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Functional characterization of a natural retinoic acid responsive element

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Retinoic acid receptor (RAR) and thyroid hormone receptor (T_3R) are thought to bind as dimers to a T_3 responsive element (T_3REpal) comprised of inverted repeats of the half-site motif GGTCA. However, a RA responsive element (βRARE) was previously identified in the promoter of the RARβ2 gene which contains two direct repeats of the motif GTTCA spaced by a six nucleotide gap. We now demonstrate the ability of RARα, β and γ to bind to and transactivate through this element and that the two direct repeats comprise the βRARE. Surprisingly, the GTTCA motifs rearranged to form a palindrome do not confer RA responsiveness to a heterologous promoter. Furthermore, no significant level of transactivation is detected by ligand-activated RAR through the Moloney murine leukaemia virus T_3RE, which comprises two direct repeats of the sequence GGTCA/C spaced by a five nucleotide gap. Similarly, T_3R does not induce gene expression through the βRARE. This study establishes the preference of T_3R to transactivate through direct repeats spaced by a five nucleotide gap as opposed to a six nucleotide gap. In contrast, RAR appears to be more flexible with respect to spacing requirements between repeats, although higher levels of transactivation are obtained through direct repeats spaced by a six nucleotide gap. Interestingly, although some elements mediate either RA or T_3 induction, changing a single nucleotide in the MoMLV T_3RE with a five nucleotide spacing creates a promiscuous RA/T_3 responsive element.

Key words: retinoic acid receptor/retinoic acid responsive element/thyroid hormone receptor

Introduction

Retinoic acid (RA) exerts a wide variety of effects in biological systems (Roberts and Sporn, 1984; Summerbell and Maden, 1990). A specific role for RA during development has been suggested by studies on limb formation where it is thought to specify the anteroposterior pattern of vertebrate limbs (Eichele, 1989). In cell culture systems, RA can influence epithelial cell growth and differentiation (Espeseth *et al.*, 1989; Collins *et al.*, 1990) and down-regulate specific proto-oncogenes and growth factors (Miller *et al.*, 1990).

To date, three different retinoic acid receptors (RAR α, β and γ) have been found, both in human and mouse (Petkovich *et al.*, 1987; Giguere *et al.*, 1987; Benbrook *et al.*, 1988; Brand *et al.*, 1988; Krust *et al.*, 1989; Zelent *et al.*, 1989). RARs have been identified and characterized as members of the steroid and thyroid hormone receptor superfamily of nuclear transcription factors (for reviews see Evans, 1988; Green and Chambon, 1988; Beato, 1989). RARs, like other members of the steroid hormone receptor family, are composed of several functionally independent domains which show a high degree of amino acid sequence homology between the different receptors (Krust *et al.*, 1986). The highly conserved DNA binding domain is composed of 66 amino acids including conserved cysteine residues which are compatible with the formation of two so-called zinc fingers (Green and Chambon, 1987). The C-terminal region is structurally and functionally complex and appears to be involved in ligand binding, transactivation and dimerization. The domain located at the N-terminal part of the proteins is hypervariable in size and amino acid composition and may have a modulatory effect on receptor function by influencing transactivation.

The steroid and thyroid hormone receptor family regulates gene expression through binding to short *cis*-acting sequences in their respective target genes. The DNA binding domain of the receptors for RA and thyroid hormone (T_3) are very similar at the amino acid sequence level (Evans, 1988). Discrimination among specific DNA binding sites for glucocorticoid, estrogen and thyroid hormone receptors has been shown to be determined by three amino acids at the base of the first finger (P box), while the second element (D box) is apparently important for determining the half-site spacing (Umesono and Evans, 1989; Mader *et al.*, 1989). RAR and T_3R contain the same amino acids at the discriminatory positions in the P box (Forman and Samuels, 1990), therefore they should be able to recognize the same responsive elements. It has been shown that the RAR can activate gene expression from the thyroid hormone responsive element (T_3RE) of the rat growth hormone promoter (Umesono *et al.*, 1988). This promoter contains two copies of an imperfect inverted repeat of the motif GGTCA. Making this sequence a perfect palindrome (T_3REpal) increases both T_3 and RA responsiveness (Glass *et al.*, 1988; Umesono *et al.*, 1988).

Responsive elements for the RAR (βRARE) have been identified in the promoter region of the RARβ2 gene in humans (de Thé *et al.*, 1990) and in the mouse (Sucov *et al.*, 1990), both contain a perfect direct repeat of the motif GTTCA. Another RARE has been found upstream of the gene for the B1 subunit of the extracellular matrix protein laminin, which contains four directly repeated GGTCA-like motifs (Vasios *et al.*, 1989, 1991). A RARE which regulates transcription of the *ADH3* gene (which encodes the γ polypeptide subunit of the enzyme human class I alcohol dehydrogenase (ADH)) was then identified (Duester *et al.*, 1991). In addition, it has been shown that RARα binds to a RARE located in the promoter region of the rate-limiting gluconeogenic enzyme phosphoenolpyruvate carboxykinase,

expression of which is regulated by RA (Lucas *et al.*, 1991). Recently, natural T_3REs containing direct repeats have been identified; one of these has been found in the third intron of the rat growth hormone gene. This T_3RE displays significantly higher affinity for the receptor protein than the T_3RE in the promoter of the same gene (Sap *et al.*, 1990). The T_3RE identified in the long terminal repeat of Moloney murine leukaemia virus (MoMLV) contains two imperfect direct repeats separated by five nucleotides (Sap *et al.*, 1989), closely resembling the βRARE found in the promoter of the RARβ gene. Finally, a very unusual T_3RE has been identified within the lysozyme silencer (S-2.4 kb) which comprises two palindromic repeats inverted with respect to the previously characterized T_3REpal; this element confers only weak RA response in F9 cells (Baniahmad *et al.*, 1990). It is remarkable that functional elements conferring response to T_3 and/or RA can be constructed from two copies of one related motif arranged as either direct or inverted repeats and that distinct factors can recognize the same or closely related target sequences.

A question arising from these observations is how specificity of transcription in response to ligand can be maintained in the cell. In this report, we present an analysis of the βRARE present in the promoter of the RARβ2 gene. We have examined the capability of the RARs to bind sequence elements comprising either direct or inverted repeats and the effect of the number and orientation of such repeats. Furthermore, we have investigated the overlap in gene regulation of RAR and T_3R acting through RA and T_3 responsive elements.

Results

RARα, β and γ bind to and transactivate through the βRARE

It has been reported that RARβ is able to form a stable complex with the βRARE in the RARβ2 promoter *in vitro*. Furthermore, RARα and β can transactivate through recognition of this βRARE (de Thé *et al.*, 1990; Sucov *et al.*, 1990). Here we extend on these observations and show by *in vitro* DNA binding analysis that the three RARs (α, β and γ) specifically recognize the βRARE (Figure 1A, lanes 1, 4 and 7). Binding to the labelled βRARE is no longer detected in the presence of 15-fold molar excess of the cold βRARE (lanes 2, 5 and 8), whereas competition with an unrelated oligonucleotide is ineffective even at a 100-fold molar excess (lanes 3, 6 and 9).

