Pergamon

PII: S0960-0760(98)00055-7

J. Steroid Biochem. Molec. Biol. Vol. 66, No. 5–6, pp. 263–269, 1998

Printed in Great Britain
0960-0760/98 $19.00 + 0.00

Direct Repeat 3-Type Element Lacking the Ability to Bind to the Vitamin D Receptor Enhances the Function of a Vitamin D-responsive Element

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In a previous study, we identified the element which allows the maximum response to $1,25(OH)_2D_3$ in concert with two vitamin D-responsive elements (VDREs) in the rat 25-hydroxyvitamin D_3 24-hydroxylase gene promoter, and designated it an accessory element [Ohyama, Y., Ozono, K., Uchida, M., Yoshimura, M., Shinki, T., Suda, T. and Yamamoto, O. Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D_3 24-hydroxylase gene. *J. Biol. Chem.*, 1996, 271, 30381–30385]. The accessory element located adjacent to the proximal VDRE is not capable of binding to the vitamin D receptor (VDR), while its nucleotide sequence resembles the consensus sequence of VDREs, direct repeat 3 (DR3). To clarify the difference between the accessory element and VDREs, the function of the accessory element was compared with that of VDREs. The mutated accessory elements with a single nucleotide substitution showed the capability of binding to the VDR *in vitro*. However, these mutants still did not act as a VDRE when driven by the heterologous SV40 promoter. The accessory element did not enhance the function of a cAMP-responsive element. The corresponding site of the accessory element in the human 24-hydroxylase is a DR4-type element, and this element did not function as an accessory element. These results indicate that a critical nucleotide sequence is necessary for the binding to the VDR and for mediating the vitamin D effect, and suggest the different regulation between the rat and human 24-hydroxylase gene.

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INTRODUCTION

The vitamin D receptor (VDR) belongs to the nuclear hormone receptor superfamily, and modulates the transcription of target genes in response to 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] by binding to an enhancer designated as a vitamin D-responsive element (VDRE) [1–4]. The VDR forms a heterodimer with retinoid X receptor (RXR) to bind to VDREs with high affinity [5–7]. RXR also heterodimerizes with other nuclear hormone receptors including thyroid hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor and NGFI-B, leading to the concept that these receptors form a subfamily, the RXR heterodimer family [8]. The potent hormone-responsive elements of the receptors belonging to this subfamily consist of a direct repeat (DR) of a hexameric AGGTCA half-site in most cases, while single half site can be the binding site of the receptors such as NGFI-B [9, 10]. The structure of VDREs is also found to be a direct repeat of half-sites with a 3-base pair intervening nucleotide (DR3) following the identification of several target genes [11–14]. The length of the spacer is reported to provide the specificity of the response elements [4, 15].

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Received 24 Oct. 1997; accepted 20 Feb. 1998.

25-Hydroxyvitamin D_3 24-hydroxylase is a key enzyme of vitamin D metabolism which hydroxylates 25-hydroxyvitamin D_3 and $1,25(OH)_2D_3$ at the position of C-24, and its activity is markedly induced by $1,25(OH)_2D_3$ [16]. Two vitamin D-responsive elements (VDREs) were recently identified in the rat 24-hydroxylase gene promoter [17–21]. Their structures are also consistent with DR3. The rat 24-hydroxylase gene is the first example which is reported to have two VDREs in its promoter.

VDREs mediate the effect of $1,25(OH)_2D_3$ by binding to VDR. In several target genes, however, the response to $1,25(OH)_2D_3$ is enhanced by DNA sequences flanking a VDRE without binding to the VDR. In the human osteocalcin gene, for example, an AP-1 site adjacent to the VDRE has a synergistic effect on the transactivational function of $1,25(OH)_2D_3$ [11]. In the rat osteocalcin gene, the DNA sequence GGTTTGG downstream from the VDRE is required for the maximal response to $1,25(OH)_2D_3$, although the transcriptional factor involved in this effect has not been determined [22].

