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Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 223–230

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Identification of a Vitamin D response element in the human insulin receptor gene promoter☆

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Abstract

The present study was designed to explore the possible presence and location of Vitamin D response elements (VDREs) in the human insulin receptor (hIR) gene promoter. To this end, the −1819 to −271 bp fragment of the hIR promoter (wild type promoter) and progressive 5′ deletions of this promoter (up to −1473 and −876 bp) were linked to the luciferase pGL2-basic vector to construct the reported plasmids: phIR (−1819)-GL2, phIR(−1473)-GL2 and phIR(−876)-GL2, respectively. U-937 cells were transiently transfected with these plasmids, and then the cells were either untreated or treated for 24 h with 10^{-8} M 1,25-dihydroxyvitamin D_3 ($1,25D_3$). Luciferase determinations revealed that, while the activity of the wild promoter was increased 1.6-fold by the hormone, the activities of progressive 5′ deletions of this promoter were enhanced 1.7-, and 1.6-fold, respectively. Thus, the region extending from −876 to −271 bp of the hIR promoter, appears to contain VDREs, and to be sufficient for induction by $1,25D_3$. In order to identify these potential VDREs, we performed a computer search of candidate sequences by homology with a *consensus* VDRE sequence. This search yielded a sequence located between −761 and −732 bp (5′CGTCGGGCCTGTGGGG**CGCCTCCGGGGG**TC3′), which includes an overlapping AP-2 like sequence, as a good candidate. Electrophoretic mobility shift assays revealed that the Vitamin D receptor (VDR) specifically recognized this sequence, since a VDR–DNA complex was able to compete with the unlabeled probe and was cleared by the specific anti-VDR antibody 9A7. These data represent the first identification of a VDRE in the hIR gene promoter.

Keywords: 1,25-Dihydroxyvitamin D_3; Vitamin D receptor; Vitamin D response element; Human insulin receptor gene promoter; Gene expression; U-937 human promonocytic cells

1. Introduction

1,25-Dihydroxyvitamin D_3 ($1,25D_3$), the most active Vitamin D metabolite, is known to regulate plasma calcium and phosphorus concentrations to levels required for normal skeleton mineralization and neuromuscular function [1,2]. Further physiological functions of this steroid hormone include effects on cell proliferation, differentiation and immunosupression, secretion of hormones, and regulation of gene expression in different cells [3,4].

$1,25D_3$ acts as a ligand for the Vitamin D receptor (VDR, NR1I1). This receptor is a member of the superfamily of nuclear receptors, which regulates gene expression as a Vitamin D-dependent transcription factor, and exerts this action by binding, preferentially as a heterodimer with the retinoid X receptor (RXR), to Vitamin D response elements (VDREs) in the promoter regions of target genes [5].

A VDRE generally consists of two direct imperfect repeats of six nucleotides separated by a three nucleotide spacer. The VDR occupies the 3′ half-site, while the RXR binds to the 5′ half-site. Several sequence variations had been detected in the 3′ half-element, the 5′ half-element, the spacer, and in the sequences flanking the VDREs [4,6]. These differences appear to be important in determining receptor-binding efficiency [7].

Although many genes have been reported to be regulated by $1,25D_3$, transcriptional regulation by this hormone has only been described in a small proportion of them, and the identification of VDREs has only been possible in a very limited number of these genes [4,6].

We recently reported the first demonstration that $1,25D_3$ increased human insulin receptor (hIR) mRNA levels, insulin binding, and insulin responsiveness of U-937 human promonocytic cells via mechanisms involving direct transcriptional activation of the hIR gene [8–10]. These effects

☆ Poster paper presented at the 15th International Symposium of the Journal of Steroid Biochemistry and Molecular Biology, "Recent Advances in Steroid Biochemistry and Molecular Biology" Munich, Germany, 17–20 May 2002.

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0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00032-3

involved no change in IR mRNA stability [8] and were mediated by an increase in VDR expression, both at the RNA and protein levels [9]. These findings suggest that the activated VDR, behaving as a Vitamin D transcription factor, binds to potential VDREs in the hIR gene promoter. However, the existence of VDREs that could account for this transcriptional induction of hIR mRNA levels by $1,25D_3$ has not yet been demonstrated in this promoter.

The aim of the present study was thus to investigate the possible existence and location of VDREs in the hIR promoter. The results indicate that a sequence, from −761 to −732 bp of this promoter, including an AP-2 like sequence, specifically binds VDR.

