A Retinoic Acid Receptor-Specific Element Controls the Retinoic Acid Receptor-β Promoter

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The morphogen retinoic acid (RA) regulates gene transcription by interacting with specific nuclear receptors that recognize DNA sequences near responsive promoters. While much has recently been learned about the nuclear receptor proteins, little is known about the genes that are directly regulated by RA and their *cis*-acting response elements recognized by these receptors. Here we have analyzed the RA receptor-β (RARβ) gene promoter that is controlled by RA. We find that a RA-responsive element (RARE) is located adjacent to the TATA box. The RARE shows a direct repeat symmetry which is essential for its function. While thyroid hormone-responsive elements can also function as RAR response elements, we show here that this RARE is activated by endogenous RARs and RARβ, but cannot be regulated by thyroid hormone receptors and other known nuclear receptors. In addition, we find that RARγ is a poor activator of this RARE. However, the response element is bound with high affinity by both RARβ and RARγ as well as by thyroid hormone receptors. Thus, interaction between specific response elements and receptors is insufficient for gene activation. (Molecular Endocrinology 4: 1727–1736, 1990)

INTRODUCTION

The morphogen retinoic acid (RA) mediates a complex pattern of gene expression during development and homeostasis. The recent isolation of specific nuclear receptors for RA by us and others (1–4) has much facilitated the further analysis of the mechanism by which RA controls complex biological programs. The three different retinoic acid receptors (RARs), α, β/ϵ, and γ are differentially expressed during mammalian development (5) and amphibian limb regeneration (6). The proteins belong to the superfamily of steroid/thyroid hormone (TR) receptors (7, 8). These regulatory proteins contain a characteristic highly conserved DNA-binding domain that encodes two zinc finger structures and a moderately conserved hydrophobic ligand-binding domain that also encodes a dimerization function (9). Interaction of the receptors with response elements near sensitive promoters is essential for the regulatory effects these proteins exert on gene transcription. While a number of responsive elements for steroid receptors and TRs have been characterized, little is known about the DNA sequences that are directing the RA response. We and others have recently found that the high homologies observed between the DNA-binding domains of TRs and RARs are of functional significance, since RARs can use TR-responsive elements (TREs) as specific response elements (10, 11). This suggested that RARs and TRs regulate gene expression via an overlapping group of responsive elements. On further analysis, however, we observed that TRs in the absence of ligand can repress the activity of RARs on the TRE (10). The ability of TRs to repress RAR activity is due to their high affinity binding to the TRE in the absence of their ligand. This dominant activity of TRs on the TRE limits the responsiveness of these elements to RA. To obtain a better understanding of how the retinoid signal regulates specific gene activation, efforts are now concentrating on the identification and analysis of promoters that directly respond to RA (12, 13). One important question is whether RA-responsive elements (RAREs) in general overlap with response elements of related receptors, thereby also responding to other receptors of the same subfamily, such as TR and estrogen receptor (ER), as reported for the palindromic TRE (10, 11; Zhang, X.-K., K. N. Wills, G. Graupner, M. Tzukerman, T. Hermann, and M. Pfahl, submitted). A second question is whether RAREs can be defined that respond preferentially to specific types of RARs (for example to RARβ/ϵ, but not to RARγ). The DNA-binding domains of all three RARs are almost identical. If the DNA-binding domain of the RARs itself contributes all the specificity of the protein-DNA interaction, one can expect that all three RARs will recognize and use essentially identical DNA sequences. However, it has

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been found recently that in the case of the TRs (Zhang, X.-K., K. N. Wills, G. Graupner, M. Tzukerman, T. Hermann, and M. Pfahl, submitted) and the ER (9, 15), the hormone-binding domain contributes significantly to DNA-binding properties of those receptors. Since the ligand-binding domains of RARs are more diverse than the DNA-binding domains, they could be important in determining response element specificity. The amino-terminal domains represent the least conserved receptor structures within one species, but are highly conserved among the same receptor types from different species and may also contribute to promoter/response element specificity, as observed for two distinct progesterone receptor forms (16). Expression of the RARβ/ϵ gene is RA dependent in the embryonal carcinoma F9 cell line, while RARα and RARγ expression in those cells does appear to be constitutive (17). RARβ/ϵ is also induced by RA in the liver (18).

Here we have cloned and analyzed the RARβ/ϵ promoter and 5′ flanking regions and identify a distinct RARE in the promoter of the RARβ/ϵ gene. Our data are consistent with those recently reported by de Thé *et al.* (13). We find, in addition, that the RARE shows a clear preference for RARβ compared to RARγ, in particular in F9 cells. The response element contains a direct repeat symmetry, which when converted to a palindromic symmetry, as observed in most nuclear receptor response elements, loses its function. The RARE is bound by both RARβ and RARγ with high affinity in the absence and presence of ligand. TRs also bind to this response element, but do not show any effect on the transcriptional regulation of the promoter.

RESULTS

Cloning and Sequence Analysis of RARβ/ϵ Promoter

One million plaques obtained from a human genomic λ library were screened with a *Bam*HI-*Xho*I fragment derived from the 5′ end of the RARβ/ϵ cDNA clone (1). Three clones hybridizing under stringent conditions were isolated. Restriction analysis of the clone with the largest insert (gRARβ-41) revealed two *Bam*HI sites in its 3′ half (Fig. 1A). Subcloning and partial sequence analysis of the nucleotides surrounding the 5′ *Bam*HI site proved that these sequences were identical to sequences surrounding the single *Bam*HI site present at the 5′ end of the RARβ/ϵ cDNA clone (1). A *Bgl*II-*Bam*HI fragment that extended 907 basepairs (bp) 5′ of the *Bam*HI site was, therefore, analyzed by DNA sequencing (Fig. 1B). A TATA box near the 3′ end of the 907-bp fragment indicated that this sequence might contain the RARβ/ϵ promoter. Other features of this sequence are shown in Fig. 1B. We observe a 318-bp open reading frame that is preceded by a stop codon and a direct repeat of a TRE half-site related sequence located 6 bp 5′ of the TATA box motive (*bold letters*, *underlined*, Fig. 1B). A *Sp*I-like sequence is located at position −380 (*boxed* in Fig. 1B).