To localize the interaction of the RARβ with the promoter of the RARβ2 gene, DNase I footprinting analysis was performed. Protection was observed over a length of ~25 nucleotides (−56 to −31 in the upper strand and −57 to −33 in the lower strand) after incubation with extracts

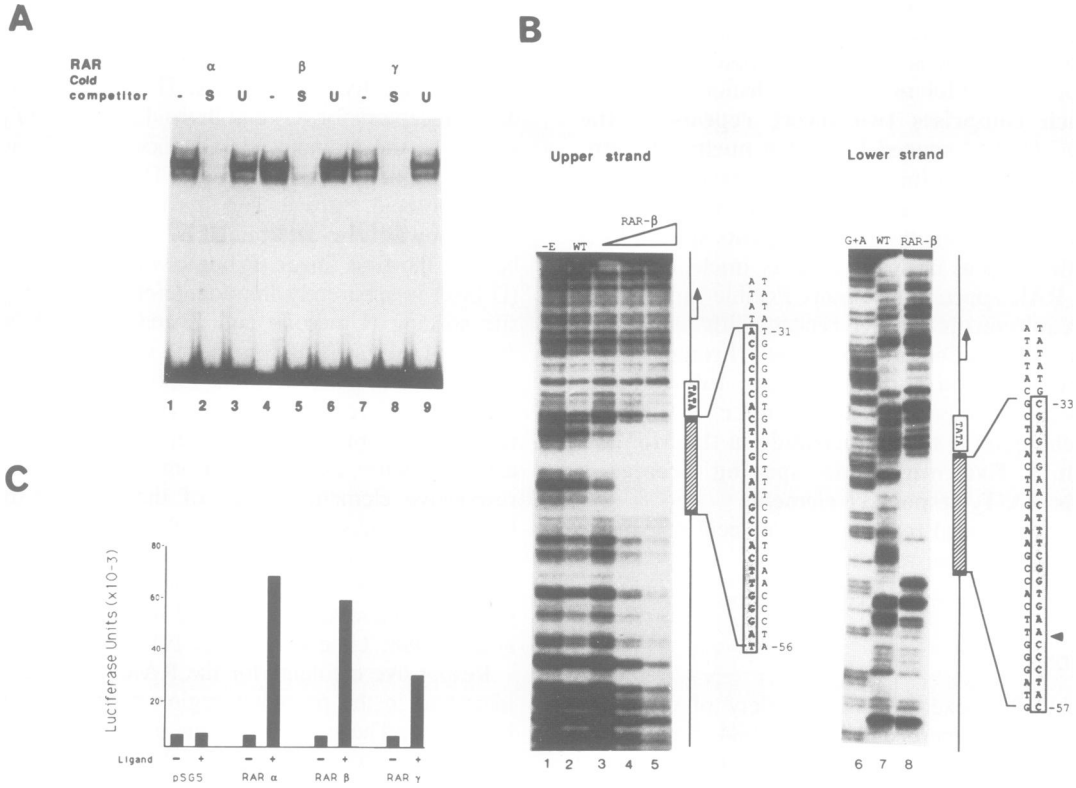

Fig. 1. (**A**) Gel retardation assay using the βRARE O1 and nuclear extracts prepared from recombinant vaccinia virus infected cells expressing either RARα (lanes 1−3), β (lanes 4−6) or γ (lanes 7−9). Competition for binding was performed using a 15-fold molar excess of unlabelled βRARE O1 oligonucleotide (lanes 2, 5 and 8) or 100-fold molar excess of an unrelated oligonucleotide (lanes 3, 6 and 9). (**B**) DNase I footprinting analysis of the RARβ2 gene promoter by increasing amounts of RARβ (lanes 3−5 and 8). Upper strand (lanes 1−5) and lower strand (lanes 6−8). Incubation of the DNA in the presence of extracts prepared from wt vaccinia virus infected HeLa cells are shown in lanes 2 and 7. Lane 1: Incubation in the absence of extract. Maxam−Gilbert G+A reaction (lane 6). A hypersensitive site is indicated by a triangle (lane 8). (**C**) Cotransfection experiments. The RARα, β and γ activate transcription through the βRARE present in the RARβ2 promoter. The reporter construct R140Luc (see Materials and methods) (5 μg/dish) was cotransfected together with an expression vector coding for either RARα, β or γ (cloned into pSG5 plasmids, 0.5 μg/dish) in the absence (−) or presence (+) of RA. Cotransfection with plasmid pSG5 served as a control, indicating the level of activation obtained by endogenous RAR.

containing RARβ (Figure 1B, lanes 3, 4, 5 and 8), but not with extracts infected with the wild type (wt) vaccinia virus (lanes 2 and 7). The same sequence is protected by RARα and γ (data not shown), which covers in both strands the perfect direct repeat contained in the βRARE. Interestingly, the DNase I protected region is adjacent to the TATA box. A single site of DNase I cleavage is observed within the protected area in the lower strand in the presence of bound RARβ (or α or γ, data not shown).

In order to compare the ability of the three RARs to transactivate the RARβ2 promoter, cotransfection experiments were performed. A reporter construct containing a fragment of the RARβ2 promoter (−124 to +14, including the βRARE, the TATA box and the site for transcription initiation) fused to the firefly luciferase gene was transfected together with expression vectors encoding the three RARs. Cotransfection of either RARα or β results in a 10- to 12-fold induction of luciferase activity in response to RA (+), whereas only half this level is obtained with RARγ (Figure 1C). Induction of transcription was not observed if the cells were cotransfected in the absence of RA (panel C, −). In the absence of exogenous RAR there is a low level of RA-dependent transactivation reflecting the level of endogenous receptor in COS cells (panel C, pSG5). Similar results were obtained using other cell lines including RK13 (rabbit kidney), HepG2 (hepatoma), RAC65 (a P19 EC derivative) and HeLa cells (data not shown). To summarize, the RARs (α, β and γ) bind to and transactivate through the βRARE present in the promoter of the RARβ gene.