2. Materials and methods

2.1. Cell culture and treatments

U-937 human promonocytic cells (mycoplasma-free) were grown in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics at 37 °C in a humidified 5% CO_2 atmosphere as previously described [11]. $1,25D_3$ (Roche) was dissolved in absolute ethanol at 10^{-5} M, and applied to the cells at a final concentration of 10^{-8} M. Untreated cells received only the vehicle. After 24 h of treatment, the cells were collected by centrifugation, and washed three times in phosphate-buffered saline to remove endogenous steroids and serum proteins. As previously demonstrated [8,9], $1,25D_3$-treatment shows no toxic effects on these cells.

2.2. Plasmids, transfections and luciferase activity

The −1819 to −271 promoter fragment of the hIR gene cloned at the BglII site of the pCAT3M vector was kindly provided by Drs. S.Y. Tsai (Department of Cell Biology, University of California) and G. Elberg (Baylor College of Medicine, Texas Medical Center). This promoter fragment, considered by these authors as the wild type promoter, was subcloned into the BglII site of the pGL2-basic vector (Promega) to create the reporter plasmid phIR(−1819)-GL2. The orientation and integrity of this insert was confirmed by restriction analysis. Digestion of this plasmid with KpnI or XhoI generated the plasmids phIR(−1473)-GL2 and phIR(−876)-GL2, respectively. In addition, the −577 to −271 promoter fragment of the hIR gene was subcloned into the *Hin*dIII site of the pGL2-basic vector to produce phIR(−577)-GL2. Transient transfections were carried out by electroporation of 20×10^6 cells in RPMI 1640 medium with the Bio-Rad gene-pulser II, essentially as described previously [10]. The cells were electroporated at 250 V, 960 μF, in a volume of approximately 800 μl of RPMI containing 50 μg of each of the above described reporter plasmids, or 50 μg of the promoterless pGL2-basic vector, together with 50 μg of the pBluescript II KS(+/−) as a carrier. As a positive control, we used 25 μg of the pGL3-control vector (Promega), which includes SV40 promoter and enhancer sequences. All transfections were performed in the absence of a VDR expression vector due to the endogenous VDR activity of the U-937 cells [9,12]. Transfection efficiency was determined using 12 μg of pCMV-βgal (Clontech), and by measuring β-galactosidase activity in the extracts [13]. After resting for 24 h, the transfected cells were either untreated or treated for a further 24 h with 10^{-8} M $1,25D_3$ before harvesting. The cells were then collected by centrifugation, and luciferase activity determined according to the instructions provided in the luciferase assay kit (Promega). Previous luciferase determinations revealed that the activity of the pGL3-control vector was about 100 times above basal levels shown by the promoterless pGL2-basic vector (assigned the arbitrary value of 1 after correction for transfection efficiency) [10].

2.3. Computer analysis of DNA sequences

A *consensus* VDRE sequence was selected by computer from a series of previously reported functional VDRE sequences in other $1,25D_3$-stimulated promoters [4,6,7,14–18]. Potential VDREs sequences in the hIR gene promoter were identified by homology with this *consensus* VDRE sequence using the SEQFIND programme generated in our laboratory [19]. Identification of potential transcription factor binding sites in the sequences flanking or overlapping these potential VDREs [20–23] was performed using the TRANSFAC system [24].

2.4. Electrophoretic mobility shift assays (EMSA)

Three oligonucleotides commercially synthesized by Cruachem were used in these assays. One of these comprised a previously identified VDRE sequence from −512 to −483 bp of the human osteocalcin gene promoter (5′TTGGTGACTCACCGGGTGAACGGGGGCATT3′) [17]. The second nucleotide, corresponded to the sequence of a potential VDRE located from −633 to −604 bp of the hIR promoter (5′GAGGCGGGGAGGCGGGCGGGCGGGGCGGG3′), and the third was a potential VDRE sequence located between −761 and −732 bp of the hIR promoter (5′-CGTCGGGGCCTGTGGGGCGCCTCCGGGGGTC3′). The annealed oligonucleotides (3.5×10^{-12} M) were 5′ end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) (NEN) using T4 polynucleotide kinase (Promega). Nuclear extracts were obtained as described by Schreibert et al. [25]. Ten micrograms of these extracts were incubated for 10 min on ice in a binding buffer containing 10×10^{-3} M Tris–HCl, pH 7.5, 1×10^{-3} M $MgCl_2$, 0.5×10^{-3} M EDTA, 50×10^{-3} M NaCl, 4% glycerol, 0.5×10^{-3} M dithiothreitol, and 10 μg poly(dI-dC) in a total volume of 20 μl. Next, 0.1–0.5 ng (100,000 cpm) of each labeled oligonucleotide was added to the reaction and the incubation was continued for 20 min at room temperature. An excess of the corresponding unlabeled

