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Characterization of an autoregulated response element in the mouse retinoic acid receptor type β gene

(vitamin A/steroid receptor/transcription)

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ABSTRACT **A sequence that confers transcriptional responsiveness to retinoic acid was identified in the promoter of the mouse retinoic acid receptor (RAR) β gene. This response element consists of a direct repeat of the sequence GTTCAC, separated by five nucleotides. Direct binding of the RAR to this sequence was demonstrated by gel retardation and immunoprecipitation assays. This element conferred retinoic acid responsiveness on heterologous promoters via all three subtypes of RAR yet failed to support transcriptional activation by the thyroid hormone, estrogen, glucocorticoid, or vitamin D receptors. Surprisingly, a high level of retinoic acid-dependent activation was seen in the absence of transfected RAR in 10 of 10 vertebrate cell lines, many functionally characterized previously as lacking endogenous receptor. This demonstrates an unusually high sensitivity of the retinoic acid response element to low levels of receptor and suggests expression of RAR in a wide variety of tissue types.**

The vitamin A derivative retinoic acid (RA) exerts profound effects in development, differentiation, and regeneration. Studies in the developing chicken limb bud have shown that RA is distributed in this tissue in a graded concentration along the anterior–posterior axis (1). Experimental perturbation indicates that this gradient bestows positional information upon the developing limb bud, suggesting that RA acts as a morphogen (2). When administered to pregnant rodents, RA causes a variety of embryonic malformations collectively known as RA embryopathy, including skeletal, heart, and craniofacial malformations (3). The embryonic period of sensitivity to RA differs for each tissue but occurs just prior to and during organogenesis, again suggesting that the process which RA affects is that of morphogenesis.

Three independent genes encode receptors for RA (RARα, -β, and -γ) and are members of the nuclear receptor gene superfamily, which includes those receptors that are responsive to steroid and thyroid hormones (4–9). These receptors all act by binding as dimers directly to target sequences, termed response elements, in the promoters of responsive genes and thereby activating or repressing transcription. The high level of amino acid homology in the DNA-binding domain of the three RARs (≈95%; ref. 9) suggests that for the most part all three recognize an identical set of target genes, although their transcriptional activation properties vary. Furthermore, the RARs contain sequences within their DNA-binding domains that suggest an overlap in response-element recognition with a subset of the nuclear receptor family, including the estrogen receptor (ER) and thyroid hormone receptor (TR) (10). Indeed, it has been shown directly that both TR and RAR are able to activate transcription through a common sequence derived from the thyroid hormone response element (TRE) of the growth hormone gene (11).

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One of the few identified primary target genes for RA transcriptional activation is the RARβ gene itself (12). This form of autoregulation might be a means of amplifying the effects of RA in responsive tissues by raising the level of receptor and therefore may play an important role in RA-dependent pattern formation and morphogenesis. In this paper, we define the sequences in the promoter of the mouse RARβ gene that confer RA responsiveness, and demonstrate that these sequences represent an extremely sensitive target specific for the three RAR subclasses. This RA response element (RARE) does not mediate significant activation by ER, glucocorticoid receptor (GR), vitamin D_3 receptor (VDR), or TR.

MATERIALS AND METHODS

Clones. A mouse liver genomic DNA library (Clontech) was screened with the *Bam*HI–*Sph* I fragment of the human RARβ cDNA clone B1-RARε (8). A *Sal* I restriction site was introduced into the genomic clone by site-directed mutagenesis; the 10-kilobase (kb) genomic fragment was then excised and cloned into the β-galactosidase vector pLSV (a derivative of pGH101; ref. 13), modified to contain a *Sal* I site and a polylinker sequence by oligonucleotide addition, to yield RAR-PL-βGAL. The 3.5-kb *Xho* I–*Sal* I fragment of RAR-PL-βGAL was subcloned into the chloramphenicol acetyltransferase (CAT) vector pUC^{PL}-CAT (14), which was modified to contain a *Sal* I site. Additional CAT constructs were generated by cloning fragments or oligonucleotides into the single *Hin*dIII site of ΔMTV-CAT, TK-CAT, or ΔSV-CAT and were sequenced prior to use. All inserts were in the sense orientation, except for ΔMTV-βRE5-CAT, which was in the opposite orientation.