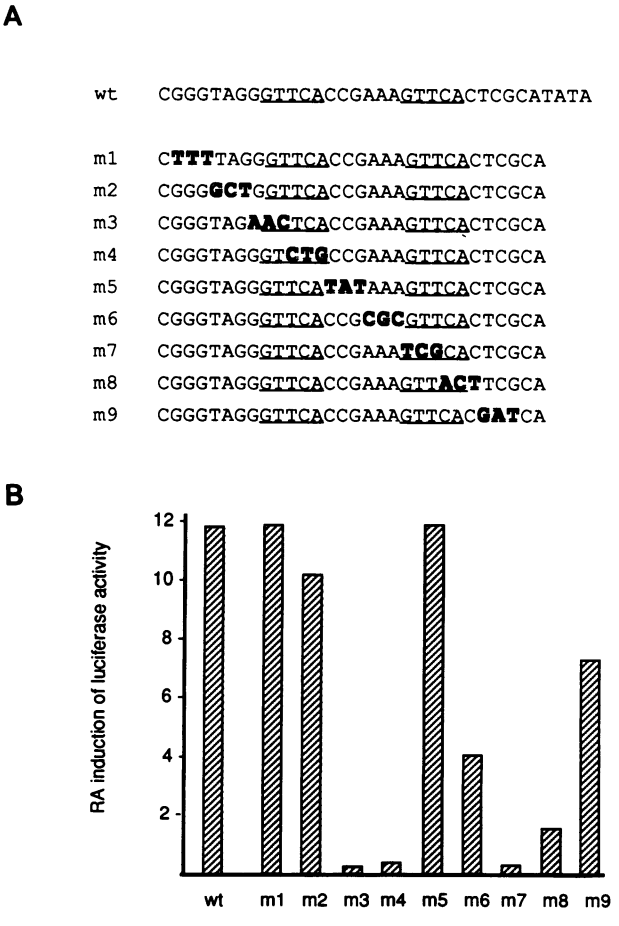

Fig. 2. Linker scanning mutagenesis of the RARβ2 gene promoter fragment. (**A**) Sequence of the RARβ2 responsive element (wt) and mutants (m1−m9) through the region comprising the βRARE. Underlined are the two direct repeats (GTTCA) and outlined the three consecutive nucleotides which have been mutated in each case. (**B**) Induction of promoter activity by cotransfection of the RARβ. The wild type promoter R140 (wt) or the different mutated forms (m1−m9) linked to the luciferase reporter were cotransfected into COS cells with the expression vector encoding RARβ. The results are presented as fold induction of promoter activity in response to RA and coexpression of RARβ.

Identification of the βRARE sequences essential for RA inducibility

To characterize the sequence requirements for binding of the RARs to their responsive element, we performed linker

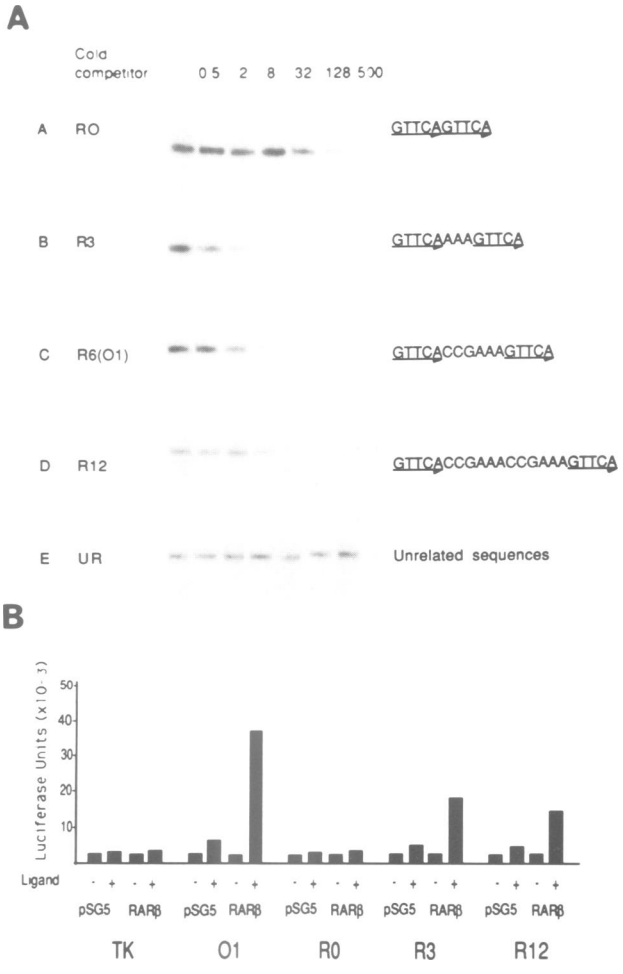

Fig. 3. Effect of the spacing between the repeats. (**A**) Competition experiments. Affinity of the RARβ for the βRARE O1 oligonucleotide *in vitro* in comparison with oligonucleotides where the distance between the repeats has been changed. In all cases, the βRARE O1 has been labelled by Klenow and allowed binding to the RAR in the presence of unlabelled R0, R3, R6(O1), R12 or a non-related oligonucleotide (NR) as competitor (panels A−E, respectively). The amount of competitor used is indicated on top of each lane as fold molar excess. Sequence of the competitor oligonucleotides is indicated to the right of each panel, and the flanking sequences are as for the βRARE O1. Panels only show the retarded complexes formed under the conditions indicated. (**B**) Activation of transcription by RAR through a single copy of the oligonucleotide inserted upstream of the TK_{109} promoter of the luciferase reporter gene. The resulting constructs were tested on transactivation in COS cells in the absence (−) or in the presence (+) of RA. Cotransfection of the plasmid pSG5 and addition of exogenous RARβ are shown. TK represents the level of activation obtained through the TK promoter.

scanning mutagenesis through the region comprising the βRARE, with mutations of consecutive groups of three nucleotides (Figure 2A). The resulting promoter mutants were fused to the luciferase reporter gene and assayed by cotransfection. The results are presented (Figure 2B) as fold induction of luciferase activity in response to RA with coexpression of RARβ. Cotransfection of the RARβ2 promoter fragment (wt in the figure) with the expression vector encoding for RARβ resulted in a ~12-fold induction of transcription. Mutations in the first GTTCA motif (m3 and m4), or in the second motif (m7 and m8), drastically reduced transactivation by ligand-activated RARβ. Mutations of sequences immediately upstream or downstream of the second repeat had a moderate effect on transactivation (m6 and m9), while sequences immediately adjacent to the first motif (m1, m2 and m5) had no apparent effect. These results confirm and extend on observations from Sucov *et al.* (1990) and, in addition, similar conclusions could be drawn from cotransfections experiments using RARα and γ (data not shown). Mutational analysis of the βRARE demonstrates that the sequences contained in both direct repeats are required for transactivation.

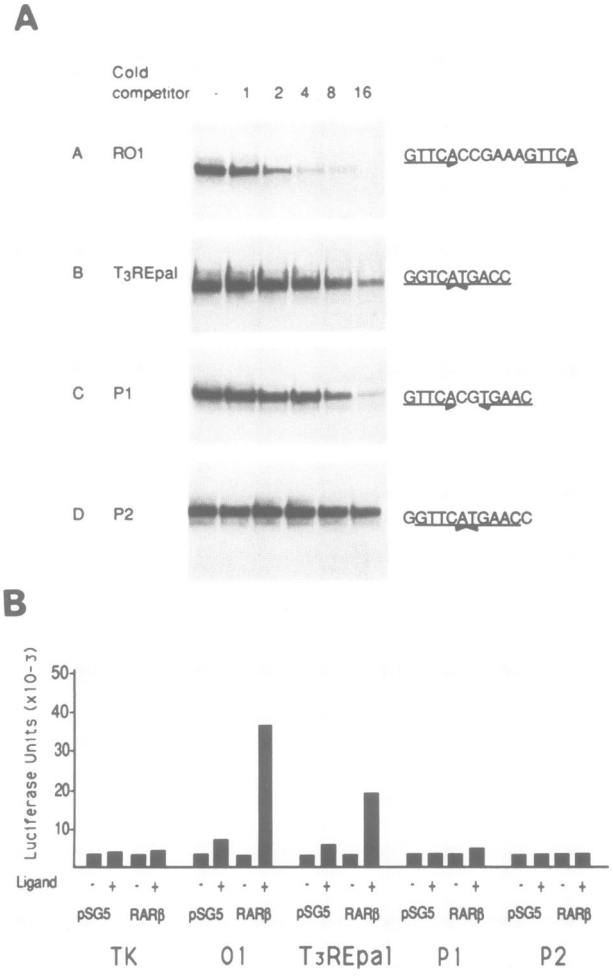

Fig. 4. Effect of the orientation of the repeats. (**A**) Affinity of the RARβ for the βRARE O1 oligonucleotide in comparison with oligonucleotides containing inverted repeats. The experiment was performed as described in the legend to Figure 3A. Sequences are indicated to the right of each panel and the amount of competitor is indicated on top as fold molar excess. Formation of retarded complexes is shown. (**B**) Cotransfection experiments. One copy of the oligonucleotides comprising direct or inverted repeats was cloned upstream of the TK_{109} promoter of the luciferase reporter gene. Conditions as indicated in Figure 3B.