oligonucleotide (50, 100, or 200×) was added as a specific competitor in each EMSA. In addition, a non-specific competitor (100×) corresponding to a DNA sequence derived from the rat tyrosine aminotransferase gene promoter [26] was also used. The unlabeled oligonucleotide of the human osteocalcin gene promoter (50×) was used as a partially related competitor in certain cases. For a stricter test of the binding specificity of the nuclear extracts, we incubated the extracts with an anti-VDR monoclonal antibody (9A7) (Alexis) that binds with high affinity, just C-terminal, to the DNA binding domain of the VDR [27]. A non-immune serum was also used. The DNA–protein complexes were resolved on 4% non-denaturating polyacrylamide gels using 0.25× TBE. The gels were then dried and subjected to autoradiography.

3. Results

In the present work, we initially determined the relative activities of the −1819 to −271 bp of the hIR promoter (wild type promoter), and that of a series of constructs of this promoter corresponding to the plasmids: phIR(−1819)-GL2, phIR(−1473)-GL2, phIR(−876)-GL2 and phIR(−577)-GL2. U-937 cells were transiently transfected with these plasmids. Luciferase determinations revealed that the basal promoter activity of the wild type promoter, considered as 100%, gradually decreased in the progressive deletions of this promoter. Thus, the fragment spanning up to −1473 bp showed 94% promoter efficiency and the fragment spanning up to −876 bp 67%. In addition, the fragment from −577 to −271 bp of the hIR promoter presented 17% promoter efficiency. These data of relative activity of each fragment are comparable to those described by other authors in transfection experiments using other cell lines [28–30]. The low efficiency of the last fragment correlates with the removal of the four GC boxes, located between −618 and −593 bp, which has already been shown to be important for the promoter activity of this gene [30,31]. In fact, the efficiency of this last fragment was similar to that of the promoterless pGL2-basic vector (around 15%). Thus, the promoter region −618 to −593 bp seems to be essential for promoter activity in U-937 cells and must be conserved. Accordingly, we only used the plasmids: phIR(−1819)-GL2, phIR(−1473)-GL2 and phIR(−876)-GL2 covering this particular region for subsequent transfections in U-937 cells.

After transfection with these plasmids, the cells were either untreated or treated for 24 h with 10^{-8} M 1,25D_3. Luciferase determinations revealed that while the promoter activity of the fragment up to −1819 bp was increased around 1.6-fold by 1,25D_3-treatment (in agreement with our previous findings) (Fig. 1), the activities of the progressive 5′ deletions of this fragment (up to −1473, and up to −876) were enhanced 1.7-, and 1.6-fold, respectively by treatment (Fig. 1). This suggests that the region from −876 to −271 bp of the hIR promoter might be sufficient for induction by 1,25D_3. In addition, these data indicate the potential presence of VDREs in this region.

To identify these putative VDREs, we first computer selected a *consensus* VDRE sequence from previous data derived from VDRE sequences in 1,25D_3-regulated genes described by several authors [4,6,7,14–18]. This *consensus* VDRE sequence (5′GGGTCANNGGGGGCA3′) was highly homologous to that of the human osteocalcin gene promoter VDRE [17]: (5′GGGTGAACGGGGGCA3′). Both had identical 3′ half-elements, and spacers, and presented a C instead of G in the 5′ half-element. We thus conducted a positive control of VDR binding by EMSA, using the natural sequence of this VDRE between −512 and −483 bp of the human osteocalcin gene promoter (5′TTGG**TGACTCA**CCGGG**TGA**ACG*G*GGGCATT3′) [17] as

