Multiple parameters determine the specificity of transcriptional response by nuclear receptors HNF-4, ARP-1, PPAR, RAR and RXR through common response elements

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Received November, 20, 1997; Revised and Accepted March 18, 1998


ABSTRACT

A number of nuclear receptors, including retinoic acid receptors (RARs), retinoid-X receptors (RXRs), hepatocyte nuclear factor 4 (HNF-4), chicken ovalbumin upstream promoter transcription factor I (COUP-TFI), apolipoprotein regulatory protein 1 (ARP-1) and peroxisome proliferator-activated receptor (PPAR), bind to response elements comprised of two core motifs, 5′-RG(G/T)TCA, or a closely related sequence separated by 1 nt (DR1 elements). The potential role of the precise sequence of the core motif as well as the spacer nucleotide in determining specificity and promiscuity of receptor–response element interactions was investigated. We show here that nucleotides at base positions 1, 2 and 4 of the core motif as well as the spacer nucleotide determine the binding preference of base positions 1, 2 and 4 of the core motif and spacer nucleotide in conferring selectivity of the core motif and spacer nucleotide in determining specificity and the precise sequence of the core motif as well as the separated by 1 nt (DR1 elements). The potential role of repression by ARP-1 correlated with the relative in transcriptional activation by HNF-4 and PPAR:RXR and PPAR:RXR heterodimers. In transfection experiments HNF-4 and ARP-1 homodimers and RAR:RXR and PPAR:RXR heterodimers. In transfection experiments transcriptional activation by HNF-4 and PPAR:RXR and repression by ARP-1 correlated with the relative in vitro binding affinity provided the element was located within the proper promoter context. Furthermore, promoter context also determined whether an element that binds to HNF-4 and PPAR:RXR with equal affinity functions as an HNF-4 response element or PPAR response element. Thus, apart from the element-specific promiscuity and preferences in affinity for the receptors, additional promoter-specific transcription factors that interact with HNF-4 and PPAR:RXR determine the specificity of transcriptional response through DR1-type elements.

INTRODUCTION

The steroid hormone receptors, thyroid hormone receptors and orphan receptors form a large family of nuclear receptors that regulate gene expression through binding to cis-acting sequences in their respective target genes (1–3). The specific DNA sequences bound by the receptors, known as response elements (REs), include direct repeats of RG(G/T)TCA with 0–5 base spacing [as in the case of thyroid hormone receptors (TR), vitamin D3 receptors (VDR), retinoic acid receptors (RARs), retinoid-X receptors (RXRs), chicken ovalbumin upstream promoter transcription factor (COUP-TFI), apolipoprotein A1 regulatory protein (ARP-1, also called COUP-TFI), hepatocyte nuclear factor-4 (HNF-4) and peroxisome-proliferator-activated receptor (PPAR) response elements; 2,4], palindromic elements of RG(G/T)TCA without any spacing [as in the case of the thyroid hormone response element (TRE); 4] and single half-sites preceded by short AT-rich sequence (2,5). The receptors bind either as monomers (as in the case of TR; 6) or homodimers (for example the estrogen receptor, HNF-4, ARP-1 and RXR; 2,4,7,8) or heterodimers (for example RAR:RXR, TR:RXR, VDR:RXR, COUP-TFI:RXR, ARP-1:RXR, PPAR:RXR and PPAR:TR; 2,4). It has been shown previously that several of these REs bind to more than one receptor type in vitro. For example, the estrogen response element of the vitellogenin gene is recognized by ER, TR and RAR (9), whereas a synthetic palindromic TRE binds RAR:RXR and TR:RXR (4). Umesono et al. (10) proposed a 3–4–5 rule in which they suggested preferential binding of VDR:RXR, TR:RXR and RAR:RXR to direct repeats of RG(G/T)TCA motifs separated by 3, 4 and 5 bases respectively, although all receptors can bind to each of these elements when they are in excess.

The direct repeats of RG(G/T)TCA with one base spacing (DR1-type) appears to be one of the most promiscuous cis-acting elements known to date, as this element binds RXR, COUP-TFI, ARP-1 and HNF-4 homodimers and PPAR:RXR, RAR:RXR, COUP-TFI:RXR and ARP-1:RXR heterodimers (4,11–14). This promiscuous binding should allow a DR1-containing promoter to be constitutively activated by HNF-4, repressed by COUP-TFI and ARP-1 and activated in response to PPAR ligands and the RXR-specific ligand 9cis-retinoic acid (9C-RA) (15–19). However, there must exist a control mechanism(s) that limits promiscuous activation through DR1 elements, since the number of genes containing DR1-type elements appears to be enormous and the majority of nuclear receptors that bind such elements are ubiquitously expressed.

Our specific interest is to study the role of the precise sequence of the core motif and spacer nucleotide in conferring selectivity and promiscuity in response element recognition of HNF-4 and ARP-1 homodimers and PPAR:RXR and RAR:RXR heterodimers. To this end we have compared the DNA binding ability of HNF-4, ARP-1, PPAR:RXR and RAR:RXR with a number of naturally

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Plasmids PPRE/tk-CAT, apoA-A/tk-CAT and C3P/tk-CAT were constructed by cloning a single copy of the PPRE, apoA-A and C3P elements into the tk promoter. These elements contain identical bases at positions 3, 5, 6 and 9–13 and purines at positions 2, 7 and 8 (Fig. 1A, last five numbers). With respect to the spacer nucleotide, A was the most frequently occurring DR1-type elements that show considerable degeneracy within the core motif and the spacer nucleotide. In addition, the role of promoter context in restricting promiscuous activation by these nuclear receptors was also investigated by studying transactivation through a common response element placed in different promoter contexts. Our results suggest that multiple independent mechanisms restrict promiscuous activation through a common response element placed in different promoter contexts. In addition, the role of promoter context in restricting promiscuous activation by these nuclear receptors was also investigated by studying transactivation through a common response element placed in different promoter contexts. Our results suggest that multiple independent mechanisms restrict promiscuous activation through a common response element placed in different promoter contexts.