The rat 24-hydroxylase gene promoter contains several degenerative half-site motifs in addition to two VDREs [21]. The contribution of half-site motifs (CGGTCA, AGGCCC and GAGTCA) other than authentic VDREs to the maximal promoter activity has been pointed out [20, 21]. We have also found that a DR3-type element juxtaposed to the proximal VDRE, which we named the accessory element, enhances the vitamin D-dependent activation through the VDRE. The sequence homology between the accessory element and VDREs are high, but the VDR failed to bind to the accessory element [21].

The promoter of the human 24-hydroxylase gene has also recently been reported [23]. The nucleotide sequence is nearly identical between the rat and human 24-hydroxylase, especially in VDREs, but the nucleotide sequence corresponding to the accessory element of the rat gene is the DR4-type in the human genes. The difference of nucleotide sequences raises the question whether the DR4 element in the human genes enhances the VDRE function.

In this study, we further investigated the differences between the accessory element and VDREs in the rat 24-hydroxylase gene promoter in terms of binding and transactivation character, and we also compared the function of the accessory element with that of the DR4-type element found in the human 24-hydroxylase gene.

MATERIALS AND METHODS

Cell culture and transfection

Human osteoblastic cells (Saos-2) were maintained in Dulbecco's Modified Eagle Medium (Nikken Bio-Medical, Kyoto) supplemented with 10% dextran-coated charcoal-treated fetal calf serum (Gibco BRL, Grand Island, NY). Transfection of 2.5 μg subjective plasmid and 1 μg pSG5hVDR, the expression vector of the human VDR cDNA (a gift of Dr. M. R. Haussler, the University of Arizona) per 1×10^6 cells was achieved by the DEAE-dextran method. Co-transfected β-galactosidase expression vector served as the internal control. The cells were incubated with 10^{-8} M $1,25(OH)_2D_3$ (Wako, Osaka) for 2 days. Each set of experiments was repeated at least three times, and the results are presented in terms of fold induction as means $\pm$ SD.

Plasmid construct and transcription activation assay

Synthetic oligonucleotides corresponding to the VDRE1, Acc E, 1A and 4A (each sequence is summarized in Table 1) were annealed and inserted into a luciferase reporter vector pGV-P2 containing SV40 promoter (Toyo Ink, Tokyo). The CRE (tgtTGACGTCAcgc) alone or fused to the accessory element (AE-CRE) was inserted into pGV-P2. The region (−367/−57) of the human 24-hydroxylase gene promoter was amplified by polymerase chain reaction (PCR) using primers (5′-ATTGTGCA-AGCGCCGGCGGCAA and 5′-AATGAGGCCAC AGAGGAGG) and subcloned into pT_7-Blue T-vector (Novagen, Madison, WI). Then, the vector was switched to pGV-P2. Each mutation at VDRE1, VDRE2 or the elements corresponding to the rat Acc E (hAE) was generated by PCR using primers carrying the mutated nucleotides (hVDRE1Mu:CGCttT-CGCTCACCT, hVDRE2Mu: CACACCCGGTG-ttCT, hAEMU:CGGTCACCCCAaaCCC). The DNA sequences of these plasmids were confirmed using an ABI 373A DNA sequencer (PE Applied

Table 1. Sequence comparison between several VDREs, the accessory element and mutated accessory elements

Elements in genes	Nucleotide sequences
Human osteocalcin VDRE	GGGTGA acg GGGGCG
Rat osteocalcin VDRE	GGGTGA atg AGGACA
Mouse osteopontin VDRE	GGTTCA cga GGTTCA
Rat 24 hydroxylase VDRE1	AGGTGA gtg AGGGCG
Human 24 hydroxylase VDRE1	AGGTGA gcg AGGGCG
Rat 24 hydroxylase VDRE2	GGTTCA gcg GGTGCG
Human 24 hydroxylase VDRE2	AGTTCA ccg GGTGTG
DR3	AGGTCA nnn AGGTCA
Rat 24 hydroxylase accessory element	CGGTCA ccg AGGCCC
Mutated Acc E(1A)	AGGTCA ccg AGGCCC
Mutated Acc E(4A)	CGGTCA ccg AGGACC

The nucleotide sequences of several VDREs including those in human osteocalcin (11), rat osteocalcin (14), mouse osteopontin (12) and rat 24-hydroxylase (proximal VDRE:VDRE1, distal VDRE:VDRE2) (17, 18) are given. The DR3 element was originally reported by Umesono et al. (15). The sequence comparison between the accessory element and VDREs prompted us to mutate the first and 4th C in the proximal and distal half-sites, respectively, to A (both are underlined) in an attempt to convert the Acc E to a VDRE.