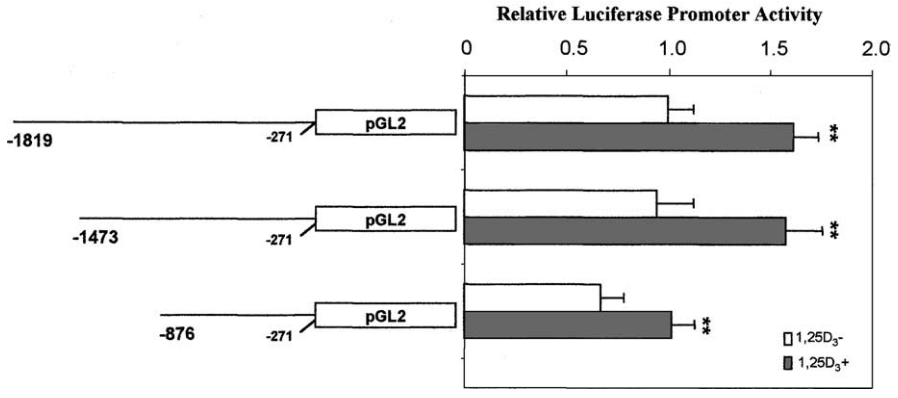

Fig. 1. Influence of 5′ deletions on stimulation of the human insulin receptor gene promoter by 1,25-dihydroxyvitamin D_3. U-937 cells were transiently transfected with the phIR(−1819)-GL2 plasmid which contains the fragment −1819 to −271 bp of the hIR promoter (wild type promoter), or with phIR(−1473)-GL2, or phIR(−876)-GL2 plasmids, which contain progressive 5′ deletions (up to −1473 and −876 bp, respectively) of the wild promoter. The transfected cells were left untreated (1,25D_3−), or treated for 24 h with 10^{-8} M 1,25D_3 (1,25D_3+). Luciferase activity is given in arbitrary units, relative to the value of 1 assigned to the wild promoter in untreated cells after correction for transfection efficiency. Values are means ± S.E.M. of at least seven transfections, $^{**}P < 0.01$.