Effect of spacing and orientation of the two direct repeats of the βRARE

The two repeated motifs in the RARβ2 promoter are located on the same face of the DNA helix, which could facilitate protein−protein interactions and hence the spacing could be important for transactivation. Oligonucleotides comprising mutants of the βRARE, where the direct repeats were separated by 0, 3 or 12 nucleotides, were synthesized. The affinity of the RARβ for the different mutated RAREs was measured as the ability of the mutated oligonucleotide (non-radiactive) to compete with the labelled wt βRARE O1 for binding of the receptor. The sequences of the oligonucleotides that have been used as competitors and the fold molar excess used are indicated (Figure 3A). All of them have the same flanking sequences, as the βRARE, and a minimum length of 22 nucleotides. The oligonucleotide containing three nucleotides between the direct repeats (R3) competes as efficiently or even slightly better than the wt βRARE, which contains a six nucleotide gap between repeats (Figure 3A, panels B and C, respectively). Therefore, RARβ binds *in vitro* to the R3 and O1 oligonucleotides with similar affinities. RARβ shows lower affinity for the oligonucleotides containing a 12 nucleotide spacer (R12) (panel D). Finally, the oligonucleotide containing two adjacent repeats (R0) is a poor competitor (panel A).

To determine the effect of the spacing between the direct repeats on transactivation, one copy of each of these oligonucleotides containing different spacers was fused to the HSV TK_{109} promoter in the luciferase reporter construct. The resulting constructs, as well as the parental TK-luciferase, were transfected into COS cells maintained in medium depleted of RA (Figure 3B). Cotransfection of the RARβ expression vector had little effect on expression under RA-depleted conditions [indicated as (−) in Figure 3B]. However, addition of RA (indicated as +) resulted in a 6- to 8-fold increase in luciferase expression directed by the O1-TK promoter. The R3-TK promoter was induced 3- to 4-fold in response to ligand-activated RARβ, however, RARβ showed similar affinity for this element, compared with the wt βRARE O1 (Figure 3A, panels B and C). R12-TK promoter was induced by ligand-activated RARβ to a lower extent, in line with the *in vitro* binding results, and R0-TK promoter was not induced at all in response to RA. Transient expression of the TK reporter

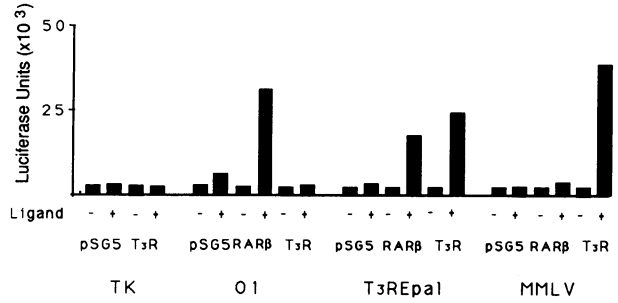

Fig. 5. RA and/or T_3 responsive elements in transactivation experiments. A single copy of each of the RA and/or T_3 responsive elements was cloned upstream of the TK_{109} promoter and cotransfected into COS cells with an expression vector encoding RARβ or T_3Rα in the absence (−) or presence (+) of the respective ligand. Sequences are indicated in Materials and methods.

did not result in activation in response to RA, either in the absence or presence of cotransfected RARβ. These results provide functional evidence that spacing between the repeats of the βRARE is important to some extent for induction of transcription by ligand-activated RARβ and that the spacing requirements for activation are similar to, but not identical with, the spacing requirements for binding *in vitro*.

Natural T_3REs identified in several promoters often consist of direct repeats of sequences related to the consensus motif GGTCA (Izumo and Mahdavi, 1988; Sap *et al.*, 1989,1990; Desvergne *et al.*, 1991). However, mutation of the T_3RE from the rat growth hormone promoter into a perfect inverted repeat leads to a substantial increase in T_3 responsiveness (Glass *et al.*, 1988) and this palindromic T_3RE (T_3REpal) confers RA responsiveness to a heterologous promoter (Umesono *et al.*, 1988). In order to study the ability of RAR to induce transcription through inverted repeats of the RA responsive half-site motif GTTCA, we prepared two different palindromic versions of this sequence, one of them with a two nucleotide gap (P1) and another one without a gap (P2). The affinity of the receptor for these inverted repeats was again determined as their ability to compete for binding of the receptor to the wt βRARE O1 (Figure 4A). The sequences of the direct repeats of the synthetic oligonucleotides are indicated on the right hand side of each panel, and they have a minimum length of 22 nucleotides. The well characterized T_3REpal has been included in this study as a reference for binding of the RAR to palindromic sequences. *In vitro* binding assays showed that the affinity of the RAR for inverted repeats, P1, and T_3REpal, was significantly reduced compared with that for the wt βRARE (Figure 4A, compare panel A with B and C) whereas P2 was unable to compete (panel D).

To examine the functional properties of these palindromic oligonucleotides on transactivation, one copy of P1 and P2, as well as the control T_3REpal, were placed upstream of the TK_{109}-luciferase expression vector and analysed for RA responsiveness in COS cells. The T_3REpal-TK promoter directed induction of gene expression in response to RA ligand-activated receptor (Figure 4B), in agreement with previous observations (Umesono *et al.*, 1988; Graupner *et al.*, 1989). In contrast, addition of RA did not result in transactivation of the reporter plasmid containing either the P1 or the P2 palindromic oligonucleotides. These results are surprising considering the sequence similarity between the T_3REpal, P1 and P2 and the comparable affinity of the RAR for the T_3REpal and the P1 *in vitro*. The level of RA-

Table I. Sequences of the oligonucleotides utilized for *in vitro* and *in vivo* analysis to study RA and T_3 differential DNA binding and transactivation.