Biosystems, Tokyo). The luciferase activities of the cell lysates were measured with luciferase assay kit (Toyo Ink) according to the manufacturer's manual. Transactivation measured by luciferase activities was standardized by the galactosidase activities of the same cells determined by a β-galactosidase enzyme assay system (Promega, Madison, WI).

Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay was basically performed as previously described [8]. The oligonucleotides indicated in the figure were labeled with [α-^{32}P] dCTP (DuPont NEN, Boston, MA) by a fill-in reaction using Klenow enzyme (New England Biolabos, Beverly, MA). The VDR and RXRα utilized for this assay were obtained from the purification of a bacterial expressed histidine-tagged system (Novagen) [35]. The reaction mixture was electrophoresed on a 5% polyacrylamide gel and visualized by BAS2000 (Fujix, Tokyo).

RESULTS

Binding of VDR/RXR heterodimer to mutated Acc Es

The nucleotide sequence of the accessory element (Acc E) is similar to that of the VDREs, but two cytosines (C) at the 1st and 4th position in the upstream and downstream half-sites, respectively, seem to be deviated from the consensus sequence of VDREs (Table 1). Thus, each C was converted to adenine (A) and designated as the mutated accessory elements 1A and 4A, respectively (Table 1). First, the binding of the heterodimer formed by VDR and RXRα to various element including the Acc E and mutated elements (1A and 4A) was investigated in a electrophoretic mobility shift assay (EMSA). As reported before, the recombinant human VDR in combination with recombinant human RXRα formed the complex when the VDRE1 was used as a probe. However, no shift band was observed when the Acc E was used as a probe in the EMSA (Fig. 1). The binding of the VDR/RXRα heterodimer to 1A was also detected in the EMSA, and a very faint retarded band was seen when the 4A element was used as a probe (Fig. 1). To confirm these data, the competition analysis was performed using an excess amount of non-radiolabeled elements in the EMSA where the VDRE1 was used as a probe. In concert with the data shown in Fig. 1, the unlabeled VDRE1, 1A and 4A competed with the complex band efficiently, in that order, while the Acc E did not compete (Fig. 2).

Failure in response of mutated Acc Es to $1,25(OH)_2D_3$

In our system, VDRE1 mediates the vitamin D effect remarkably when fused to the Acc E, as reported previously [21]. In contrast to the data obtained in the VDR binding assay, both mutated Acc Es (1A and 4A) did not respond to 10^{-8} M $1,25(OH)_2D_3$ when transfected into Saos-2 cells in the context of the SV40 promoter-driven luciferase reporter vector (Fig. 3). Two copies of Acc E (AE-AE) did not mediate the $1,25(OH)_2D_3$ function either, confirming that Acc E does not work as a VDRE (Fig. 3).

To investigate the enhancing effect of the Acc E on response elements other than VDREs, the Acc E fused to the cAMP-responsive element (CRE) was examined. The CRE mediated the 8-bromo cAMP effect by 2.4-fold, but the response was not significantly changed when CRE was fused to the Acc E (2.1-fold). The Acc E did not enhance the effect of cAMP on the CRE (Fig. 4).

No apparent Acc E in the human 24-hydroxylase gene

The nucleotide sequence flanking the VDRE1 in the rat 24-hydroxylase is very similar to that in the human gene. Interestingly, the nucleotides in the human gene which corresponding to the Acc E in the rat gene are structurally the DR4-type, CGGTCA cccc AGGCCC [Fig. 5(A)].

Mutated VDRE1 in the human 24-hydroxylase markedly reduced the responsiveness to $1,25(OH)_2D_3$, similar to mutated VDRE2 fused to heterologous promoter. The introduction of mutation into the DR4 element slightly reduced the fold induction, suggesting that the DR4 element did not work well as an accessory element [Fig. 5(B)].