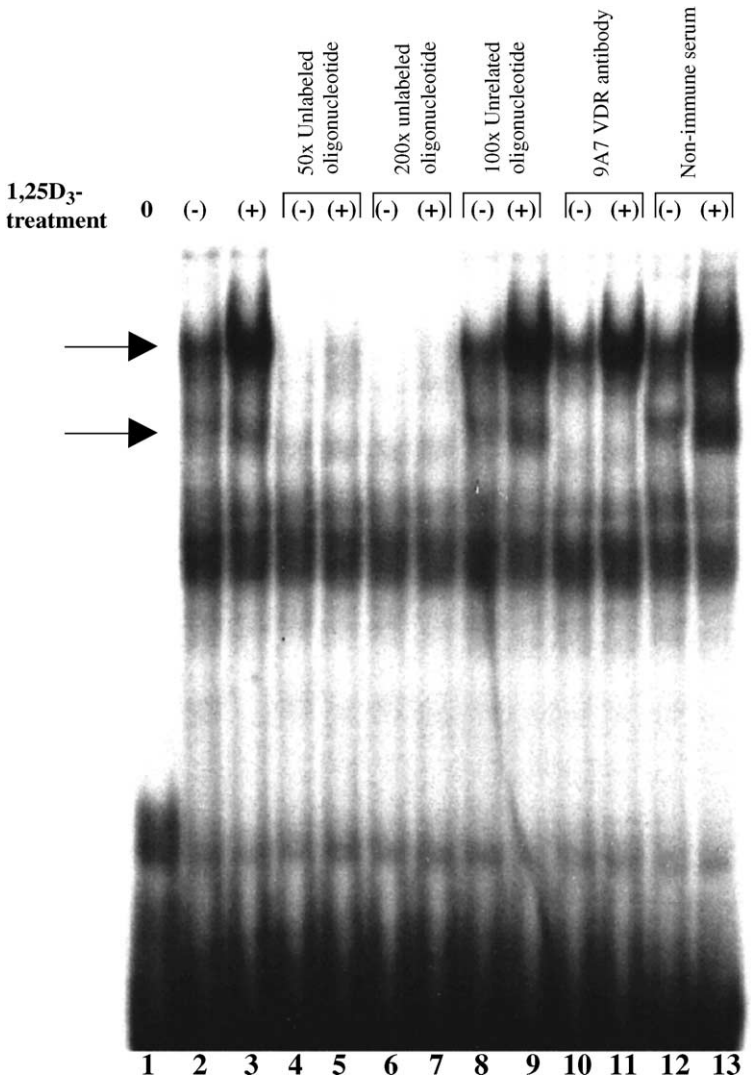

Fig. 2. Positive control of Vitamin D receptor binding by electrophoretic mobility shift assays. Nuclear extracts of U-937 cells, either untreated or treated for 24 h with 10^{-8} M 1,25-dihydroxyvitamin D_3, were incubated with the labeled −512 to −483 bp sequence of the human osteocalcin gene promoter as oligoprobe. This sequence contains a Vitamin D response element and an AP-1 like sequence immediately upstream of the VDRE. Lane 1, no nuclear extracts; lane 2, nuclear extracts from untreated cells; lane 3, nuclear extracts from treated cells; lanes 4 and 5, a 50× excess of unlabeled oligonucleotide; lanes 6 and 7, a 200× excess of unlabeled oligonucleotide; lanes 8 and 9, a 100× excess of unrelated oligonucleotide; lanes 10 and 11, 1 μl of the anti-VDR monoclonal antibody 9A7; lanes 12 and 13, 1 μl of non-immune serum. Arrows indicate a VDR complex (lower arrow) and an AP-1 complex (upper arrow).

an oligoprobe, along with nuclear extracts of both untreated and 1,25D_3-treated cells. This sequence includes two AP-1 like sequences, one immediately upstream from the 5′ half-element [17], and the other located between the two half elements [23]. Consistently with previous reports [21,23], we observed formation of two complexes (Fig. 2), an AP-1 complex (upper arrow) and a VDR complex (lower arrow). Hormone treatment enhanced the intensity of these complexes. The addition of excess (50 and 200×) unlabeled oligonucleotide abolished both complexes, while the addition of excess (100×) unrelated oligonucleotide was unable to compete with the complexes. The formation of the VDR complex was effectively blocked by the anti-VDR monoclonal antibody 9A7. Addition of a non-immune serum showed no effect on the VDR complex. Thus, as expected, this sequence was able to bind VDR in nuclear extracts of U-937 cells, confirming the viability of our methodology.

We next performed a computer search of candidate VDREs sequences by homology with the *consensus* VDRE sequence in the region extending from −876 to −271 bp of the hIR promoter. The results of our search indicated no sequence identical to this VDRE *consensus* in this region of the hIR promoter, and neither were any sequences found to show 1, 2, 3, or 4 base variations with respect to this *consensus* sequence. Nevertheless, we detected 10 sequences showing a difference of five bases from the *consensus* (Table 1). Comparison of the 3′ half-element of each of these sequences with the 3′ half-element of the

Table 1
Candidate VDRE sequences of the human insulin receptor gene promoter with five variations of the *consensus* VDRE sequence (5′GGGTCANNGGGGGCA3′)

−500	5′GGCCCGCACGGGGCC3′	−486
−607	5′CGGGACCGGGCGGCA3′	−593
−616	5′GGGGCGGGCGGGAC3′	−602
−620	5′GGGCGGGGCGGGGCG3′	−606
−625	5′GAGGCGGGCGGGGCG3′	−611
−678	5′CGGTCCCGGCGCGCC3′	−664
−712	5′GGGCTGTAGGGCGCG3′	−698
−722	5′AGGAGACTCGGGGCT3′	−708
−746	5′GCGCCTCCGGGGGTC3′	−732
−758	5′CGGGCCTGTGGGGCG3′	−744

These sequences were detected using the SEQFIND programme as indicated in Section 2.

VDRE *consensus* indicated no complete homology in each case. Nevertheless, the first five of the six making up the 3′ half-element were identical to those in the sequences: −500/−486, −620/−606, −625/−611, −722/−708 and −758/−744. Further, in sequences −620/−606, −625/−611 and −758/−744, the sixth base was purine yet in the remaining two sequences (−500/−486 and −722/−708) it was pyrimidine. Given that the substitution of purine by purine is spatially more favorable that purine by pyrimidine and that the presence of pyrimidine at position 6 of the 3′ half-element had been described in a VDRE that is susceptible to negative control by $1,25D_3$ [32], we decided to focus only on sequences −620/−606, −625/−611 and −758/−744, as potential VDREs.

When we explored the location of sequences −620/−606 and −625/−611 in the hIR promoter, both were found to be included inside the four GC boxes positioned from −618 to −593 bp of the hIR promoter. These boxes are binding sites for the transcription factor Sp1 [30,31]. EMSA analysis using as oligoprobe the hIR promoter sequence −633 to −604 bp (5′GAGGCGGGGAGGCGGGCGGGGCGGGGCGGG3′), which include both −620/−606 and −625/−611 sequences, yielded negative results as shown in Fig. 3.