RARβ/ϵ Gene Is Regulated by a RAR-Responsive Promoter

To investigate whether the sequences up-stream of the *Bam*HI site contained a promoter, the *Bgl*II-*Bam*HI fragment was inserted up-stream of a chloramphenicol acetyltransferase (CAT) reporter gene (Fig. 2) that does not contain a promoter. This reporter gene construct was transiently transfected into CV-1 cells. A low level of CAT activity was measured that could be increased 2-fold when the cells were grown in the presence of RA. An approximately 10-fold increase in CAT activity was measured when the cells were cotransfected with various amounts of a RARβ expression vector in the presence of RA, while in the absence of RA, only a 2-fold increase in CAT activity was observed. A somewhat lower increase in CAT activity was observed when a RARγ expression vector was cotransfected (Fig. 2B). These data demonstrate that the *Bgl*II-*Bam*HI fragment contains both a functional promoter and a RARE.

To further define the putative RARβ/ϵ gene promoter and signals required for a direct RA response, three subfragments of the *Bgl*II-*Bam*HI sequence, 1) *Bgl*II-*Sma*I, 2) *Sma*I-*Bam*HI and, 3) *Sma*I-*Eco*RI, were inserted up-stream of the CAT reporter gene. When transfected alone or together with RAR expression vectors into CV-1 cells, both the *Sma*I-*Eco*RI as well as the *Sma*I-*Bam*HI fragments showed activation of the CAT gene, whereas the *Bgl*II-*Sma*I fragment did not (Fig. 2C). While most of the induction by RARβ is RA dependent, a much higher level of RA-independent activation of CAT is observed by RARγ, consistent with earlier observations, showing that RARγ has a higher basal level activity (19). Our data suggest that the *Sma*I-*Eco*RI fragment contains a promoter, a transcription initiation sequence, and a RARE. However, the adjacent 3′ sequences (contained in the *Eco*RI-*Bam*HI sequence) contribute to the overall efficiency of the RA-responsive promoter, since the *Sma*I-*Bam*HI construct confers higher levels of CAT activities than the *Sma*I-*Eco*RI construct (Fig. 2). The fold induction by all three active fragments is approximately the same, suggesting that the RARE is located in the Sma-*Eco*RI fragment. Since the *Sma*I-*Bam*HI fragment and the *Bgl*II-*Bam*HI fragment show almost identical promoter activity, our data also suggest that all essential promoter elements that direct transcription in CV-1 cells are encoded within the *Sma*I-*Bam*HI fragment.

Analysis of the RARβ/ϵ Promoter RARE

The RA-responsive *Sma*I-*Eco*RI construct contains only 32 nucleotides up-stream of the TATA box, including a 22-bp sequence with a direct repeat that shows homology to a TRE/RARE (20). We synthesized this DNA sequence and derivatives of it (see Fig. 3) and inserted these sequences into the multiple cloning site of the

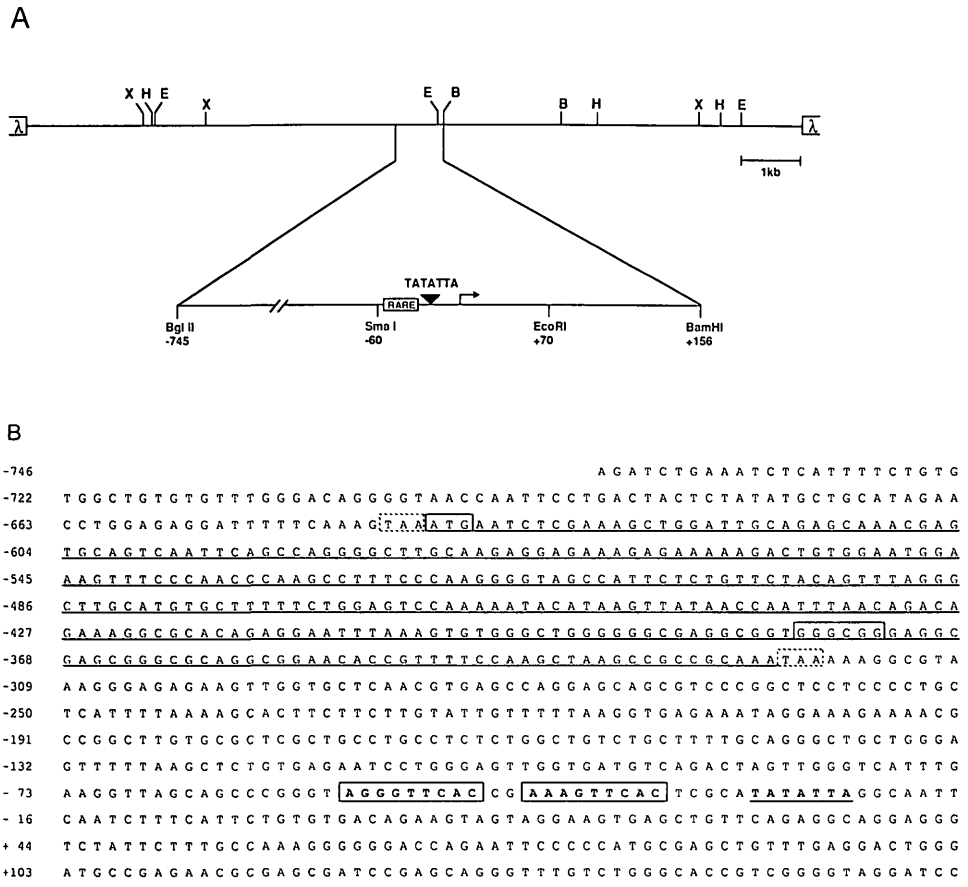

Fig. 1. A, Restriction Map of the Genomic Clone gRARβ-41 Coding for the 5′ Region of the RARβ/ϵ Gene

