 10-fold molar excess.

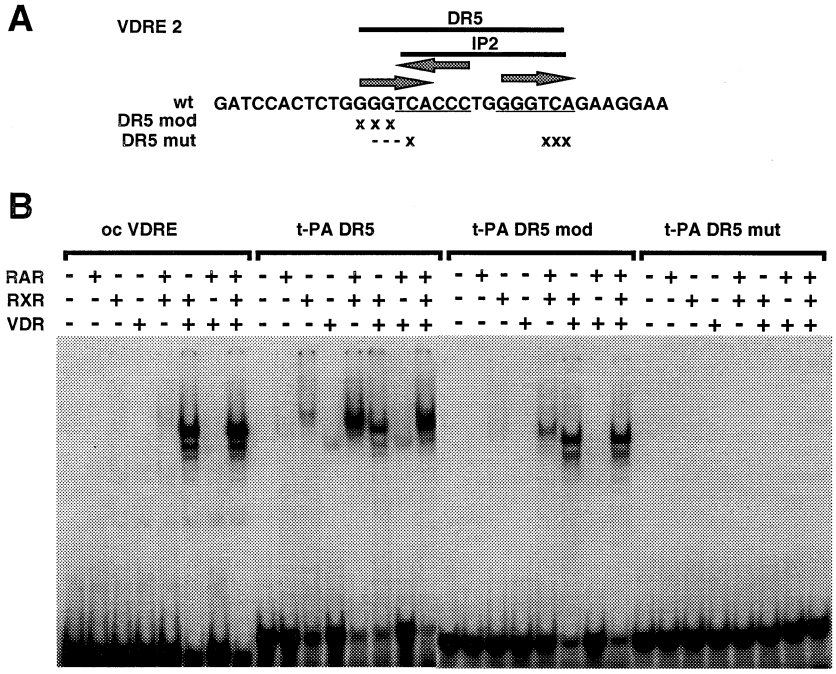

Fig. 5. Binding of VDR/RXR and RAR/RXR heterodimers to *VDRE 2*. A: Sequence of *VDRE 2* (bp −7338 to −7304) and its putative half sites (arrows). Mutated half sites are indicated with a bold cross. Putative binding elements are indicated at the top of the sequence. B: EMSA was performed as in Fig. 3B with the labeled oligonucleotides *oc VDRE*, *t-PA DR5*, *t-PA DR5 mod* and *t-PA DR5 mut* (sequences are provided in Table 1). Myc-hVDR, myc-hRXRα or hRARβ proteins were added as indicated above the lanes.

(lanes i, k), which was supershifted and stabilized by addition of an antibody directed against the myc tag (lanes j, l). The role of the different half sites was evaluated in EMSA competition experiments. Binding of myc-hVDR/myc-hRXR to *oc VDRE* was challenged by competition with oligonucleotides representing wild type (wt) or mutated sequences of *VDRE 1* (Fig. 4C). In contrast to the unrelated *t-PANF-I* oligonucleotide, *VDRE 1 wt* was able to compete for VDR/RXR binding to the *oc VDRE* (lanes 7, 8), although slightly less efficiently than *oc VDRE* itself (lanes 3, 4). Mutation of either of the three half sites (*VDRE 1 mut4*, *VDRE 1 mut6* and *VDRE 1 mut7*) affected competition efficiency (lanes 9–14). Combined mutation of the half sites constituting either the *Pal3* or the *IP9* element further reduced binding of VDR/RXR heterodimers to the *VDRE 1* element (*VDRE 1 mut8* and *VDRE 1 mut9*; lanes 15–18) suggesting that VDR/RXR binding to *VDRE 1* could be mediated through both elements.

Since a RA-responsive element (*RARE*) in the avian β_3 integrin promoter also mediated a response to $1,25(OH)_2D_3$ [30], we investigated the ability of VDR/RXR to interact with the *RARE t-PA DR5* in the *t-PA* enhancer (bp −7319 to −7303; *VDRE 2*) [17]. Indeed, myc-hVDR/myc-hRXR could bind to an oligonucleotide comprising the *t-PADR5* sequence in EMSA (Fig. 5B). This *DR5* element overlaps an inverted palindromic element separated by 2 bp (*IP2*; Fig. 5A). Specific mutation of the *DR5* element, leaving the *IP2* intact (*DR5 mod*), disrupted RAR/RXR binding, but not myc-hVDR/myc-hRXR binding. Upon mutation of the *IP2* sequence (*DR5 mut*) the interaction with myc-hVDR/myc-hRXR was also disrupted.