Mutagenesis. RARβ sequences were modified by site-directed mutagenesis, using single-stranded plasmid DNA derived from Bluescript vectors (Stratagene) grown in the *Escherichia coli* dut^- ung^- strain CJ236 (Bio-Rad), by standard procedures. Mutagenesis was used to introduce a *Sal* I restriction site into the coding region of the RARβ genomic clone and to delete the βRARE. All mutations were confirmed by sequencing.

Cells. Cells were transfected in 10-cm dishes with 10 μg of DNA containing 5 μg of reporter plasmid, 1–2 μg of the reference plasmid (RSV-LUC, RSV-βGAL, or pCH110), pGEM-4 as carrier DNA, and, when used, 1 μg of Rous sarcoma virus (RSV)-derived receptor expression vector. Cells were harvested 1 day after addition of ligand. RA was added to a final concentration of 1 μM; other ligands were added to 0.1 μM. Control plates received solvent only. All CAT assays represent equivalent amounts of β-galactosidase

Abbreviations: RA, retinoic acid; RAR, RA receptor; RARE, RA response element; ER and ERE, estrogen receptor and its response element; GR and GRE, glucocorticoid receptor and its response element; TR and TRE, thyroid hormone receptor and its response element; VDR, vitamin D_3 receptor; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.

activity; β-galactosidase experiments were normalized to luciferase activity.

DNA Binding Assays. COS cells were transfected with receptor expression vectors or with control plasmids by the DEAE-dextran method, harvested, and lysed by freeze–thaw in 20 mM Hepes, pH 7.8/0.5 M KCl/20% (vol/vol) glycerol/2 mM dithiothreitol/0.5 mM EDTA/0.5 mM EGTA. For gel shift experiments, 4 μg of total protein was preincubated 20 min at room temperature in 20 mM Hepes, pH 7.8/80 mM KCl/20% glycerol/0.2 mM dithiothreitol with 2 μg of poly(dI-dC). Competitor oligonucleotide and labeled βRE2 oligonucleotide were added, and the mixture was incubated 30 min at room temperature and then electrophoresed through a 5% acrylamide/5% glycerol gel in 45 mM Tris/45 mM boric acid/1 mM EDTA. For immunoprecipitation experiments, 15 μl of lysate was added to 100 μl of 20 mM Tris, pH 7.8/2 mM dithiothreitol/0.5 mM EDTA containing 1 ng of probe and 2 μg of poly(dI-dC). After a 20-min incubation on ice, Pansorbin (Calbiochem) coated with anti-GR antibody (15) was added and the mixture was further incubated on ice 30 min. After two washes in 20 mM Tris, pH 7.8/75 mM NaCl/2 mM dithiothreitol/0.1% Nonidet P-40, bound DNA was eluted by resuspension in 0.1 M NaCl/1% SDS and heating 5 min at 65°C, then measured by liquid scintillation counting. RSV-GRR encodes amino acids 1–420 of the human GR derivative GRnx and amino acids 87–462 of the human RARα derivative RARnx (5). The sequence of the glucocorticoid response element (GRE) oligonucleotide has been described (16).

RESULTS

Identification of a RARE in the Promoter of the Mouse RARβ Gene. A mouse liver genomic DNA library screened with a human RARβ cDNA probe yielded a genomic fragment containing ≈10 kb of upstream sequence, the complete first exon, and 10 kb of the first intron. The upstream region was fused in-frame just downstream of the RARβ translation initiation codon to a β-galactosidase reporter gene (Fig. 1*a*) and introduced into mouse F9 teratocarcinoma cells (data not shown), which express high levels of endogenous RAR, or into monkey CV-1 kidney cells cotransfected with RAR expression vector. Enzyme activity was induced in both cases upon addition of RA, indicating that this region of genomic DNA contains a RA-responsive promoter.

Deletions from the 5′ end of RAR-PL-βGAL revealed that sequences mediating RA induction reside within the 2-kb *Nhe* I–*Sac* II fragment (Fig. 1*a*). To localize the RARE, subfragments of this region and then oligonucleotides were cloned into the enhancer-dependent CAT reporter plasmid ΔMTV-CAT, which contains the mouse mammary tumor virus promoter with the natural GREs deleted (17). The sequence represented by βRE1 (Fig. 1*b*) was found to confer RA inducibility (see below) and is termed the βRARE. Similar results were obtained when truncated thymidine kinase or simian virus 40 promoters were used (data not shown). The sequences shown in Fig. 1*b* reside close to the transcription initiation site, which has been mapped in the human RARβ gene to 27 bp downstream of the TATA box (18). The human and mouse RARβ genes are identical in sequence in this region (18).