A human genomic placenta library was screened with a *Bam*HI-*Xho*I fragment of the 5′ region of the RARβ/ϵ cDNA as a probe (see *Materials and Methods*). One clone, gRARβ-41, was further analyzed. The *Bgl*II-*Bam*HI fragment which contains the RARE (*boxed*) and the TATA box are enlarged. The sequence of this region is shown in B. The restriction enzyme sites are *Eco*RI (E), *Bam*HI (B), HindIII (H), and *Xba*I (X). B, DNA sequence of the RARβ/ϵ promoter region. For sequencing, subfragments of the clone gRARβ/ϵ-41 were cloned into Bluescript M13- (Stratagene). The DNA sequence of the *Bgl*II-*Bam*HI fragment from clone gRARβ/ϵ (A) is shown. A direct repeat in the RARE is shown in *boxed bold characters*. The TATA box is shown in *underlined bold characters*. Up-stream of the RAR promoter is an open reading frame (*underlined*) framed by stop codons (*dotted boxes*). Within this open reading frame is located a *Sph* site (*boxed*).

reporter gene vector pBLCAT2 that contains a thymidine kinase (tk) promoter (21). The direct repeat construct ($RARE_{wt}$; that contains the sequences *boxed* in Fig. 1B) responds to RA and RARs very similarly as the constructs containing the RARβ/ϵ promoter, although the tk promoter is approximately 10-fold stronger. A clear RA response is observed in the absence of cotransfected receptors in CV-1 cells. The RA response is increased 3-fold when RARβ is cotransfected. RARγ, as observed previously, leads only to a small increase (1.5- to 2-fold; Fig. 3). A double insert of the RARE sequence further increases responsiveness to RA in the absence and presence of cotransfected RARβ. Neither the single nor the double $RARE_{wt}$ construct can be activated by TRβ. For comparison, the RA-responsive TRE_{pal} (10, 11, 20) is shown, which is activated approximately equally well by TRβ, RARβ/ϵ and RARγ. In the absence of cotransfected RARs, little activation of the TRE_{pal} is observed, while both the single and, in particular, the double $RARE_{wt}$ are highly RA responsive in the absence of cotransfected RARs (Fig. 3). The RAR-responsive TRE contains a palindromic symmetry, while the RARE delineated here is a direct repeat (see Fig. 3). To determine the functional significance of the observed RARE symmetry, two sequences ($RARE_{pal}$ and $RARE_{rev}$) were investigated in which the RARE was converted into a palindrome by inverting the second repeat (see Fig. 3). Neither one of these palindrome sequences could function as a RARE (Fig. 3). Thus, the direct repeat structure of the minimal RARE defined here is essential for its function.

The RARE Shows RARβ/ϵ Specificity in F9 Cells

F-9 embryonal carcinoma cells have been shown to express endogenous RARα and RARγ in the absence of RA, while RARβ is induced by RA (17, 22). We investigated the ability of the RARE to function in these cells, since the RARβ/ϵ promoter may contribute an essential signal for the differentiation response of the