Whether the DR4-type element found in the human 24-hydroxylase gene promoter fused to the

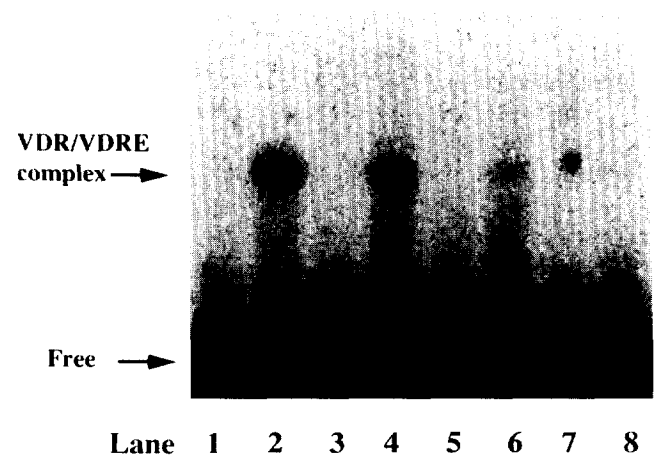

Fig. 1. The binding of the *E. coli*-derived recombinant human VDR with or without human RXRα to VDRE1, 1A, 4A and Acc E was examined in EMSA. The binding of the VDR and RXRα to VDRE1 and 1A and weak binding of them to 4A were observed, while no complex was detected when the Acc E was used as a probe. Lanes 1–8: same amount of VDR; lanes 2, 4, 6 and 8: same amount of RXRα. Probe: lanes 1 and 2: VDRE1, lanes 3 and 4: 1A, lanes 5 and 6: 4A, lanes 7 and 8: Acc E.

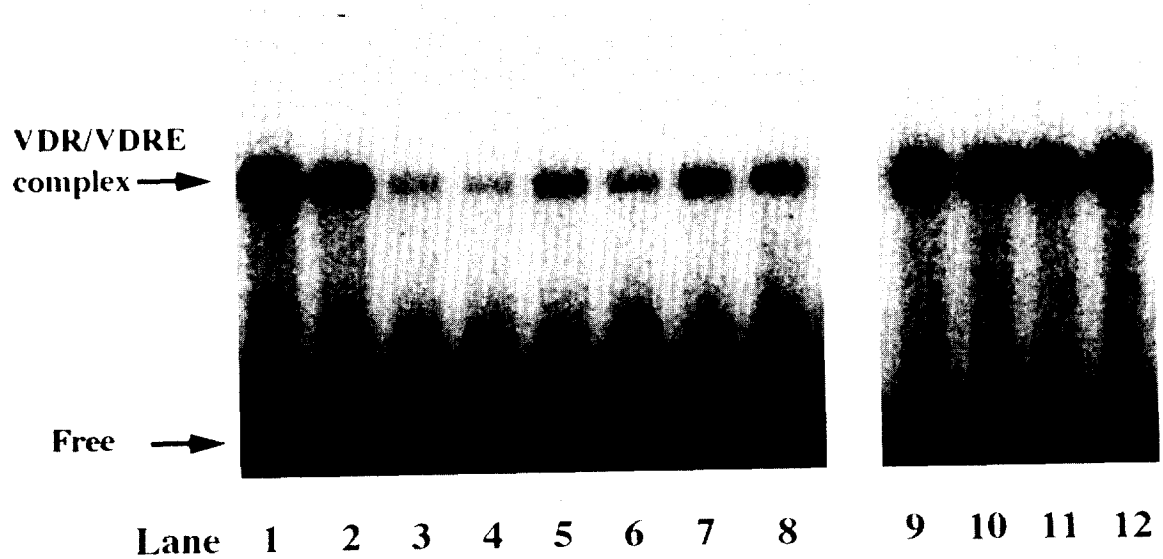

Fig. 2. The competition of cold oligomers corresponding to VDRE1, 1A and 4A was examined in an EMSA. The human VDR and RXRα were incubated with ^{32}P-labeled VDRE1 in all lanes. A 5-times higher amount of cold oligomers was added in lanes 3 and 4 (VDRE1), 5 and 6 (1A) and 7 and 8 (4A) (lanes 1 and 2; no competition). The VDR/RXR/VDRE1 complex band was made faint by the excess cold oligomers (VDRE, 1A and 4A) in that order. In contrast, cold excess oligomer carrying the Acc E sequence (lane 11 and 12) did not compete the VDR/RXR/VDRE1 complex (lane 9 and 10 as a control).