The next step was to check for the possibility of *cis*-elements adjacent to the −758/−744 sequence in the hIR promoter using the TRANSFAC system [24]. We detected an overlapping downstream AP-2 like sequence. Positive results were obtained by conducting EMSA on nuclear extracts of untreated and $1,25D_3$-treated cells and the natural sequence extending from −761 to −732 bp of the hIR promoter (5′CGTCGGGCCTGTGGGG**CGCCTCCGGGGG**TC3′) including the overlapping AP-2 like sequence, as the oligoprobe. As shown in Fig. 4, a major complex was observed (arrow), the intensity of which was enhanced by hormone treatment. This complex was efficiently competed with the addition of (100×) unlabeled oligonucleotide. The complex was highly specific since it was cleared by 9A7, the specific anti-VDR antibody, while it was unaffected by a non-immune serum.

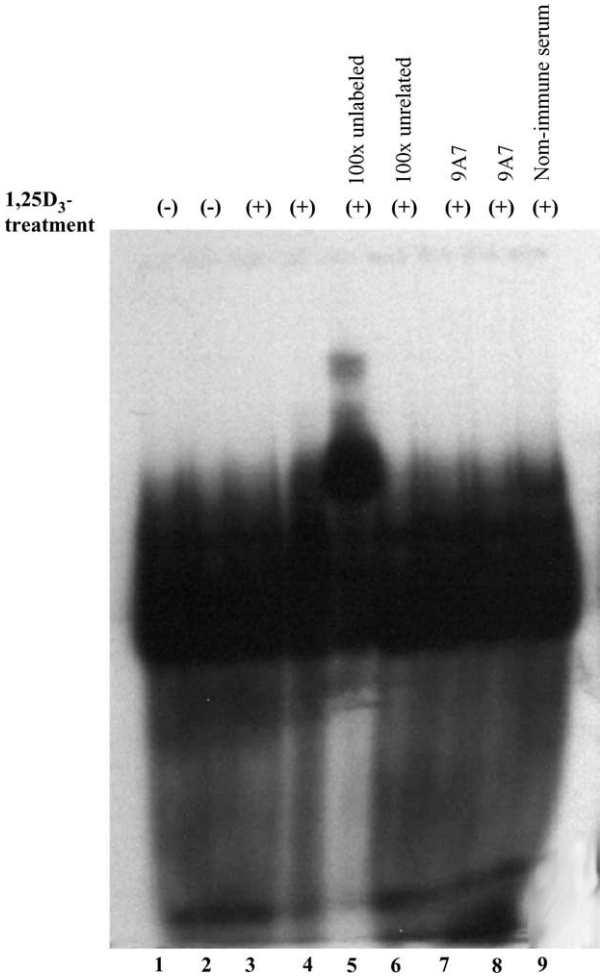

Fig. 3. Electrophoretic mobility shift assays using the labeled sequence of the human insulin receptor gene promoter from −633 to −604 bp as oligoprobe, and nuclear extracts of U-937 cells, untreated or treated for 24 h with 10^{-8} M 1,25-dihydroxyvitamin D_3. This sequence contains two overlapping sequences whose first five bases of the 3′ half-element are identical to those of the 3′ half-element of a *consensus* VDRE sequence deduced by us from previously reported functional VDRE sequences in other $1,25D_3$-regulated genes. Lanes 1 and 2, nuclear extracts from untreated cells; lanes 3 and 4, nuclear extracts from treated cells; lane 5, a 100× excess of unlabeled oligonucleotide; lane 6, a 100× excess of unrelated oligonucleotide; lanes 7 and 8, 2 and 5 μl, respectively of the anti-VDR monoclonal antibody 9A7; lane 9, 2 μl of non-immune serum.

Moreover, slight competition for the complex was shown after adding an excess (50×) of a partially related oligonucleotide, the VDRE of the human osteocalcin gene promoter [17]. This oligonucleotide only differs in that it contains an A instead of a G in the last position of the 3′ half-element.