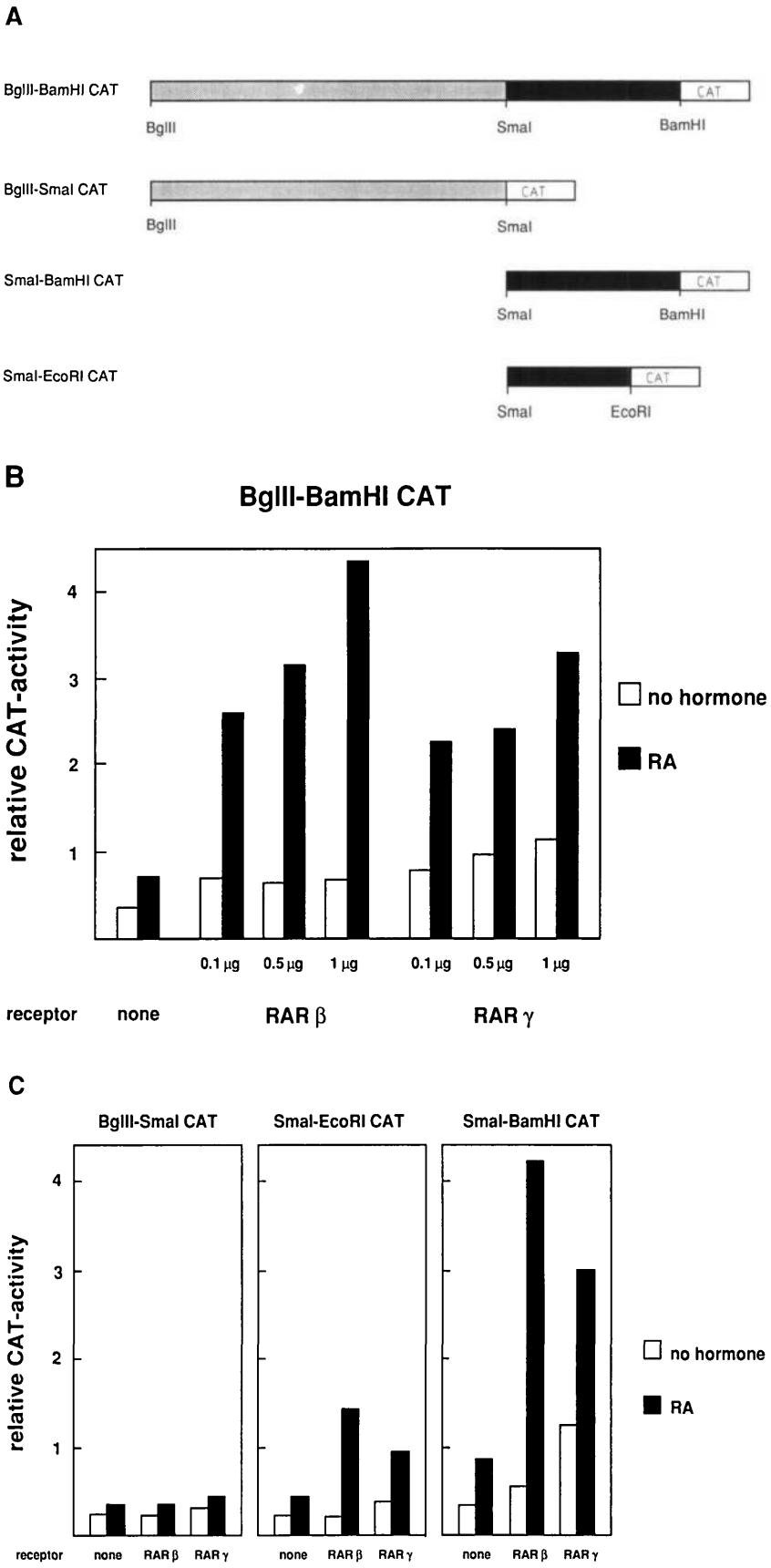

Fig. 2. Analysis of the RARβ/ϵ Promoter

A, Schematic illustration of the reporter plasmids used in this study. The *Bgl*II-*Bam*HI fragment or the subfragments *Bgl*II-*Sma*I, *Sma*I-*Eco*RI, and *Sma*I-*Bam*HI were fused to the CAT reporter gene in the vector BS-CAT. B, Transient transfection studies. Concentration-dependent activation of the RARβ/ϵ promoter by RARβ and RARγ. CV-1 cells were cotransfected with 2 μg

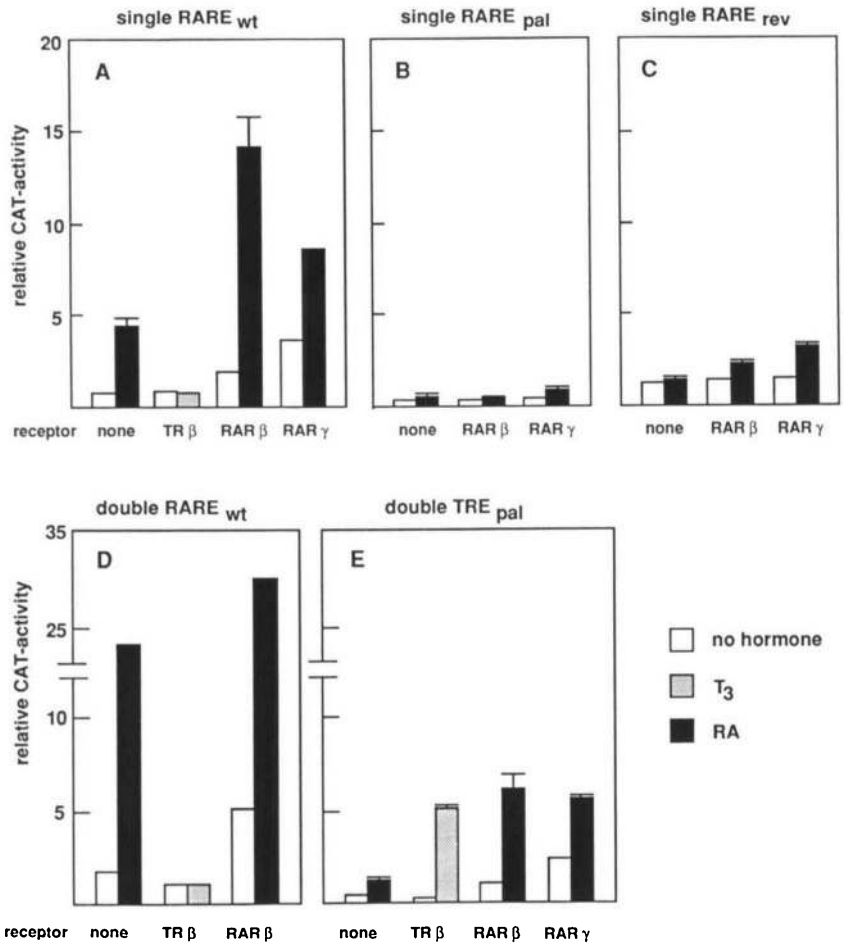

Fig. 3. The RARβ Promoter Contains a Highly Responsive and Specific Element for RARs

The synthetic oligonucleotides shown below (A through E; containing *Bgl*II and *Hin*dIII linkers) were cloned into pBLCAT2 at position −105 of the tk promoter. The derived reporter plasmids containing RARE-derived or TRE inserts were transiently transfected into CV-1 cells, alone or together with expression vectors for TRβ, RARβ, or RARγ. Cells were treated with hormone (T_3, 50 nM; RA, 600 nM), and 24 h later assayed for CAT activity. A, Single $RARE_{wt}$ (GATCTGT*AGGGTTCACCGAAAGTTCAC*TCA). B, Single $RARE_{pal}$ (GATCTGT*AAGGTTCACCGGTGAACCTT*ACA). C, Single $RARE_{rev}$ (AGCTTGT*AGGGTTCACCGGTGAACTTT*TCA). D, Double $RARE_{wt}$ (GATCTGT*AGGGTTCACCGAAAGTTCAC*TCAGATCTGT*AGGGTTCACCGAAAGTTCAC*TCA). E, Double TRE_{pal} (GATC*TCAGGTCATGACCTGA*GATC*TCAGGTCATGACCTGA*).

F9 cells. Both, the synthetic RARE construct ($RARE_{wt}$) and the RARE promoter fragment (*Sma*I-*Bam*HI) respond to RA in F9 cells (Fig. 4A). However, only cotransfected RARβ/ε is able to activate CAT in the presence of RA above the level observed in the absence of cotransfected receptors on both RARE constructs. RARγ does not activate the RARE constructs above the level induced by the endogenous receptors, whereas with the TRE-CAT construct, both RARγ and RARβ/ε are potent activators (Fig. 4). Increasing the amount of cotransfected RARγ did not increase the level of CAT activity from the RARE construct (data not shown). Thus, the RARE shows a differential RAR response in F9 cells, such that the endogenous RARs are more efficient activators than exogenous RARβ, while exogenous RARγ does not show any measurable activation activity. In contrast, on the TRE_{pal} the exogenous receptors RARβ and RARγ are as efficient activators as the endogenous RARs (Fig. 4).