In conclusion, two *VDRE*s have been identified in *t-PA0.5*: *VDRE 1* consists of a *Pal3* and an *IP9* element, while *VDRE 2* is an *IP2* element which overlaps a functional *RARE*.

3.5. Functionality of the vitamin D-responsive elements

The *VDRE 1* and *VDRE 2* (*t-PADR5*) sites were mutated in the *t-PA2.0-TK-CAT* reporter construct to the *VDRE 1 mut1* and *DR5 mut* sequences (Table 1). These mutated reporter constructs were evaluated for their $1,25(OH)_2D_3$ responsiveness in ROS 17/2.8 cells co-transfected with expression vectors for VDR and RXR as above. Mutation of *VDRE 1* or *VDRE 2* reduced $1,25(OH)_2D_3$ induction as compared to wt *t-PA2.0-TK-CAT* activity (Fig. 6, c or d versus b). Mutation of the two sites simultaneously (Fig. 6, e) resulted in a further decrease of $1,25(OH)_2D_3$ induction, although the response was not completely blunted.

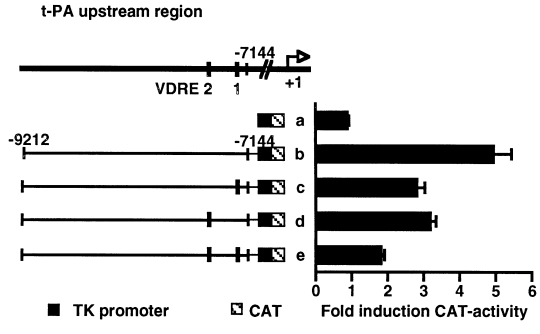

Fig. 6. $1,25(OH)_2D_3$ responsiveness of mutated *t-PA VDRE*s in the *t-PA2.0-TK-CAT* reporter construct in ROS 17/2.8 cells. A: Schematic representation of the *t-PA2.0-TK-CAT* reporter construct. Mutations are indicated with a bold bar. B: Transient transfection of ROS 17/2.8 cells with *TK-CAT* (a), *t-PA2.0-TK-CAT* (b), *t-PA2.0VDRE1mut-TK-CAT* (c), *t-PA2.0DR5mut-TK-CAT* (d), *t-PA2.0VDRE1/DR5mut-TK-CAT* (e), and expression vectors for RXR and VDR. Following stimulation with 10 nM $1,25(OH)_2D_3$, cells were harvested and CAT activity was determined. Results are presented as described in the legend for Fig. 3.

In summary, mutation of *VDRE 1* and *VDRE 2* reduces the $1,25(OH)_2D_3$ induction of the *t-PA-CAT* reporter constructs to the same level as *t-PA1.9-TK-CAT*, indicating that the responsible *VDRE*s in *t-PA0.5* (bp −7650 and −7144) have been identified. The minor residual induction could be caused by additional *cis*-elements upstream of bp −7650 and by the *IP9* element in *VDRE 1*, which was not affected by the *VDRE 1* mutation.

4. Discussion

$1,25(OH)_2D_3$ was shown to induce the expression of the endogenous *t-PA* gene in rat ROS 17/2.8 osteosarcoma cells and of a *lacZ* gene linked to the 9 kb upstream sequence of the human *t-PA* gene in primary osteoblasts derived from t-PAlacZ transgenic mice. Two *VDRE*s located in the *t-PA* enhancer at −7.2 kb are involved in this response.

It was known that $1,25(OH)_2D_3$ stimulates plasminogen activator activity [10,11], but this was mainly attributed to a decrease in PAI-1 activity [31]. We show that $1,25(OH)_2D_3$ also upregulates *t-PA* gene expression at the mRNA and the protein level in rat and murine osteoblasts. This induction could be mimicked by the use of reporter constructs containing the upstream sequence of the human *t-PA* gene, implying that $1,25(OH)_2D_3$ regulation is conserved among species. A sequence homologous to the *t-PA* enhancer is not known yet in mouse and rat.