	1	2	3
RARE O1	CGG**GTTCA**CCGAAA**GTTCA**CTCG		
OL-1	CAG**GTTCA**TTTTCA**GTTCA**TTGC		
OL-2	CAG**GTTCA**TTTTCA**GTTCC**TTGC		
OL-3	CAG**GGTCA**TTTTCA**GTTCA**TTGC		
OL-4	CAG**GGTCA**TTTTCA**GGTCA**TTGC		
OL-5	CAG**GGTCA**TTTTCA**GTTCC**TTGC		
OL-6	CAG**GTTCA**TTTTCA**GGTCC**TTGC		
OL-7	CAG**GGTCA**TTTTCA**GGTCC**TTGC		
OL-8	CAG**GTTCA**TTT CA**GTTCA**TTGC		
OL-9	CAG**GTTCA**TTT CA**GTTCC**TTGC		
OL-10	CAG**GGTCA**TTT CA**GTTCA**TTGC		
OL-11	CAG**GGTCA**TTT CA**GGTCA**TTGC		
OL-12	CAG**GGTCA**TTT CA**GTTCC**TTGC		
OL-13	CAG**GTTCA**TTT CA**GGTCC**TTGC		
MoMLV-T_3RE	CAG**GGTCA**TTT CA**GGTCC**TTGC		

The direct repeats were surrounded by the sequences of the T_3RE MoMLV. Mutations of nucleotides in the direct repeats (numbered from 1 to 3) spaced by either six (OL-1 to OL-7) or five (OL-8 to OL-13) nucleotides are indicated in a shaded box.

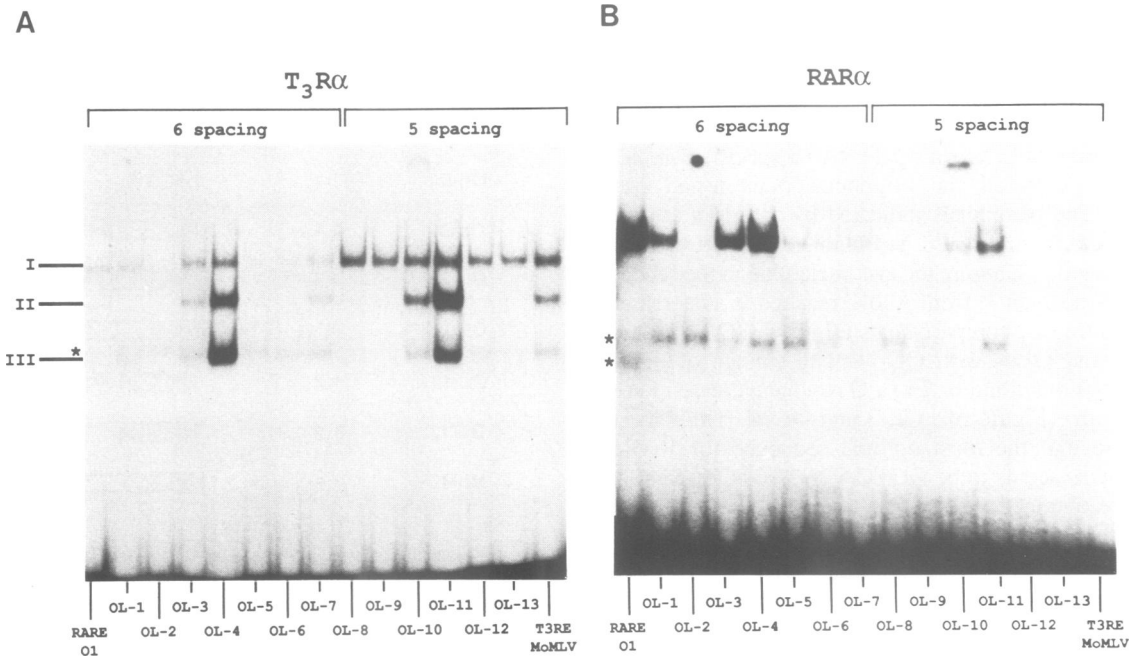

Fig. 6. Gel retardation assays showing binding of T_3R (panel **A**) and RAR (panel **B**) containing extracts to the oligonucleotides listed in Table I. Whole cell extracts were prepared from COS cells infected with recombinant vaccinia virus expressing chicken T_3Rα or human RARα. Stars indicate non-specific shifts.

dependent expression was higher if the wt βRARE O1 was located upstream of the TK_{109} promoter than for any of the artificial palindromes (Figure 4B). In summary, it seems that RAR does not activate transcription through inverted repeats of the half-site motif GTTCA.

Determination of a RAR or T_3R responsive element by the sequence or the spacing between the repeats

Given that RAR can bind to and activate transcription through a promoter containing the T_3REpal, a perfect inverted repeat of the half-site motif GGTCA, we tested whether RARβ could function through this motif arranged as direct repeats. For this purpose, we tested the natural T_3RE present in the long terminal repeat of MoMLV which contains two imperfect direct repeats of the motif GGTCA with a five nucleotide gap between them. The observation that RAR could bind, although weakly, to the T_3RE MoMLV (see Figure 6B, MoMLV), raised the question of whether this T_3RE can also mediate RA response. Surprisingly, transfection assays have shown that ligand-activated RAR does not significantly activate gene expression through the T_3RE MoMLV (Figure 5). Similarly, ligand-activated T_3R does not induce gene expression through the RARβ2 promoter (Figure 5 and Hoffmann *et al.*, 1991); however, MoMLV functions as a strong T_3RE. This striking result prompted a further comparison of the sequence requirements of RAR and T_3R for DNA binding *in vitro* and transactivation.

The βRARE O1 and the MoMLV T_3RE differ in the sequence of the direct repeats (GTTCA and GGTCA/C, respectively) and the spacing between the repeats (six and five nucleotide gap, respectively, see Table I). The spacing analysis described above suggested that transactivation by RAR was not drastically affected by the spacing between the repeats (see Figure 3B, R3 and R12), therefore, we initially examined the effect of single nucleotide changes in the repeats (arbitrarily referred to as positions 1, 2 and 3, see Table I). We have located the different direct repeats in the context of the MoMLV, in order to assess exclusively the effect of the nucleotide changes in the repeats both *in vitro* and *in vivo*.

By gel retardation assays a single RARα specific complex was detected with all the oligonucleotides tested (see Figure 6B). The complexes indicated by asterisks are not specific for RAR, and could be obtained with wt extracts (data not shown). Changing the last nucleotide in the second direct repeat (position 3) from A to C resulted in a decreased binding (Figure 6B, compare lanes OL-1 with OL-2, OL-3 with OL-5 and OL-4 with OL-7). The change of the T residue at positions 1 and/or 2 to a G residue increased RAR binding *in vitro* (Figure 6B, OL-3 and OL-4). Thus, these data indicate that the most optimal sequence for RARα binding *in vitro* seems to be GGTCA-N_6-GGTCA. Similar results were obtained when RARβ and γ were tested for binding to these oligonucleotides *in vitro* (data not shown). Analysis of the binding requirements of T_3Rα was more complex as the T_3Rα formed three specific complexes (referred to as I, II and III) with the oligonucleotides (Figure 6A and Sap *et al.*, 1989). We tentatively interpret complex III as the result of the binding of one T_3R monomer, complex II of two receptor molecules and complex I of one T_3R molecule and an unknown polypeptide (T.H.Bugge and H.G.Stunnenberg, unpublished observations). Substituting the T residue at positions 1 and 2 for a G residue strongly enhanced complex formation (OL-4), whereas this effect was neutralized by the A to C residue change at position 3 (OL-5 to OL-7).