The RARE Binds RARs and TRs with High Affinity

We have shown previously that binding of nuclear receptors to their specific response element can be ligand

of the reporter plasmid and various amounts of the receptor expression vector. Cells were treated with 1 μM RA (■) or no hormone (□), and 24 h later assayed for CAT activity. A representative experiment is shown. C, Activation of different RARβ/ε promoter constructs by RARβ and RARγ. CV-1 cells were cotransfected with 2 μg of the reporter plasmids and 1 μg of the receptor expression vectors. Cells were treated with 1 μM RA (■) or no hormone (□), and 24 h later assayed for CAT activity. A representative experiment is shown.

independent (10). In the case of the TRs, binding to the TRE in the absence of ligand is of functional significance and is important for restricting the TRE response to thyroid hormone (10; Zhang, X.-K., K. N. Wills, G. Graupner, M. Tzukerman, T. Hermann, and M. Pfahl, submitted). We have used the gel shift assay to analyze the binding of *in vitro* synthesized receptors to the *Sma*I-*Eco*RI fragment. RARβ, RARγ, and TRβ bind strongly to the DNA fragment in the presence and absence of ligand. Binding is completely inhibited by a 50-fold molar excess of synthetic RARE, but not by a random DNA sequence of comparable size. We also investigated binding to the synthetic RARE and the TRE_{pal} using the same protein extracts. While specific binding of the RARs to the TRE_{pal} can barely be observed, binding of the RARs to the RARE is very efficient (Fig. 5, A and B). However, TRβ binds about equally well to the RARE (data not shown) and the TRE (Fig. 5C). The reduced binding by TRβ to the TRE in the presence of ligand has been found previously (Zhang, X.-K., K. N. Wills, G. Graupner, M. Tzukerman, T. Hermann, and M. Pfahl, submitted). Thus, RARE binds RARs with high affinity independent of whether the RARs are associated with their specific ligand. The affinity of the RARs for the single RARE appears to be much higher than that for the double TRE, while TRβ binds to both elements with high affinity.

Control of the RARβ/ϵ Promoter Is Restricted to RARs

To determine whether the RARβ/ϵ promoter can also be regulated by other receptors and/or transcription factors, we cotransfected TRα, TRβ, ER, and the human glucocorticoid receptor (GR) with the *Bgl*II-*Bam*HI reporter gene construct into CV-1 cells. None of these receptors was able to activate this promoter significantly in the absence or presence of their specific ligand (Fig. 6A). Since TRβ binds the RARE with high affinity, we investigated the possibility that TRβ can repress RAR activity by competing for the RARE. When we cotransfected RARβ together with TRβ, we did not observe significant inhibition of RAR activity in the presence or absence of ligand (Fig. 6B). A small repression effect was observed when the TRβ was present in a 2- to 4-fold excess over RAR in the presence of the heterologous ligands. This effect was also observed with ER and GR receptors and could be due to a competition for functionally limiting transcription factors, as observed for the ER by others (23). The proto-oncoproteins c-*jun* and c-*fos* also did not show any significant effect on RAR activity in CV-1 cells. A small enhancing effect by c-*jun* was observed, but did not appear to be specific for the RARβ promoter, since another similar CAT construct that lacked the RAR promoter was also induced (data not shown).

DISCUSSION

We have cloned and characterized a RARE in the promoter of the RARβ/ϵ gene. This response element is located adjacent to the TATA box and thereby is part of a natural minimal RA-responsive promoter that in

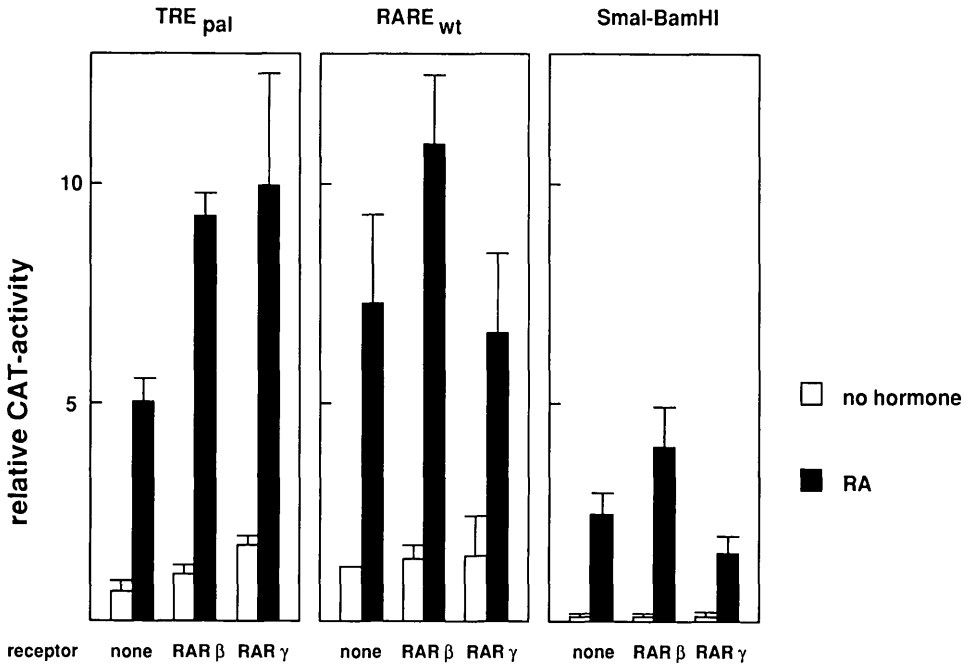

Fig. 4. Differential Activation of the RARE by RARs in F9 Cells

Comparison of TRE and RARE activation by RARs in F9 cells. F9 cells were cotransfected with 2 μg receptor and 4 μg of the reporter plasmid TRE_{pal}-CAT, $RARE_{wt}$-CAT, or Sma-*Bam*HI-BS-CAT. Cells were grown in the presence of 0.1 μM RA (■) or no hormone (□) and assayed for CAT activity 24 h later.