$1,25(OH)_2D_3$ induction was studied in three lines of transgenic mice containing 9 kb or 2.1 kb of the human *t-PA* gene upstream sequence linked to the *lacZ* gene. Probably, because of position effect variegation, only a small part of the primary osteoblast population derived from individual calvariae from both 9kbht-PAlacZ lines expressed the *lacZ* gene. Both the number of osteoblasts expressing the *lacZ* gene and the β-galactosidase activity were increased 2-fold after $1,25(OH)_2D_3$ treatment. This suggests that $1,25(OH)_2D_3$ statistically increases the chance that an osteoblast expresses the *lacZ* gene (i.e. the *t-PA* gene), supporting the probability model for enhancer function. In contrast to the classical model, into which enhancers are thought to increase the rate of transcription of a given gene in each cell, the probability model favors an on/off state of gene expression which is modulated by enhancers [32]. It is assumed that enhancers do so via disruption of the surrounding heterochromatin. The probability model is based on observations with reporter constructs containing enhancers in front of heterologous promoters that were stably transfected into cells and assayed for resistance to neomycin or for β-galactosidase expression [33,34]. Our experiment is one of the first demonstrations that the on/off state of a gene is modulated through an enhancer positioned at its natural location, namely 7.2 kb upstream of its homologous promoter. Osteoblasts derived from the 2.1kbht-PAlacZ line do not express any β-galactosidase, suggesting that the enhancer is necessary for the basal level of expression (the on state) in these osteoblasts.

Since there is a RA- and steroid hormone-responsive enhancer located at −7.2 kb from the human *t-PA* gene, it was investigated whether this enhancer was also involved in $1,25(OH)_2D_3$ induction [17,18]. Transient transfection of ROS 17/2.8 cells with *t-PA* reporter constructs showed that the enhancer linked to its natural or the heterologous *TK* promoter was able to mediate a $1,25(OH)_2D_3$ response. Evaluation of a 3′ deletion mutant revealed the involvement of the region between bp −7650 and −7144 (*t-PA0.5*) in this induction. Two sequences (*VDRE 1* and *VDRE 2*), located in this region, were shown to interact with VDR in EMSA.

VDR/RXR binding has been observed to *DR3* elements, as in the mouse osteopontin gene [35], to *IP9* elements as in the human calbindin D_{9k} gene [36], and to *DR4* and *DR6* type of elements, as in the rat Pit1 and human phospholipase C-γl genes [37,38]. *VDRE 1* (bp −7175 to −7146) belongs to the family of complex *VDRE*s which consists of multiple half sites. Mutational analysis of the *VDRE 1* sequence showed that half sites at bp −7175, bp −7166 and bp −7151 are involved in VDR/RXR binding. These three binding sites are structured as a *Pal3* element overlapping an *IP9* element. A similar structure comprising an *IP9* and *DR3* is present in the rat osteocalcin promoter [36,39].

VDR/RXR heterodimers also interacted with an *IP2* sequence (*VDRE 2*; bp −7315 to −7302) overlapping the *t-PA DR5* element, previously shown to mediate the RA induction of the human *t-PA* gene [17]. Specific mutagenesis analysis demonstrated that the *DR5* element interacted with RAR/RXR heterodimers and that the *IP2* element interacted with VDR/RXR heterodimers. Responsive elements mediating RA and $1,25(OH)_2D_3$ induction have been described already for the human osteocalcin promoter (*DR6*) [40], the human 25-hydroxyvitamin D_3-24-hydroxylase promoter (two *DR3* elements) [41] and the avian β_3 integrin promoter (*DR3*+ *DR9*) [30]. In contrast to the responsive elements of the first two promoters, the *VDRE* and the *RARE* can be discriminated in *t-PA VDRE 2* and in the avian β_3 integrin promoter: RAR/RXR binds to a *DR5* and a *DR9* respectively, while VDR/RXR binds to an *IP2* and a *DR3* respectively. Further studies are required to investigate whether, just like for the avian β_3 integrin promoter, there is crosstalk between the $1,25(OH)_2D_3$ and the RA pathways.

Despite the weak interaction with VDR/RXR in EMSA, both *VDRE 1* and *VDRE 2* are directly involved in the $1,25(OH)_2D_3$ response, since mutation of either of them in *t-PA2.0-TK-CAT* strongly affects the induction. However, we cannot exclude that *VDRE*s located outside *t-PA0.5* (bp −7650 to −7144) are also involved in the $1,25(OH)_2D_3$ induction of the *t-PA* gene.

In conclusion, besides a *RARE* (*t-PA DR5*) and a steroid hormone responsive unit, comprising four *GRE*s, we have identified two functional *VDRE*s within the far upstream *t-PA* enhancer, demonstrating the convergence of an additional hormonal pathway on this unique regulatory element conveying modulation of *t-PA* gene expression.

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