To assess the effect of these oligonucleotides on transactivation, one copy of each of these were inserted upstream of the TK_{109} promoter in front of the luciferase reporter gene, and cotransfected into COS cells with an expression vector encoding RARβ or T_3Rα (Figure 7, black and shaded bars, respectively; from βRARE O1 to OL-7). As expected, substitution of the sequences surrounding the GTTCA repeats of the βRARE for those of the MoMLV T_3RE (OL-1), only affected RA-dependent transactivation to a low extent. Substitution of the GTTCA half-site motifs of the βRARE for these of the MoMLV, creating a MoMLV T_3RE element with a six nucleotide gap (OL-7), resulted in a slight decrease on RA-dependent transactivation. When the sequence of the direct repeats was changed into the consensus half-site motif GGTCA (OL-4), RA induction was comparable with that obtained with OL-1, although RAR showed an increased affinity for OL-4 *in vitro*, in comparison with OL-1. Interestingly, T_3Rα did not induce gene expression through any of the oligonucleotides containing a six nucleotide gap between the repeats. This

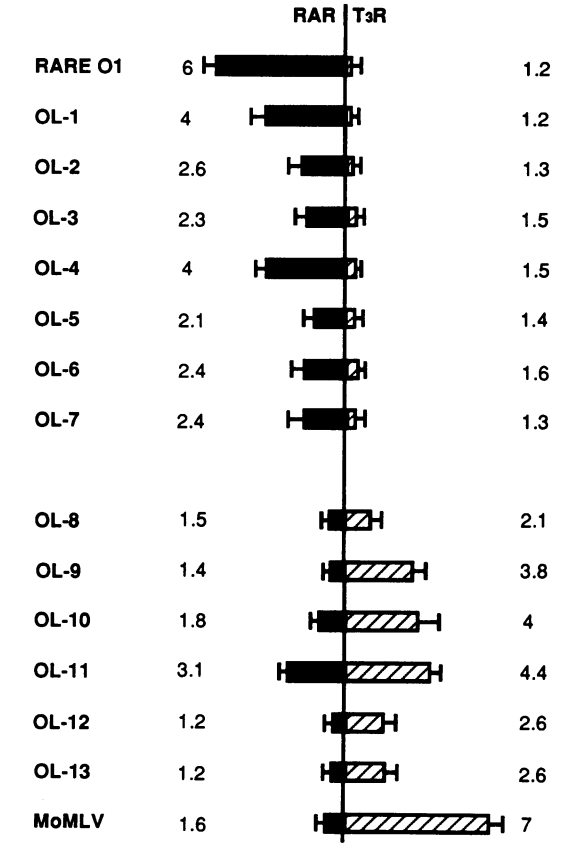

Fig. 7. Transient transfection assays of RAR and T_3R with reporter constructs containing one copy of the oligonucleotides listed in Table I. The oligonucleotide corresponding to the MoMLV T_3RE is indicated only as MoMLV in the figure. One copy of each of the oligonucleotides was inserted upstream of the TK_{109} promoter of the luciferase reporter gene and cotransfected into COS cells with expression vectors encoding either human RARβ or chicken T_3Rα. The results of the transactivation experiments are presented as fold induction of luciferase activity in response to ligand-activated RARβ (−) or T_3R (+).

is in clear contrast to the DNA binding of $T_3R\alpha$ to OL-4 (see Figure 6A). These results indicate that the differences in sequence composition of the direct repeats of the βRARE and the MoMLV T_3RE do not seem to be responsible for the differential response of both elements to RA and T_3 induction *in vivo*. Therefore, we decided to analyse the influence of the spacing in different oligonucleotides containing the same direct repeats with a five instead of a six nucleotide gap between them.

Overall RARα, β and γ bind with reduced affinities to the oligonucleotides with direct repeats spaced by five as opposed to six nucleotides, whereas the reverse is true for $T_3R\alpha$ (Figure 6). Again the change of the T residue at positions 1 and 2 to a G residue increased binding of both RARα and $T_3R\alpha$ (OL-11, panels A and B). Furthermore, the change of the A to C residue (at position 3) strongly decreased binding in all cases (OL-9, panels A and B). The formation of T_3R complexes III and II was shown to be dependent on the sequence composition of the direct repeats whereas complex I was only slightly affected.

Interestingly, spacing the GTTCA half-site motifs by a five nucleotide gap (Figure 7, OL-8) resulted in reduction of RA-dependent transactivation, in comparison with OL-1, while $T_3R\alpha$ was now able to induce gene expression to a low extent. As previously observed (see also Figure 5), T_3-dependent transactivation resulted in ~7-fold induction of luciferase activity through the wt MoMLV T_3RE, but this element did not significantly respond to RA induction (Figure 7, indicated as MoMLV in the Figure). Finally, when the half-site motifs GGTCA were spaced by a five nucleotide gap (OL-11), both RAR and T_3R were able to transactivate, indicating that a single nucleotide change in the MoMLV T_3RE converts it into a promiscuous RA and T_3 responsive element.

In summary, these results suggest the requirements of a five nucleotide gap between both half-site motifs (either GTTCA or GGTCA) in order to determine T_3-dependent transactivation. However, RAR seems to be more flexible with respect to the spacing between the direct repeats, although a clear preference for direct repeats spaced by six nucleotides on transactivation can be observed. The sequence composition of the direct repeats either GTTCA or GGTCA appears to be irrelevant to distinguish a RA from a T_3 responsive element in transactivation experiments, although it modulates the level of induction.

Discussion

The sequence of the βRARE identified in the promoter region of the RARβ2 gene contains two perfect direct repeats of the motif GTTCA (de Thé *et al.*, 1990; Sucov *et al.*, 1990), which are related to other hormone responsive elements identified in several gene promoters (Beato, 1989). In this report we have shown that the three RARs (α, β and γ) bind *in vitro* to and induce gene expression through this βRARE. Both direct repeats of the βRARE are required for transactivation of the RARβ gene by ligand-activated RARs. Linker scanning mutagenesis shows that mutations directed towards one of the two motifs specifically abolish transactivation, while other mutations have a minor effect. DNase I footprinting analysis indicates that RARs protect the direct repeats comprising the βRARE in both strands. A striking feature of this βRARE is the location of the DNase I protected region immediately adjacent to the TATA box. In contrast, other hormone responsive sequences that have been characterized are normally positioned between 100 and 1000 bp from the initiation site (Jantzen *et al.*, 1987; Ham and Parker, 1989).

By analogy with other members of the receptor family, it is reasonable to predict that RAR binds DNA as a dimer. For instance, homodimer formation has been shown for the rat glucocorticoid (Tsai *et al.*, 1988) and human estrogen (Kumar and Chambon, 1988) receptors, where sequences required for dimerization seem to be contained within the ligand binding domain (Fawell *et al.*, 1990). The finding that G residues on T_3REs are contacted symmetrically suggested that T_3R binds DNA as a dimer (Glass *et al.*, 1988). However, natural T_3REs and RAREs are often found to comprise direct instead of inverted repeats. It is surprising that both kinds of elements (direct or inverted repeats) can serve as functional RA and/or T_3 responsive elements.