Characterization of the βRARE. Previous work showed that a TRE can mediate RA-dependent transcriptional activation by transfected RARs in CV-1 cells. Nontransfected cells show no response (ref. 11; also see Fig. 2*a*). Thus, in the course of testing βRARE sequences for transactivation properties, we were surprised when ΔMTV-CAT constructs βRE1, -2, and -3 showed robust RA-dependent induction in the absence of cotransfected RAR expression vector (Fig. 2*a*). Cotransfection of RARα expression vector increased induction 2-fold (e.g., see Fig. 5). This suggests that CV-1 cells express a low level of endogenous RAR near saturation

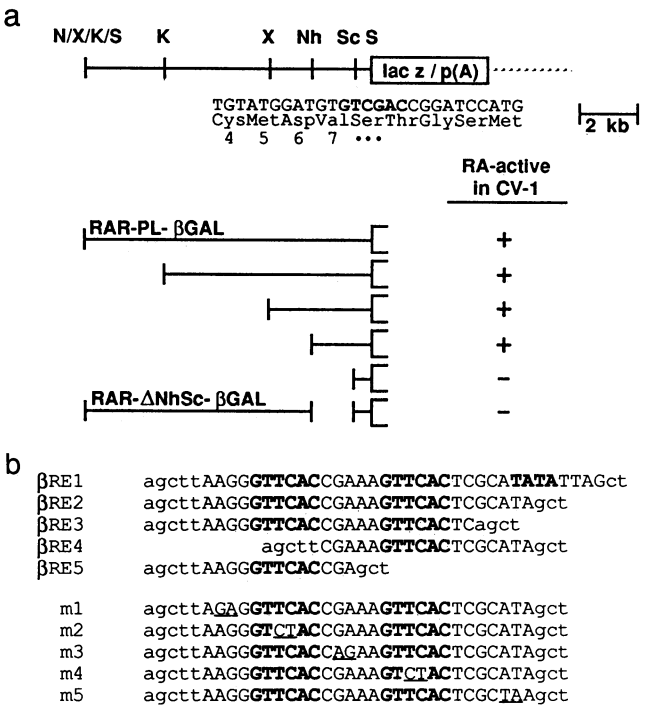


FIG. 1. Identification of the βRARE. (*a*) Deletion analysis of the RARβ promoter. The sequence at the junction between the mouse RARβ gene and the β-galactosidase (*lacZ*) reporter gene is as shown. Numbered amino acids correspond to the native RARβ translation product. Plasmids were transfected into CV-1 cells and assayed for β-galactosidase activity either without or with the addition of RA. Not all constructs shown were tested at the same time. Negative responses were 2-fold induction or less; positive inductions were 7-fold or greater. Restriction sites: N, *Not* I; X, *Xho* I; K, *Kpn* I; S, *Sal* I; Nh, *Nhe* I; Sc, *Sac* II. p(A), poly(A). The dotted line represents plasmid sequence. (*b*) Sequences of oligonucleotides used in these experiments. The terminal lowercase bases are foreign to the RARβ gene and were included to allow insertion into the single *Hin*dIII site of the ΔMTV vector. The 6-base-pair (bp) direct repeats and the TATA box are in bold type. Oligonucleotides m1–m5 represent internal mutations in the βRARE sequence; the underlined bases indicate changes from βRE2.

for activation of vectors containing the βRARE, but below a threshold for activation of the TRE. A survey of several different cell lines [CV-1, F9 and P19 (mouse teratocarcinomas), CHO, HeLa, NIH 3T3 (mouse fibroblasts), Rat2 fibroblasts, HT1080.T (human lymphoid cells), chicken embryo fibroblasts, and quail QT6 cells] indicated that all were able to efficiently transactivate the βRARE in a RA-dependent fashion in the absence of transfected RAR expression vector (data not shown).

Inspection of the sequences of βRE 1-3 (Fig. 1*b*) identifies a tandem repeat of the 6-bp motif GTTCAC. Constructs containing single copies of either the 5′ or the 3′ motif (βRE4 and βRE5) are functional but require cotransfected RARα, -β, or -γ expression vectors to be activated (Fig. 2*b*). This not only indicates that the βRARE is a target of all three RAR subtypes but also demonstrates that these half-sites can serve as a minimal RARE in the context of the ΔMTV promoter. One interpretation of these results is that a single element is a low-affinity target requiring high levels of receptor for activation, whereas the tandem repeat represents a high-affinity binding site that is able to respond to the low level of endogenous RARs present in CV-1 and other cells.