VDRE1 mediates the vitamin D effect was examined, and the results were compared with those of the DR3-type Acc E. As shown in Fig. 6, the DR4-type element was not active in enhancing the function of VDRE1.

DISCUSSION

The rat and human 24-hydroxylase genes, exclusively to date, are known to contain two VDREs in the promoter region. However, two VDREs are not sufficient to achieve the maximum response to $1,25(OH)_2D_3$ in the rat 24-hydroxylase gene. Another DR3-type element, designated the Acc E, is necessary as reported previously [21]. The Acc E has a nucleotide sequence very similar to that of VDREs, but does not bind to the VDR. The difference between the Acc E and VDREs was investigated in this study. The substitution of only one nucleotide in the Acc E (1A and 4A, Table 1) was sufficient to

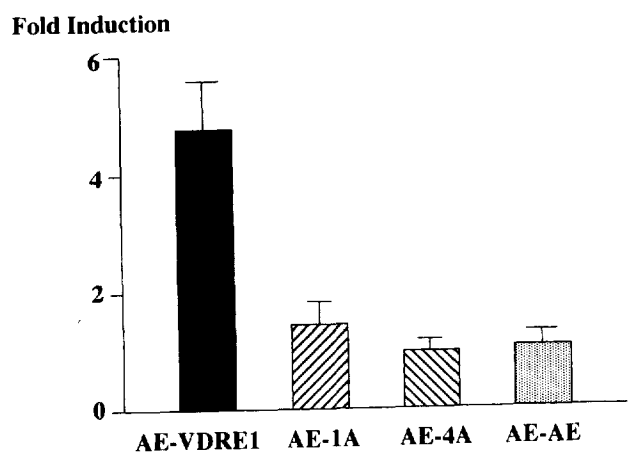

Fig. 3. The response of four elements (AE-VDRE1, AE-1A, AE-4A and 2 copies of AE; each element fused to the accessory element) inserted in the SV40 promoter-driven luciferase vector to 10^{-8} M $1,25(OH)_2D_3$ was analyzed in Saos-2 cells. In contrast to the apparent response of AE-VDRE1, AE-1A, AE-4A and AE-AE did not mediate the $1,25(OH)_2D_3$ action.

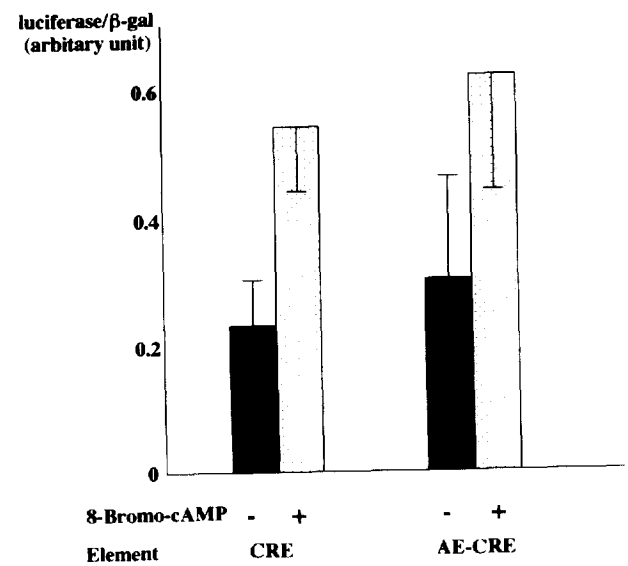

Fig. 4. The response to 0.5 mM 8-bromo cAMP was examined in CRE and AE-CRE (the cyclic AMP response element fused to the accessory element) in Saos-2 cells. 8-bromo cAMP clearly enhanced the luciferase activity through both CRE and AE-CRE, but no significant difference was observed between them.