By *in vitro* and *in vivo* studies we have shown that RARβ binds with high affinity to a RARE comprised of direct repeats with either three (R3) or six (R6 or βRARE) nucleotide gaps between the repeats, while increasing the separation to 12 nucleotides (R12) diminishes the ability for binding. In agreement with these results, a RARE containing a direct repeat of the motif GGTCA with a three nucleotide gap was recently identified in the mouse cellular retinol binding protein I (mCRBPI) promoter. This has lower RA inducibility than that of a similar reporter gene construct containing the mouse βRARE promoter region (Smith *et al.*, 1991). Although R3 and R6 show similar affinity in binding to the receptor *in vitro*, this is not paralleled by their abilities to mediate transactivation, since the level of RA induction obtained through R6 (wt βRARE) is higher than that through R3, and both are higher than through R12. A lack of spacing between the direct repeats, as in the R0 mutant, abolishes binding and RA responsiveness. These reporter constructs (R0, R3 and R12) were also assayed for T_3 responsiveness. T_3R did not significantly affect promoter activity through any of these elements, neither in the presence nor in the absence of hormone (data not shown). After submission of this work, a study was published concluding that direct repeats of the motif GGTCA with a three nucleotide gap spacing conferred negative regulation by the T_3R (Näär *et al.*, 1991). However, we did not observe this effect with R3 (GTTCA with a spacing of 3 bp), suggesting that sequence composition also plays a role in distinguishing regulation by RAR and T_3R.

One possible receptor–DNA interaction taking place on these elements might be envisaged by considering that each direct repeat is bound by a separate receptor dimer. In this instance, the βRARE could be considered as two imperfect inverted repeats, each comprising one strong and one very weak half-site. The weak binding sites would affect the degree of binding and transactivation to some extent, as observed in the mutants m6 and m9 (Figure 2). Thus, RAR would bind to the βRARE as two dimers, while binding to palindromic motifs (T_3REpal, P1 or P2) would be only as a single dimer. However, binding of RAR to both direct and inverted repeats generates a protein complex with identical gel mobility (data not shown). This suggests that the inverted as well as the direct repeats are bound by a single receptor dimer, unless one assumes that complexes comprising two or four RAR molecules migrate to the same position on non-denaturing gels.

The present results could then be explained by the fact that increasing the spacing between the direct repeats increases the distance between the two receptor molecules, making direct protein–protein contacts more difficult. Location of both repeats too close together would preclude binding. The functional distinction between the different RAREs examined in this report suggests that the presence of a gap between the direct repeats is important in order to achieve optimal binding of RAR. However, this does not guarantee optimal transactivation (compare binding and transactivation for R3 and R6(O1) in Figure 3, P1 in Figure 4). It is currently unclear what structural arrangements allow receptor dimer binding to either direct or inverted repeats. We propose that RAR binds as a dimer to DNA sequences comprised of both perfect direct repeats (as in the βRARE) and inverted repeats (as in T_3REpal). Thus, the receptor dimer must be flexible to allow independence between the different domains so that the DNA binding domain can turn with respect to the dimerization domain in order to recognize both direct and inverted repeats in the responsive element.

Maintenance of differential gene expression in response to RA and T_3

The available information on T_3R and RAR and their responsive elements makes the maintenance of differential gene expression in response to RA and T_3 in the cell an intriguing issue. High amino acid sequence homology, particularly in the DNA binding domain, facilitates transactivation by both ligand-activated receptors through the common responsive element T_3REpal (Umesono *et al.*, 1988; Graupner *et al.*, 1989) and formation of heterodimers between RARα and T_3Rβ on the T_3REpal (Glass *et al.*, 1989). We have observed that the binding affinities of RAR for the T_3REpal and for the P1 are very similar (Figure 4A). However, although T_3REpal functions as a RA responsive element (Figure 4B), P1 does not mediate transactivation by RAR. In contrast, RAR does not bind to P2, a perfect inverted repeat of the motif GTTCA, suggesting that the sequence composition is crucial in DNA binding by RAR through inverted repeats. The palindromic constructs (P1 and P2) were also tested in transfection experiments to establish the ability of T_3R to transactivate through these elements. Interestingly, ligand-activated T_3R was not able to regulate the level of luciferase gene expression through inverted repeats of the motif GTTCA (data not shown), again indicating the important role of sequence composition for gene regulation by T_3R through palindromic sequences. In conclusion, neither RAR nor T_3R regulate gene expression through inverted repeats of the half-site motif GTTCA.

T_3R can form specific protein–DNA complexes with the βRARE and with the MoMLV T_3RE (see Figure 6A). Although binding of the T_3R does not result in T_3-dependent induction of transcription through the βRARE O1 and OL-4, the MoMLV T_3RE functions as a strong element in transactivation (see Figures 5 and 7). Conversely, the complex between RAR and the MoMLV T_3RE can be detected *in vitro*, but it does not significantly induce gene expression. These results imply a clear distinction between DNA binding and transactivation by RA and T_3 receptors. This is in contrast to the recently published observations where a good correlation was reported between results obtained in *in vitro* DNA binding and *in vivo* transactivation experiments (Umesono *et al.*, 1991).

T_3R and RAR are capable of binding with high affinity to a family of related sequences, however, a distinction could be observed between these receptors both *in vitro* and *in vivo*. Although RAR induces gene expression to higher levels through repeats spaced by six nucleotides, it is also able to transactivate through direct repeats with a 3, 5 or 12 nucleotide gap betweem them (Figures 3B and 7 and Smith *et al.*, 1991). Transactivation studies establish the preference of T_3R to induce gene expression through direct repeats spaced by a five nucleotide gap compared with a six nucleotide gap (Figure 7). Moreover T_3R is unable to regulate luciferase gene expression through direct repeats of the motif GTTCA with a spacing of 0, 3 or 12 bp (data not shown). Thus, T_3R is less flexible in its spacing requirements for binding to direct repeats than RAR. These results are in contrast to previous studies where T_3R was shown to function through T_3REs comprising inverted repeats irrespective of the presence of a gap (up to six nucleotides) between the motifs (Glass *et al.*, 1988). Apparently, the spacing requirements are less restricted when the motifs are oriented as inverted repeats than in a direct configuration. After submission of this manuscript, similar analyses on gene expression regulation by RAR and T_3R were reported (Umesono *et al.*, 1991; Näär *et al.*, 1991). They emphasized that the specificity of hormonal response depends on the unique spacing of the direct repeats, corresponding to a five nucleotide gap for T_3R and a six nucleotide gap for RAR (following the spacing nomenclature of our report) (Umesono *et al.*, 1991). These results correlate to a certain extent with our findings, however, as demonstrated with the mutants reported here (R3, R5 and R12) and with the wild type RARE found in the CRBPI promoter (Smith *et al.*, 1991), RAR shows more flexibility in spacing requirements between direct repeats.