The βRARE Is Required for RA Induction of the RARβ Gene. The 3.5-kb *Xho* I–*Sal* I promoter fragment of the RARβ gene (Fig. 1*a*) was cloned so as to direct expression of a CAT reporter construct. When introduced into F9 teratocarci-

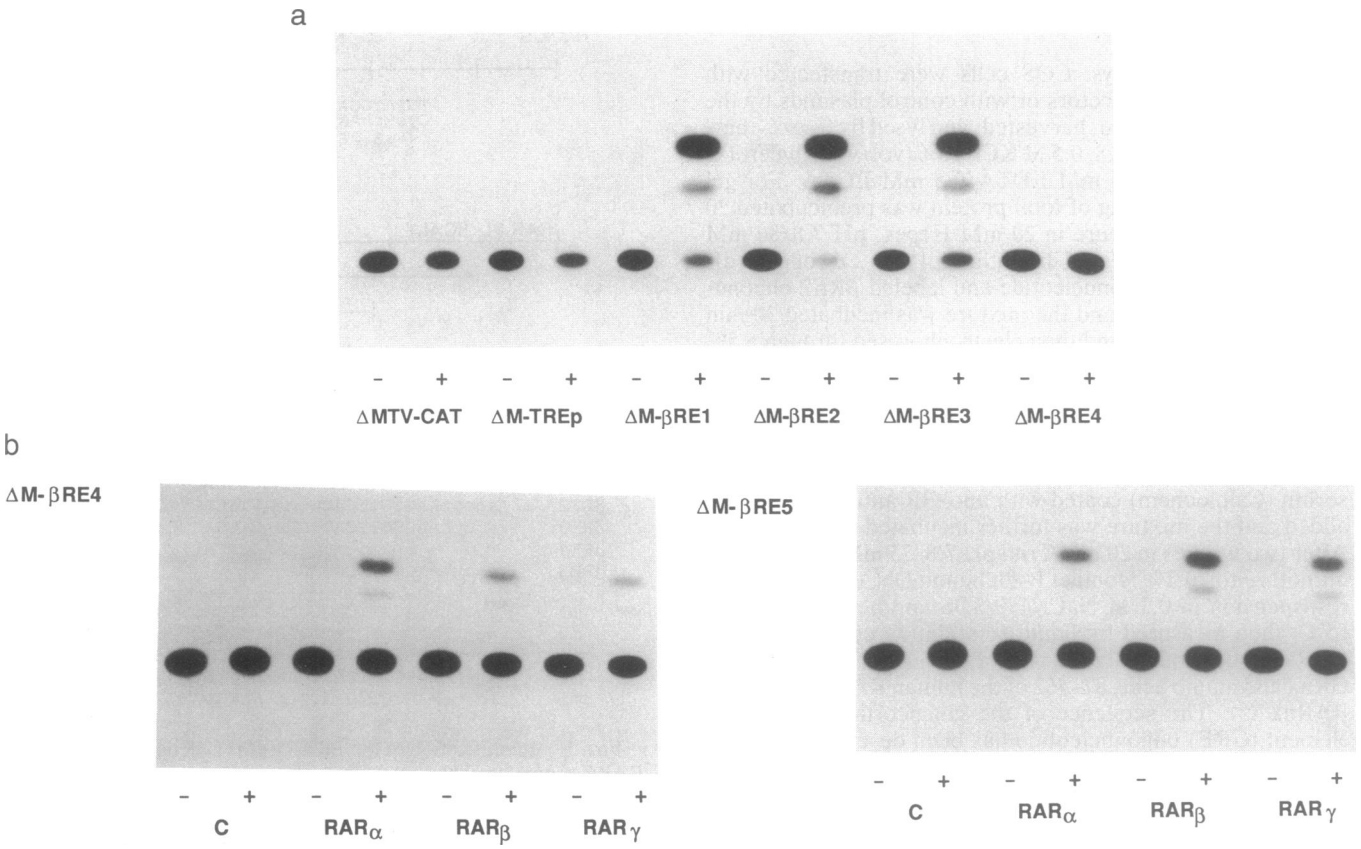


FIG. 2. Characterization of the βRARE. (*a*) The βRARE confers RA induction in CV-1 cells in the absence of transfected receptor. The indicated ΔMTV-CAT constructs were introduced into CV-1 cells without RAR expression vector, and the transfected cells were treated as indicated either with solvent (−) or with RA (+). TREp is a palindromic variant of the rat growth hormone TRE, which has been shown to also be a RARE when cotransfected with RAR expression vectors (see text). (*b*) Half-site sequences independently confer RA responsiveness. The indicated reporter plasmids were introduced into CV-1 cells with RAR expression vectors or with a control construct encoding an antisense message (C), and the transfected cells were treated with solvent (−) or RA (+). Induction of the CAT reporter gene was assayed by incubating cell extracts with [^{14}C]chloramphenicol. Chloramphenicol (lowest spot in each lane) and its acetylated products (upper spots) were separated by thin-layer chromatography and detected by autoradiography.

noma cells, this construct showed a 15- to 20-fold induction upon addition of RA (Fig. 3). A 24-bp deletion that excised the βRARE [as represented by βRE3 (Fig. 1*b*)] but left the TATA box intact abolished RA induction (Fig. 3), indicating that sequences important in RA induction of the RARβ gene are not repeated elsewhere in the 3.5-kb promoter fragment.