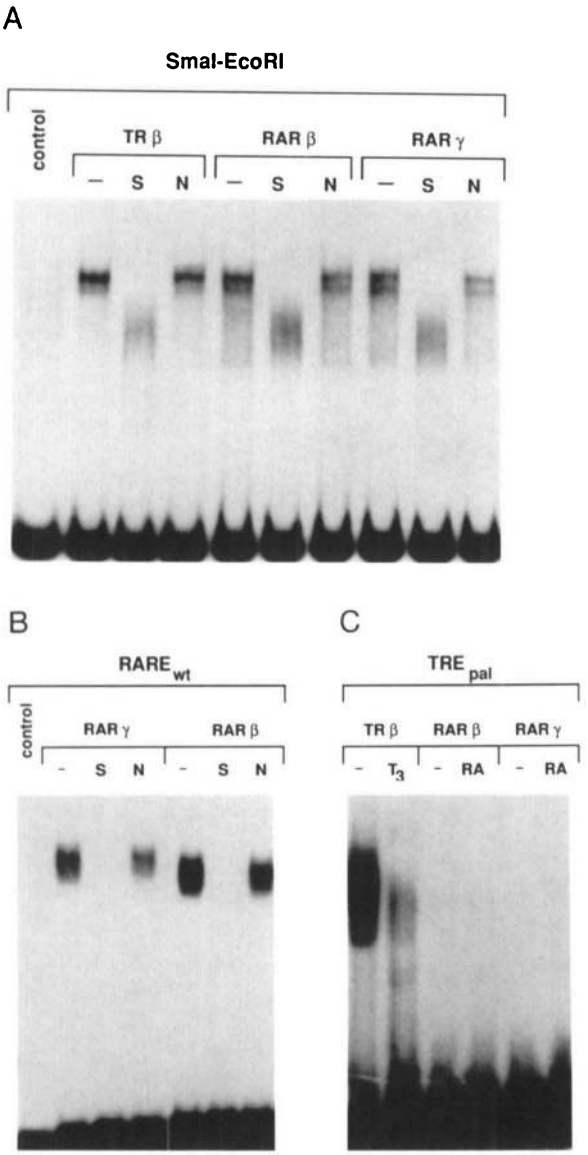

Fig. 5. Specific Binding of TRβ, RARβ, and RARγ to RARE and TRE

Receptor proteins synthesized by *in vitro* transcription/translation were incubated with the ^{32}P-labeled *Sma*I-*Eco*RI DNA fragment derived from the RARβ/ϵ promoter (A), a RARE$_{wt}$ (B; see Fig. 3), or double TRE$_{pal}$ (C; see Fig. 3) in the presence or absence of their respective ligand (10^{-7} M). For competition experiments, a 50-fold excess of unlabeled specific RARE fragment (S) or nonspecific DNA fragment with comparable length (N) or no DNA (–) was included in the binding assay. The DNA-protein complexes were analyzed on a 5% nondenaturing polyacrylamide gel.

CV-1 cells may not require other factors beyond those associated with a TATA box. These data are consistent with the recent report by de Thé *et al.* (13). We show, in addition, that contrary to the TRE, this RARE is specific for RARs and is not a response element for the closely related TRs. Interestingly, we found that TRs bind to this sequence with high affinity. However, this binding does not allow TRs to repress RAR activation of the RARE. From the gel retardation analysis it is also apparent that both RARs bind the RARE much more strongly (10- to 20-fold) than they bind the TRE in the presence and absence of ligand. The RAR-RARE interaction may, therefore, be strong enough to prevent competitive interference of TRs. This is consistent with a model developed for the TRE regulation of gene transcription by TRs, in which the unliganded receptor binds its own response element with high affinity, which allows it to function as a response element-specific repressor (10; Zhang, X.-K., K. N. Wills, G. Graupner, M. Tzukerman, T. Hermann, and M. Pfahl, submitted) or silencer (24). Future experiments will have to show whether this is also the case for the RARs.

The majority of efficient receptor response elements have an inverted repeat symmetry (20, 25, 26), although direct repeats are also found in glucocorticoid response elements (27). The function of the RARE defined here is dependent on the direct repeat symmetry, since palindromic versions of the RARE do not allow activation of transcription (Fig. 3). Vasios *et al.* (12) have identified another RA-responsive DNA region in the laminin B1 promoter. This promoter shows a relatively delayed response to RA (28, 29). Two symmetrical regions were described that are similar or identical to half-sites of the estrogen response element and the TRE, but show less similarity with the here-identified RARE. One of these regions also contains a direct repeat. A comparison of the specificities and efficiencies of these RA-responsive regions with the RARE defined here will be of interest.

Receptors have been shown to bind as dimers (9, 30, 31). The ability to interact with palindromic sequences (TRE$_{pal}$) and direct repeat sequences (RARE) would then suggest that receptors may have more than one interface for subunit-subunit interaction. This is also supported by the finding that synergistic DNA binding of RARs and TRs is observed with a double TRE$_{pal}$ compared to a single TRE$_{pal}$ (Zhang, X.-K., K. N. Wills, G. Graupner, M. Tzukerman, T. Hermann, and M. Pfahl, submitted).