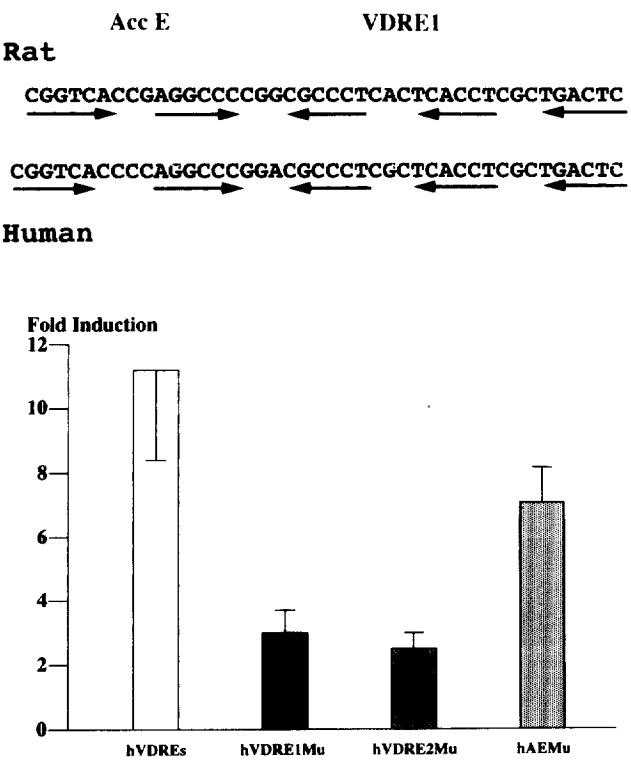

Fig. 5. (A) Sequence comparison between the rat and human 24-hydroxylase gene around the region containing VDRE1 and the Acc E. The Acc E in the rat gene corresponds to the DR4-type element in the human 24-hydroxylase gene. (B) The fragment (−367/ − 57) of the human 24-hydroxylase gene inserted in the SV40 promoter-driven luciferase vector (hVDREs) was examined in terms of $1,25(OH)_2D_3$ inducibility. Three mutants possessing a mutation at the proximal VDRE (hVDRE1Mu), the distal VDRE (hVDRE2Mu) and the DR4-type element (hAEMu) were also examined. The hVDREs responded to 10^{-8} M $1,25(OH)_2D_3$ by 11.4-fold in Saos-2 cells, but the introduction of a mutation at either VDRE1 or VDRE2 significantly decreased the response. The response of hAEMu to 10^{-8} M $1,25(OH)_2D_3$ was slightly decreased ($p < 0.05$), but was still a good response compared to those of hVDRE1Mu and hVDRE2Mu.

convert the Acc E to a VDR-binding site. These data suggest that the adenine (or guanine) at the first position and adenine (or thymine) at the fourth position of the half-site is required for the VDR to bind to DNA.

In contrast to the results of binding to the VDR, neither 1A nor 4A fused to the Acc E (AE-1A and AE-4A) mediated the $1,25(OH)_2D_3$-dependent transcription activation. Therefore, these substitutions were still not enough for the elements to function as an active VDRE, at least in the heterologous promoter tested in this study. In addition, these data confirmed that the Acc E enhances the response of a VDRE to $1,25(OH)_2D_3$ in a manner distinct from that of the just increased copy numbers of the VDREs. The reason why these mutated elements, 1A and 4A, are unable to mediate the $1,25(OH)_2D_3$ effect must be considered. The low affinity of these elements to the VDR binding, leading to no detection of a VDR/RXR/DNA complex band in the EMSA is one possibility. To activate the transcription, the VDR requires the interaction of coactivators, as do other nuclear hormone receptors [24–28]. The interaction of the VDR with coactivators might be impaired in the mutated Acc E. The discrepancy between receptor binding and transcription activation is also reported in LXR, and suggested that flanking nucleotides are important for transactivation [29].

The significance of the DR3 structure of the Acc E was supported by the mutational analysis where the mutation at each half-site, not an intervening site, lost the enhancing activity [21], and by the result that the DR4 element in the human gene exhibited no apparent enhancing effect (Fig. 6). This conclusion is also supported by the data that the proximal and distal VDREs worked as VDREs almost equally in the human 24-hydroxylase gene [Fig. 5(B)], while the VDRE1 adjacent to the Acc E contributes more to $1,25(OH)_2D_3$ responsiveness in the rat 24-hydroxylase gene [21].