Direct Binding of the RAR to the βRARE. To demonstrate that the sequences described above are direct binding sites for the RAR, cell extracts were examined by gel retardation assays with ^{32}P-labeled βRE2 (Fig. 4*a*). Mock-transfected cells showed an endogenous activity that bound the labeled oligonucleotide; unlabeled βRE2 or TRE (a known RAR binding site; ref. 11) competed with labeled βRE2 for binding to this activity, whereas an unlabeled GRE did not. This activity presumably represents the endogenous RAR. Cells transfected with a RARβ expression vector showed an increase in this binding activity (Fig. 4*a*). To unambiguously demonstrate that binding is a consequence of the transfected receptor, a hybrid receptor was created (termed GRR) that introduces a unique GR epitope in the amino terminus of the RAR. This hybrid is RA-dependent and exhibits the response element specificity of the RAR in transfection experiments (D. Mangelsdorf and R.M.E., unpublished observations) and has the advantage that it can be selectively immunoprecipitated with antibody to the GR epitope. Cell extracts containing the hybrid receptor bound labeled βRE2 oligonucleotide with the expected specificity (Fig. 4*b*). Thus, the βRARE serves as a direct target of the RAR.

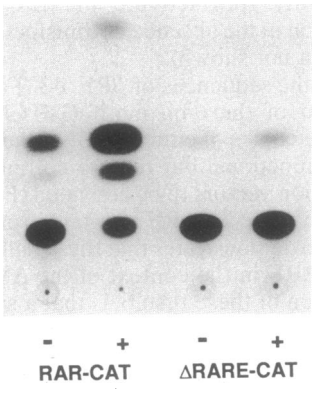
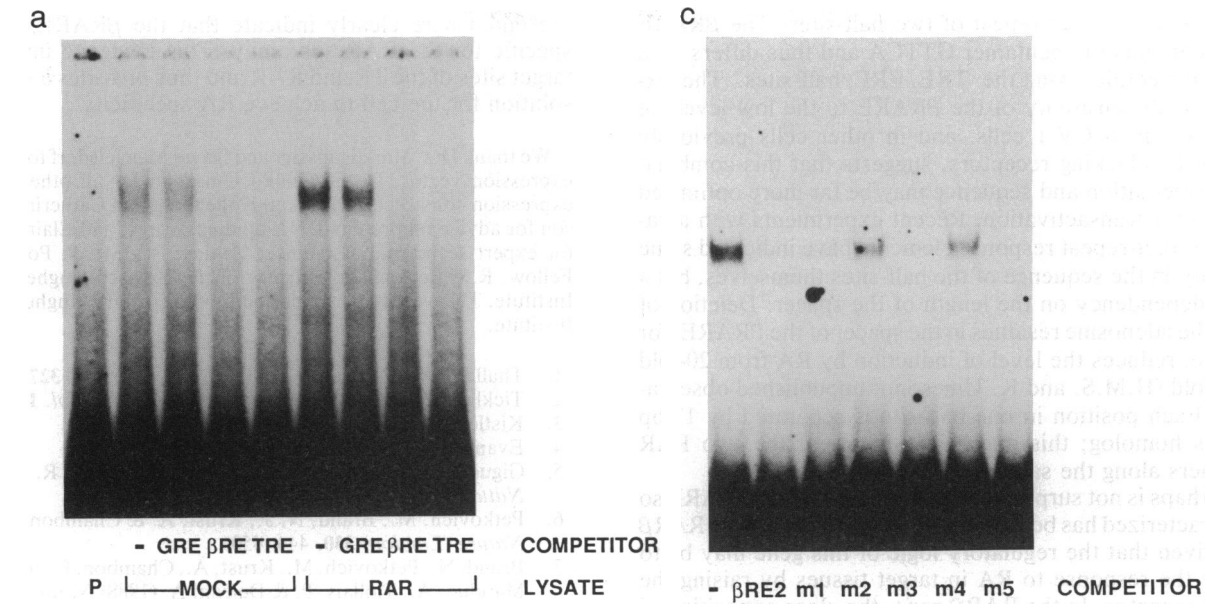