While TRs and RARs bind strongly to the RARE, their activation capacities differ. RARβ is a stronger activator than RARγ on the RARE, in particular in F9 cells, while TRβ does not activate the RARE. This is independent of whether natural promoter constructs (*Bgl*II-*Bam*HI) or synthetic constructs are used (Fig. 2). Thus, specific DNA binding in the presence of ligand is not sufficient for gene activation. The DNA sequence bound by the receptors may, therefore, influence their activation configuration, as has been observed for other transcription factors (32). Since the DNA-binding domains of the two investigated RARs do not differ substantially (1, 17), it can be assumed that other features present in RARβ, but not in RARγ, are essential for function of the investigated RARE. Thus, it is possible that the RARγ-2 isoform recently reported (19, 33), which contains an amino-terminal domain more closely related to the RARβ, is a more effective RARE activator. Alternatively, the ligand/dimerization domains may de-

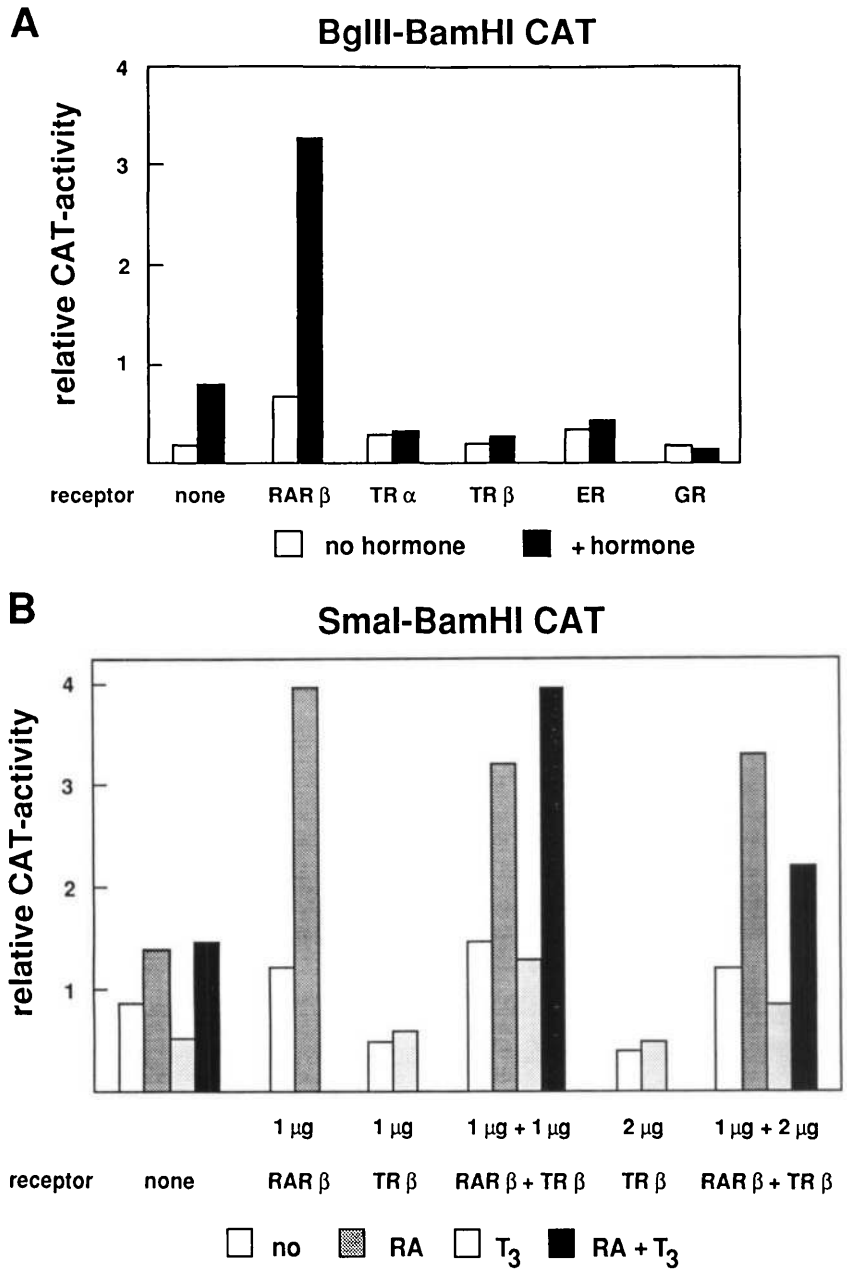

Fig. 6. A, Analysis of the RARβ Promoter with Heterologous Receptors

CV-1 cells were cotransfected with 2 μg *Bgl*II-*Bam*HI-BS-CAT reporter gene and 1 μg receptor. Cells were induced by 1 μM RA (RARs), 100 nM T_3 (TRs), 10 nM estradiol (ER), 100 nM dexamethasone (GR; ■), or no hormone (□), and 24 h later assayed for CAT activity. A representative experiment is shown. B, Cotransfection of RARβ and TRβ. CV-1 cells were cotransfected with 2 μg of the *Sma*I-*Bam*HI-BS-CAT construct and the indicated amount of the receptor expression vectors. Cells were induced by 1 μM RA, 100 nM T_3, or both, and 24 h later assayed for CAT activity. A representative experiment is shown.

termine differential response element activation, since they control receptor dimerization and, thereby, DNA binding.

The high endogenous activity observed for all RARE constructs in F9 cells could be due to the presence of RARα, which has been shown to be an efficient activator of a triple version of this RARE in HepG2 cells (13). However, it cannot be excluded that the most efficient RARE activators are neither of the RARs, but belong to the RXR class, a new type of retinoid receptor recently identified (34). This would be consistent with the observation that the RARE functions well in CV-1 cells in the absence of cotransfected RARs, in contrast to the TRE that does not show any significant response in the absence of cotransfected receptors in these cells (Fig. 3E) (10). This effect becomes particularly striking when the double RARE construct is used (Fig. 3D). Alternatively, the high affinity of RARs for the RARE may allow low levels of RARs known to be present in CV-1 cells (our unpublished results) to activate the

RARE, but not the TRE, which has a much lower affinity for RARs (Fig. 5). The differential response of the receptors for the different elements observed here reveals some of the complexity of the RA response network in which RARs can function as master regulators, as they can regulate their own synthesis and, thereby, maintain a differentiated state of a cell.