The mechanism whereby the Acc E enhances the hormone-inducibility remains to be elucidated. In our preliminary experiment, specific binding distinct from the VDR to this element was detected using a nuclear extract from the human osteoblastic cell line MG63 cells or HeLa cells in an EMSA, but the identification of the factor(s) is still underway (data not shown). The requirement of other DNA element for maximum transactivation has recently been reported in the rat osteocalcin gene by Sneddon *et al.* [22]. This element does not resemble the Acc E described in the present report. The elements are quite different in

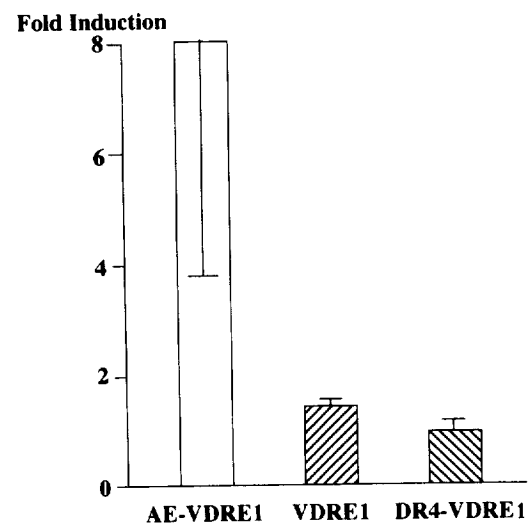

Fig. 6. The DR3-type Acc E and the DR4-type element found in the rat and human 24-hydroxylase genes, respectively, were each fused to the VDRE1 of the rat 24-hydroxylase gene inserted in the SV40 promoter-driven luciferase vector, and these plasmids were examined in terms of the response to $1,25(OH)_2D_3$. In contrast to the Acc E, the DR4-type element did not enhance $1,25(OH)_2D_3$ effect mediated by VDRE-1.

their sequences, and functionally the Acc E did not enhance the mediating effect of CRE (Fig. 4), in contrast to the enhancing effect of the element in the rat osteocalcin promoter on the response of CRE [22]. Sneddon *et al.* suggested that the transcription factor which they did not identified mediates the effect. Although further study is necessary, it is likely that the Acc E is a binding site of a certain transcription factor and specifically enhances the effect of a VDRE.

The requirement of elements other than VDRE for the maximum response is not unique in the rat 24-hydroxylase. In the human osteocalcin promoter, an AP-1 site adjacent to a VDRE allows the promoter to respond to $1,25(OH)_2D_3$ intensively [8]. The enhancement or synergism between a VDRE and other transcription factors including SpI and Oct-1 were also reported in the heterologous promoter [30].

Requirements for the maximum response to a nuclear hormone have also been reported in other transcription factors belonging to the nuclear hormone receptor superfamily. For example, the strong androgen induction of the mouse sex-limited protein gene is reported to require an androgen response element as well as several sites to which Oct-1 can bind [31]. The hepatic phosphoenolpyruvate carboxykinase (PEPCK) gene has a complex glucocorticoid response unit consisting of a tandem array of two accessory factor-binding sites (AF1 and AF2) and two adjacent glucocorticoid receptor (GR)-binding sites [32]. AF1 and AF2 do not function as glucocorticoid response elements by themselves, but the mutation of either AF1 or AF2 results in a 50–70% reduction in the stimulatory effect of glucocorticoid. Hepatic nuclear factors 4 and 3 are recently identified as factors which bind to AF1 and AF2, respectively [33]. Ligand-dependent transactivation by TR and RAR is markedly potentiated by the hematopoietic cell-specific bZip protein p45/NF-E2 [34]. CBP is reported to mediate the positive cross-talk between them. Therefore, the elucidation of the cross-talk between receptors and other transcription factors is necessary to understand the mechanism of transcription mediated by nuclear hormone receptors.

In conclusion, we further characterized the accessory element (Acc E) in the rat 24-hydroxylase gene. This specificity of the structure of Acc E may be a key to identify the interacting factor, although the transcriptional factor which binds to the DR3-type element is not yet known, except for the VDR [29].

Acknowledgements—We are grateful to Miss Tomoko Hayashi and Mrs. Noriko Tsuda for their technical assistance. We thank Miss Satsuki Nozu for helping to prepare this manuscript.

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