The present results indicate that RA and T_3 receptors seem to be able to induce gene expression through responsive elements containing direct repeats of the half-site motif GTTCA or GGTCA. It is interesting to note that some elements are responsive to only RA or T_3 induction, as the βRARE O1 and the MoMLV T_3RE, respectively, while the change of one nucleotide in the MoMLV T_3RE can convert this sequence into a promiscuous responsive element responding to both RA and T_3 (see Figure 7, OL-11). This again indicates the flexibility of RAR in spacing requirements and is in contrast to the rule proposed by Umesono *et al.* (1991), which is restricted to a six nucleotide gap as spacer between direct repeats in the RARE. In this study the functional differentiation between a RA and a T_3 responsive element has focussed on the distinction between the βRARE and the T_3RE MoMLV, and in the analysis of some distance and orientation mutants. Our results show that although the distance between repeats can distinguish the hormone responsiveness of a particular element, a full understanding of RA- and T_3-induced gene expression requires knowledge of both the sequence and orientation of the repeats in a given promoter context.

Materials and methods

Preparation of extracts from recombinant vaccinia virus infected cells

Recombinant vaccinia virus expressing the RARα, β or γ were constructed and amplified using standard procedures (de Magistris and Stunnenberg, 1988; Stunnenberg *et al.*, 1988). The cDNAs encoding each of the three receptors were under the transcriptional control of the late promoter of the

11K-protein gene. Screening for positive plaques was performed by pulse labelling of recombinant vaccinia infected cells and by immunoprecipitation using anti-RARα, β or γ antibodies. For extract preparation HeLa spinner cells infected with the corresponding recombinant virus were washed twice with ice cold phosphate-buffered saline (PBS). The cell pellet was resuspended in 5 vol (5×10^8 cells correspond to ~1 ml) of a buffer containing 10 mM HEPES pH 7.9, 5 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT and 0.1 mM PMSF. Protease inhibitor was added freshly before use. The cells were incubated for 5 min on ice, pelleted, and this procedure repeated once. The cells, but not the nuclei, were broken by Dounce homogenization 10–20 strokes with pestle B, centrifuged for 5 min at 1500 r.p.m. and the supernatant collected (cytoplasmic extract). The crude nuclear pellet was resuspended in 2 vol of a buffer containing 20 mM HEPES pH 7.9, 420 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 1 mM glycerol, 1 mM DTT, 0.1 mM PMSF (two strokes with Dounce) and incubated 45 min on ice on a rotator wheel in the cold room. The nuclear extract is cleared by centrifugation at 35 000 r.p.m. for 1 h, quick-frozen in aliquots using liquid N_2 and stored at −80°C. The protein concentration (as determined by Bradford assay) was 0.7–1.0 mg/ml.

For the gel retardation assays shown in Figure 6, whole cell extracts were prepared by lysis of COS cells in 50 mM Tris–HCl pH 7.5, 420 mM NaCl, 5 mM $MgCl_2$ and 0.5% (v/v) Triton X-100. Clarified lysates were frozen in liquid nitrogen and stored at −70°C.

Gel retardation assays

The oligonucleotide containing the βRARE O1 (GGGTAGGGTTCACC-GAAAGTTCACTCG) was synthesized with single-stranded 5′ extensions to generate *Sal*I and a *Xho*I compatible overhangs and used as a probe after end labelling by Klenow. Nuclear extracts (1 μl) were incubated in a binding buffer containing 15% glycerol, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 0.1 mM EDTA, poly(dI-dC) (0.2 mg/ml final concentration), and 0.01% Triton for 15 min on ice. Subsequently, 10 000 c.p.m. of probe were added (together with the unlabelled competitor in the case of the competition experiments) and the binding reaction continued for another 20 min on ice. The reaction was loaded on pre-cooled 5% acrylamide gels containing 0.5×TBE as running buffer. The gels were run in the cold at 150 V, dried and exposed.

Plasmid construction

The RARβ2 gene promoter fragment from *Hin*fI (−124) to *Alu*I (+14) was cloned into pXP(1,2) (Nordeen, 1988), thereby replacing the TK promoter, containing the luciferase reporter gene, resulting in the reporter construct R140Luc. RARβ cDNA (β2) was cloned as a *Sac*I–*Bam*HI fragment into the expression vector pSG5 (Green *et al.*, 1988) and RARα and γ were kindly provided by Dr Chambon (in pSG5). The different mutants through the βRARE (m1–m9) were obtained by site-directed mutagenesis using the *in vitro* mutagenesis system version 2 from Amersham. One copy of each of the oligonucleotides corresponding to the distance or the palindromic mutants was inserted upstream of the TK promoter (−109 to +52) at the *Sal*I site of the expression vector pXP(1,2).

Transient expression assays

COS cells were passaged 24 h before transfection at ~0.5×10^{-6} cells per 6 cm dish and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Fresh medium supplemented with charcoal-stripped serum was added 2 h before transfection of the cells.

The reporter luciferase gene, R140Luc, (5 μg), together with the RSV-CAT (0.5 μg), as internal control for effficiency of transfection, and the expression vectors encoding for RARα, β or γ (0.5 μg) were transfected into cells by calcium phosphate precipitation. After overnight exposure to the precipitate, the cells were incubated for 24 h in fresh medium containing charcoal-stripped serum with or without 10^{-6} M retinoic acid. Cells were harvested and the luciferase activity measured (Reinberg *et al.*, 1987) and normalized for CAT expression (Gorman *et al.*, 1982). A representative of several experiments is shown.

DNase I footprinting

Probe fragment was prepared by cutting at the *Bam*HI and *Sal*I sites of R140Luc (polylinker sites of the plasmid) and labelling using Klenow enzyme. The resulting 150 bp fragments labelled on the upper or lower strand were incubated with 0, 2, 4 or 8 μl of RARβ extract, or 4 μl of wild type extract. Binding reactions were performed as indicated above for the gel retardation assays in a total volume of 50 μl. After 20 min incubation on ice, 50 μl of a solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ were added and the mix incubated for 1 min at room temperature. 2 μl of an appropriate dilution of DNase I (1/200 to 1/1000 dilution from a DNase I stock of 10 mg/ml) were added and digestion allowed to proceed for 1 min at room temperature. The reaction was stopped by addition of a solution containing 200 mM NaCl, 20 mM EDTA pH 8, 1% (w/v) SDS, and 250 μg/ml of carrier DNA. The resulting fragments were analysed on 6% denaturing sequencing gels. Maxam and Gilbert G+A reaction was performed essentially as described by Sambrook *et al.* (1989).

Oligonucleotides

The following oligonucleotides were used in this study: βRARE O1:
5′-TCGACGGGTAGGGTTCACCGAAAGTTCACTCGC-3′
MoMLV T_3RE: 5′ TCGACAGGGTCATTTCAGGTCCTTGC 3′
R0: 5′ TCGACGGGTAGGGTTCAGTTCACTCGC 3′
R3: 5′ TCGACGGGTAGGGTTCAAAAGTTCACTCGC 3′
R12: 5′ TCGACGGGTAGGGTTCACCGAAACCGAAAGTTCACTCGC 3′
T_3REpal: 5′ AGCTTCAGGTCATGACCTGAAGCA 3′
P1: 5′ AGCTTCAGTTCACGTGAACTGAAGCA 3′
P2: 5′ AGCTTCAGGTTCATGAACCTGAAGCA 3′
The series of oligonucleotides from OL-1 to OL-13 have exactly the same flanking sequences as indicated for the MoMLV T_3RE.

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