MATERIALS AND METHODS

Isolation of Genomic Clones

A genomic human placenta library (Stratagene, La Jolla, CA) was screened with a *Bam*HI-*Xho*I fragment (position 8–545) of the RARϵ clone BI-RAR (1) under stringent conditions in 6 × sodium saline citrate (SSC), 0.5% sodium dodecyl sulfate (SDS), 0.1% BSA, Ficoll, polyvinylpyrrolidone, and 200 μg/ml denatured salmon sperm DNA at 65 C in 2 × SSC-0.1% SDS for 30 min (twice), followed by one wash in 0.2% SSC-0.1% SDS for 15 min. The insert of one positive clone, gRARβ-41, was subcloned in fragments into the plasmid vector Bluescript M13- (Stratagene) for restriction enzyme mapping and sequencing. A 907-bp *Bgl*II-*Bam*HI fragment was sequenced.

DNA Sequence Analysis

All sequencing reactions were performed using the dideoxynucleotide chain termination method (35) with the sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH). Both DNA strands were sequenced.

Construction of Reporter and Expression Plasmids

A Bluescript-CAT (BS CAT) vector plasmid was constructed by insertion of the *Xho*I-*Kpn*I fragment of the pBLCAT2 plasmid (21) into the multiple cloning site of Bluescript (Stratagene). The inserted fragment contains the CAT gene as well as the small t intron and the polyadenylation signals from SV40. Four different BS-CAT reporter plasmids were constructed that contained either the complete 907-bp *Bgl*II-*Bam*HI fragment or portions of it, as indicated in Fig. 2.

RAR Expression Vectors

PECE-RARϵ/β has been previously described (10). A full-length RARγ cDNA clone was isolated from a human placental library (Lehmann, J. M., and M. Pfahl, unpublished). PECE-RARγ was obtained by cloning an *Eco*RI-*Sma*I fragment containing the complete coding region of RARγ into the *Eco*RI and *Sma*I sites of the pECE expression vector (36).

Tissue Culture, Transient Transfection, and CAT Assay

CV-1 cells were plated at 1.3 × 10^6/dish 16–24 h before transfection in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum. A modified calcium phosphate precipitation procedure was used for transient transfection (37) and was previously described (38). Briefly, 2 μg reporter plasmid, 3 μg β-galactosidase (β-gal) expression vector (pCH 110, Pharmacia, Piscataway, NJ), and variable amounts of receptor expression vector were mixed with carrier DNA (Bluescript) to 20 μg total DNA/plate. The β-gal activity was determined as previously described (39) with a modification to be run in microtiter plates. CAT activity was determined using [^{3}H]acetyl coenzyme-A as substrate (40). To normalize for transfection efficiency, CAT activities determined were corrected for β-gal activity.

The Gel Retardation Assay

One to 5 μl *in vitro* translated receptor protein were incubated with the ^{32}P-labeled TRE monomer or dimer in a 20 μl reaction mixture containing 10 mM HEPES buffer (pH 7.9), 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl, 10% glycerol, and 1 μg poly(dI-dC) at 25 C for 20 min. The reaction mixture was then loaded on a 5% nondenaturing polyacrylamide gel containing 10 mM HEPES, pH 7.9, and 3.3 sodium acetate, pH 7.9.

Preparation of Receptor Proteins

Receptor proteins were synthesized by *in vitro* translation/transcription as previously described (10, 41, 42). Receptor cDNA was cloned into the Bluescript vector. One microgram of linear template DNA was used for *in vitro* transcription in a 50-μl reaction mixture containing 40 mM of each ribonucleotide, 1 U RNase inhibitor (Stratagene), and 10 U of either T7 or T3 RNA polymerase (Stratagene). The transcription reaction was incubated at 37 C for 30 min. The reaction was diluted 10-fold with 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, and 10 mM NaCl and incubated with 1 U RNase-free DNase-I (Bethesda Research Laboratories, Gaithersburg, MD) at 37 C for 15 min to remove the DNA template. Receptor proteins were synthesized *in vitro* using the rabbit reticulocyte translation system (Promega, Madison, WI). Approximately 1 μg RNA was mixed with 35 μl nuclease-treated reticulocyte lysate, amino acid mix (20 μM of each), and 1 U RNase inhibitor in a total volume of 50 μl. The translation reaction was carried out at 30 C for 60–90 min. The synthesized receptor protein was then aliquoted and stored at −70 C. Alternatively, 2.5 μl [^{35}S]methionine (>1200 Ci/mmol; New England Nuclear, Boston, MA) was included in the translation reaction. The labeled receptor protein was then analyzed on SDS-polyacrylamide gel electrophoresis. The translation efficiency of each receptor was determined by measuring the amount of [^{35}S]methionine incorporated into the protein.

Preparation of Specific DNA Fragments

Three different DNA fragments were used: the *Sma*I-*Eco*RI fragment derived from the RARβ promoter; the RARE$_{wt}$, which is a 11-bp direct repeat response element (AGGGTTCACCGAAAGTTCAC) derived from RARβ promoter (−55 to −36); and the TRE, which is a 16-bp perfect palindrome response element (TCAGGTCATGACCTGA) (20). Both RARE$_{wt}$ and TRE oligonucleotides flanked by *Bgl*II adaptor sequences were synthesized using an Applied Biosystems (Foster City, CA) DNA Synthesizer and purified by polyacrylamide gel electrophoresis. Oligonucleotides were annealed and radioactively labeled using the Klenow fragment of DNA polymerase. To obtain TRE dimer, the synthetic TRE monomer was ligated, and double stranded dimer TRE was purified by polyacrylamide gel electrophoresis. In these dimers, the two TREs were separated by 4 bp of the *Bgl*II restriction site. Labeled RARE monomer or TRE dimer was purified by gel electrophoresis and used for the gel retardation assay.

Acknowledgments

We thank Waldemar Lernhardt for providing the genomic λ library, Michaela Pfahl for plasmid preparations, and Nancy Lyman and Kathleen Sweeting for preparing this manuscript.

Received June 20, 1990. Revision received August 8, 1990. Accepted August 17, 1990.

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This work was supported by NIH Grant DK-35083.

* Supported by a fellowship from the Deutsche Forschungsgemeinschaft.

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