FIG. 3. The βRARE is unique in the RARβ promoter. CAT constructs bearing 3.5 kb of the wild-type promoter sequence (RAR-CAT), or a deletion construct that lacks 24 bp containing the βRARE (ΔRARE-CAT) were transfected into F9 cells, and the cells were treated with solvent (−) or RA (+).

The importance of the direct repeats of the βRARE in RAR recognition was established using the gel retardation assay. Mutant oligonucleotides (m1–m5; Fig. 1*b*), each with changes in two bases from the parental βRE2 sequence, were used as competitors for binding of RAR extract to a labeled βRE2 probe. Mutations in either of the repeats reduced the ability of these oligonucleotides to act as competitors, whereas mutations elsewhere in the sequence were much less consequential (Fig. 4*c*).



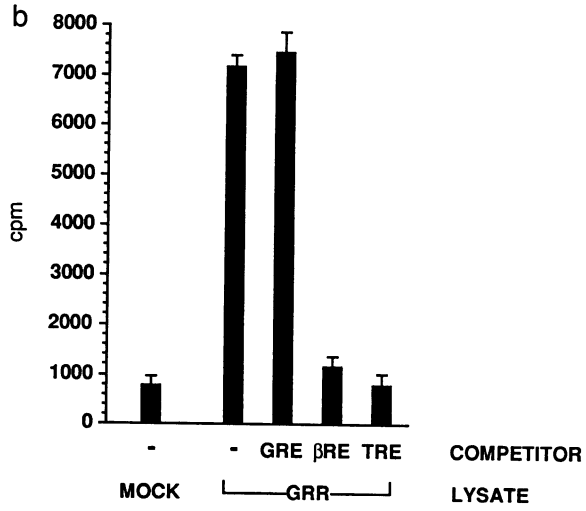


FIG. 4. Protein–DNA binding assays with βRARE sequences. (*a*) Gel shift assay. Extracts of COS cells transfected with RSV-RARβ, or of mock-transfected cells, were incubated with labeled βRE2 oligonucleotide with or without added unlabeled competitor, then subjected to electrophoresis. The same mass of protein (4 μg) was used in all experimental lanes. Competitors were present at a 25-fold excess over the labeled probe. TRE, TREp; P, probe alone. (*b*) Immunoprecipitation assay. Extracts of mock- or RSV-GRR-transfected COS cells were incubated with labeled βRE2 oligonucleotide with or without competitor (present at 25-fold excess); radioactivity immunoprecipitated by anti-GR antibody was determined. All values shown reflect the average of duplicate measurements, after subtraction of background (determined by parallel analysis with lysis buffer only and no added cell extract), which in this experiment measured 770 cpm. (*c*) Importance of the direct repeats in RAR recognition. Oligonucleotides shown in Fig. 1*b* were used as competitors (present in 12-fold excess) in binding assays with RARβ extracts and labeled βRE2 as probe.