4. Discussion

Our research group is presently engaged in exploring hIR gene expression regulation by different hormones, in particular, steroid hormones such as glucocorticoids [11,33],

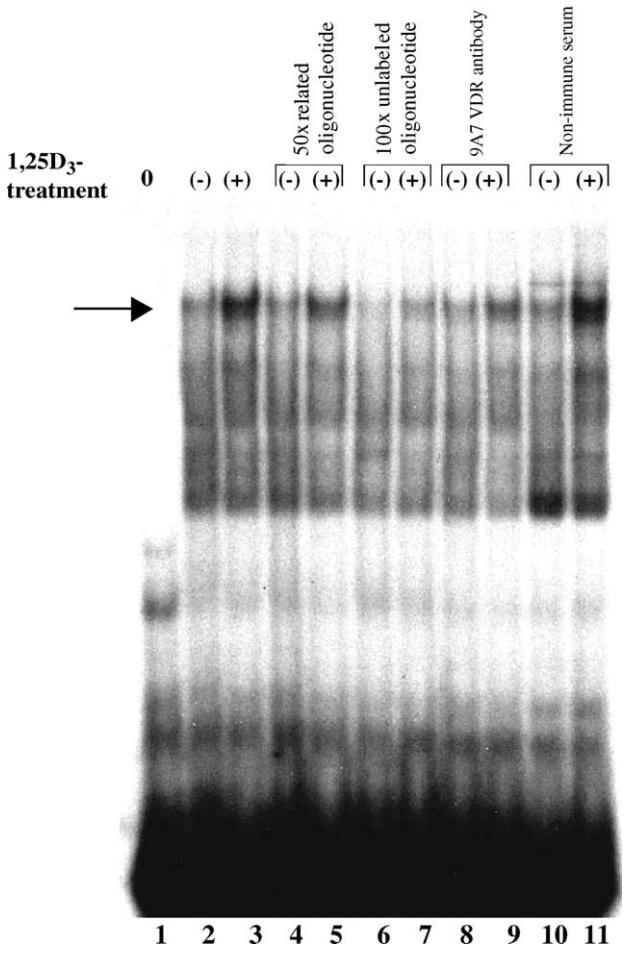

Fig. 4. Electrophoretic mobility shift assays using the labeled sequence, −761 to −732 bp, of the human insulin receptor gene promoter as oligoprobe, and nuclear extracts of U-937 cells, untreated or treated for 24 h with 10^{-8} M 1,25-dihydroxyvitamin D_3. This sequence contains a putative VDRE and an overlapping downstream AP-2 like sequence. Lane 1, no nuclear extracts; lane 2, nuclear extracts from untreated cells; lane 3, nuclear extracts from treated cells; lanes 4 and 5, a 50× excess of a partially related oligonucleotide; lanes 6 and 7, a 100× excess of unlabeled oligonucleotide; lanes 8 and 9, 2 μl of the anti-VDR monoclonal antibody 9A7; lanes 10 and 11, 2 μl of non-immune serum. Arrow indicates a VDR complex.

mineralocorticoids [34,35], estrogens [36] and $1,25D_3$ [8–10] in U-937 human promonocytic cells.

Concerning $1,25D_3$, we were previously able to demonstrate that the capacity of this hormone to potenciate hIR expression and insulin action in U-937 cells was transcriptionally regulated [10]. This was tested by transiently transfecting U-937 cells with the reporter plasmid phIR(−1819)-GL2, which contains the −1819 to −271 bp fragment of the hIR promoter (wild type promoter) linked to the luciferase pGL2-basic vector. We observed that treatment of these transfected cells with $1,25D_3$ induced an approximately 1.8-fold increase in the activity of this promoter. This range of induction was consistent with the 1.8-fold increase previously reported by us in both hIR gene expression and IR number [8,9] and with the enhanced cellular responsiveness to insulin in terms of glucose transport (1.3-fold increase) and glucose oxidation (1.6-fold increase) induced by $1,25D_3$treatment [9,10].

In agreement with our previous findings, here we observed that $1,25D_3$ provoked a 1.6-fold increase in the activity of the wild hIR promoter (Fig. 1). In addition, we noted similar $1,25D_3$ induction of the activity shown by sequential 5′ deletions of this promoter. Thus, the fragment spanning up to −1473 bp showed a 1.7-fold increase in activity, and the fragment spanning up to −876 bp was related to an induction of around 1.6-fold (Fig. 1). These data suggest that the region from −876 to −271 bp of the hIR gene promoter might be sufficient to account for this induction by $1,25D_3$. In addition, our data suggest the presence of potential VDREs in this region.

The −876 to −271 bp region is somewhat larger than that suggested by others (from −692 to −345 bp) to contain sufficient information for efficient transcription of the hIR gene [28,29]. This region includes a cluster of four GC boxes (from −618 to −593 bp) that are binding sites for the transcription factor Sp1 [30,31]. Further, the fragment denoted Fragment-C2 (from −874 to −674 bp), which appears to be essential for the expression of the IR gene during differentiation from myoblasts to myocytes in BC3H-1 cells [37], is also in this region, as are four putative glucocorticoid response elements [28,38]; one proximal (from −363 to −341 bp) and three central (from −679 to −654 bp) (from −706 to 688 bp) and (from −754 to −725 bp). Moreover, using the TRANSFAC system [24], we were also able to identify a potential binding site for the AP-1 factor (from −877 to −867 bp), and three potential binding sites for factor AP-2, (from −436 to −425 bp) (from −444 to −433 bp) and (from −745 to −734 bp), in this same region. The first two AP-2 like sites coincide with a cluster of three GC boxes located between −436 and −400 bp of the hIR promoter. This cluster appears to be non-functional in vivo [28,30].

In an effort to identify the presence of VDREs in this −876 to −271 bp region, we first performed a computer search of candidate VDREs sequences by homology with a *consensus* VDRE sequence (5′GGGTCANNGGGGGCA3′), which we compiled from data cited by authors reporting functional VDREs in various genes [4,6,7,14–18]. This *consensus* sequence was slightly different to that (5′PuGGTCANNPuPuGTTCA3′) proposed by Colnot et al. [6], and Haussler et al. [4]. Our *consensus* and the latter have identical 5′ half-elements, but ours contains two GG instead two TT, in the 3′ half-element. The spacers are identical, with G at position 3, which appears to be important in VDR binding.

Computer analysis failed to reveal any sequence homologous with our VDRE *consensus* or sequences with 1, 2, 3, or 4 variations with respect to this *consensus*. Nevertheless, we detected 10 candidate VDREs sequences with five variations of this *consensus* in the region under study (Table 1). Of these, only the −758/−744 bp sequence (5′CGGGCCTGTGGGGCG3′)

together with the overlapping AP-2 like sequence (5′CGTCGGGCCTGTGGGG**CGCCTCCGGGGG**TC3′), which contains the well-characterized GCC N3 GGG motif [39], proved to be a binding site for VDR when probed in EMSA (Fig. 4).

The VDRE site of this sequence has a G in the second position of each half-site, which had been described as the target of a side-chain contact by a conserved arginine residue within the recognition alpha helix of the DNA-binding domain of the VDR [40]. Moreover, the other G may also establish contacts with the VDR [6], emphasizing the physiological significance of this residue. VDR binds this site sharing 2 bp with the AP-2 site. Possible cross-talk between the two transfactors and their stabilization requirements will need to be clarified in further studies, but the fact that a unique complex was detected by EMSA lends support for the possibility that the VDR–DNA complex includes the AP-2 site.

Among other described cases of adjacent *cis*-elements and their associated transfactors that appear to modulate transcriptional activation by $1,25D_3$, the cooperation of a VDRE with an adjacent AP-2 like sequence has been described, although this case, involving $1,25D_3$ regulation of the rat 25-hydroxyvitamin D_3 24-hydroxylase gene [15,20], differs from ours. In this example, two VDREs separated by approximately 100 bp plus the cooperation of an adjacent downstream AP-2 like sequence separated by only 4 bp of the proximal VDRE, are all required for full transcriptional activation of this gene. The two VDREs act in an additive manner and not synergistically. The proximal VDRE appears to be stronger than the distal one due to the presence of the accessory element. The VDR fails to bind the AP-2 like sequence and the Vitamin D effect is not mediated via this site [15,20]. A further case, in which an apparent cooperation had not yet been explored, is the transcriptional activation of the Cdk inhibitor p21 by $1,25D_3$ [41], and AP-2 [42]. $1,25D_3$ specifically binds a VDRE located between −779 and −764 bp of the p21 promoter and induces differentiation, while AP-2 specifically binds to a region that spans −121 to −95 bp of this promoter, negatively regulating cell-cycle progression.

In conclusion, as far as we are aware, ours is the first report of the identification of a VDRE in the hIR gene promoter that could account for the transcriptional induction of this gene by $1,25D_3$ detected in U-937 cells. This VDRE is overlapped by a downstream AP-2 sequence. Together, these *cis*-elements may form a locus that can respond to $1,25D_3$ via activation of VDR. This locus could mediate cross-talk between Vitamin D and insulin signalling pathways in U-937 cells.

Acknowledgements

The authors are indebted to Drs. S.Y. Tsai and G. Elberg for their generous gift of the hIR promoter. B. Maestro holds a fellowship at the Complutense University of Madrid.

This work was supported by research funds from the DGES, grant (BMC2000/0765); and the CAM, grant (08.6/0010/98).

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