Activation of the βRARE Is Selective for the RAR. Many previously characterized response elements are targets of more than one type of receptor: both the RAR and the TR are able to activate a TRE (11); the RAR, TR, and ER all activate the estrogen response element (ERE) of the vitellogenin gene (K. Umesono and R.M.E., unpublished observations); the progesterone, mineralocorticoid, and glucocorticoid receptors all activate the GRE (19). Thus, it might be expected that the response element of the RARβ gene would reciprocally be responsive to the TR, ER, and/or other members of the receptor superfamily. Cotransfection of the TR, ER, GR, or VDR in CV-1 cells with construct βRE1 failed to result in appreciable activation in the absence or following addition of the appropriate ligand (Fig. 5), although these receptors can efficiently activate their cognate response elements. The response element of the RARβ gene therefore mediates transcriptional activation specifically and selectively by the RAR.

DISCUSSION

The RARs have previously been shown to bind to TREs and EREs that contain an inverted repeat of the sequence GGTCA (10). The RARE of the RARβ gene is instead

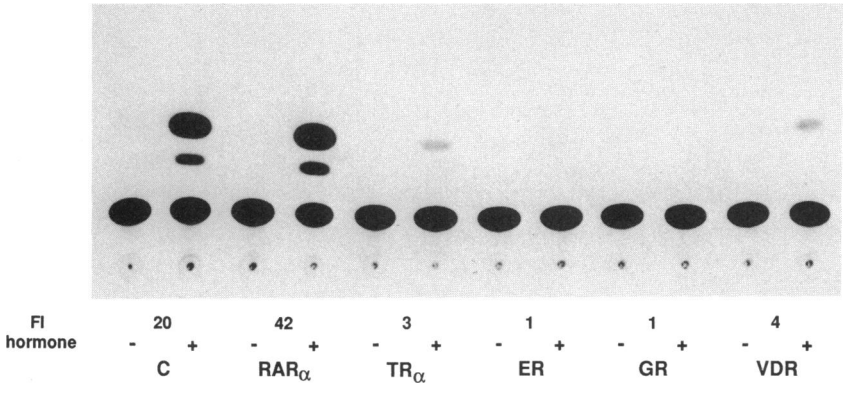


FIG. 5. Specificity of transactivation of the βRARE with various receptors. CV-1 cells were cotransfected with RSV expression vectors encoding the indicated receptors (C represents a control expression construct encoding an antisense message) and reporter construct ΔMTV-βRE1-CAT. Solvent only (−) or ligands (+) were added (final concentration, 1 μM RA or 0.1 μM triiodothyronine, estradiol, dexamethasone, or 1,25-dihydroxyvitamin D_3) and CAT activity in cell extracts was assayed. FI, fold induction.

composed of a direct repeat of two half-sites. The βRARE repeat contains the pentamer GTTCA and thus differs by a single nucleotide from the TRE/ERE half-sites. The extremely high sensitivity of the βRARE to the low levels of RAR present in CV-1 cells, and in other cells previously described as lacking receptors, suggests that this combination of orientation and sequence may be far more optimized for receptor transactivation. Recent experiments with a variety of direct-repeat response elements have indicated some flexibility in the sequence of the half-sites themselves, but a strong dependency on the length of the spacer. Deletion of one of the adenosine residues in the spacer of the βRARE, for example, reduces the level of induction by RA from 20-fold to <2-fold (H.M.S. and K. Umesono; unpublished observations). Each position in one half-site is separated by 11 bp from its homolog; this may serve to orient the two RAR monomers along the same face of the DNA helix.

It perhaps is not surprising that the most efficient RARE so far characterized has been found in the promoter of the RARβ gene, given that the regulatory logic of this gene may be to amplify the response to RA in target tissues by raising the level of receptor. In the RARβ gene, the close apposition of the RARE to a TATA box may endow this gene with even greater sensitivity to transcriptional activation, although the experiments described here with heterologous promoters would not detect such an effect. The observation that many, if not all, cultured cells express RAR at levels sufficient to activate the βRARE suggests that in addition to its importance in differentiation, RA (and RARβ) may have a role in homeostasis, similar to glucocorticoid and thyroid hormones. On the other hand, we have observed that the level of expression of the RARβ promoter varies depending on cell type, from fairly low in CV-1 cells to very high in F9 cells. This is apparently not a consequence of either the type or amount of receptor in these cells and therefore suggests that additional factors are regulating the expression of the RARβ gene and that the response to RA may be restricted to particular cell types.

The demonstration that TREs could function as RAREs led to the suggestion that these related receptors might recognize overlapping sets of response elements. The experiments presented here clearly indicate that the βRARE is highly specific for the RAR subfamily. This evidence unlinks the target sites of the TR and RAR and thus provides a structural solution for the cell to achieve RA specificity.

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