**MATERIALS AND METHODS**

**Plasmid construction**

Plasmids PPRE/tk-CAT, apoA-A/tk-CAT and C3P/tk-CAT were constructed by cloning a single copy of the PPRE, apoA-A and C3P sequences of the ACO, apoAI and apoCIII genes respectively (1, 3, 20; exact sequences are shown in Table 1) into the tk promoter. Note that the orientation of repeats in relation to the tk promoter was the same in all constructs. Plasmids PPRE/tk-CAT, apoA-A/tk-CAT, mCRBPII(C3P)/CAT, mCRBPII(DR1G)/CAT and mCRBPII(DR1Gm7A)/CAT were constructed by a two step procedure. First, the RE3 sequence of mCRBPII/CAT3mut5 was deleted and a BglII site was introduced in its place by PCR-mediated mutagenesis. PPRE, apoA-A, C3P, DR1G and DR1Gm7A sequences (12; as shown in Table 1) were introduced into the BglII site by PCR-directed mutagenesis. Note that the orientation of the repeats in relation to the mCRBPII promoter TATA box (14) was the same in all plasmids and is identical to that in tk-CAT constructs.

Expression vectors for RARα, RXRα, HNF-4, ARP-1 and PPAR have been described previously (14, 15).

**Cell transfection and CAT assays**

Transfection of Cos-1 and CV1 cells using the calcium phosphate procedure was as described previously (14). The concentrations of T-RA, 9C-RA and WY-14,1643 were 5 × 10^{-8} M, 5 × 10^{-8} M and 10^{-5} M respectively.

**Electrophoretic mobility shift assays (EMSA)**

Preparation and incubation of in vitro transcribed–translated receptors with 32P-labeled oligonucleotide probes (50 fmol) and EMSAs were as described previously (14). In competition experiments unlabeled oligonucleotides were added along with 32P-labeled probes. The amount of radioactivity in DNA–protein complexes and free DNA was calculated using a radioanaytical imaging system (Fuji).

**RESULTS**

**Binding of RARα, RXRα, HNF-4, COUP-TFI, ARP-1 and PPAR to DR1-type elements**

DR1-type elements in a number of genes were compiled to analyze whether there is any consistent pattern with respect to the position of degenerate bases in core motifs and a preferred base in the spacer. As shown in Figure 1, DR1 elements can be classified into four groups: (i) elements with two perfect RG(G/T)TCA repeats; (ii) elements with two imperfect repeats; (iii) elements with a perfect RG(G/T)TCA 5′-motif and an imperfect 3′-motif; (iv) elements with an imperfect 5′-motif and a perfect 3′-motif. With respect to the spacer nucleotide, A was the most frequently observed base, followed by G, T and C. Since elements with 5′ imperfect RG(G/T)TCA were the most common DR1 elements, we chose to study five elements of this group in greater detail. These elements are the C3P element of the apoCIII gene (11), the apoA1 element of the apoAI gene (12), the RARE2 element of the mouse CRABPII gene (21), the PPRE element of the rat acetyl CoA oxidase gene (13) and the RE3 element of the mouse CRBPII gene (14). These elements contain identical bases at positions 3, 5, 6 and 9–13 and purines at positions 2, 7 and 8 (Fig. 1A, last five elements; see also Table 1 for assignment of numbers to bases within the elements). A synthetic DR1 with G as the spacer nucleotide (DR1G; 14) was also included in the study. Since the apoA1 response element contains overlapping DR1 and DR2 elements, an assay was also performed with an oligonucleotide containing only the DR1 element (apoA-ADR1).

Binding of RARα and PPAR on their own was not detected with any of the probes (Fig. 1B, lanes 1 and 6 respectively). RXRα binding was seen only with the synthetic DR1G element (lane 2). HNF-4, COUP-TFI and ARP-1 bound with various
Figure 1. Nuclear receptor binding to naturally occurring DR1-type elements. (A) Sequence comparison of naturally occurring DR1-type hormone response elements. DR1 elements from different promoters are sub-grouped based on the presence of degenerate bases (indicated in plain text) in the RG(G/T)TCA motif (indicated in bold). A summary of nuclear receptor binding to these elements is also shown (indicated as +). Wherever the data on receptor binding is not available that space is left blank. (B) Binding of RARα, RXRα, HNF-4, COUP-TFI, ARP-1 and PPAR to various DR1-type elements. EMSAs were performed with in vitro transcribed-translated receptors and indicated probes. Only DNA–protein complexes are shown. Lanes 1–6 contain rabbit reticulocyte lysate containing either RARα (lane 1), RXRα (lane 2), HNF-4 (lane 3), COUP-TFI (lane 4), ARP-1 (lane 5) or PPAR (lane 6). In lanes 7–11 RXRα along with either RARα (lane 7), HNF-4 (lane 8), COUP-TFI (lane 9), ARP-1 (lane 10) or PPAR (lane 11) were added. Lane 12 contains rabbit reticulocyte lysates in which a control RNA (bromo mosaic virus RNA) was translated. An arrowhead indicates a specific DNA–protein complex.

Contribution of the 5′-motif of DR1 elements in determining the affinity for various receptors

Most of the divergence in elements with imperfect 5′-motifs are at position 1, 2 and 4 (4, 5 and 10 of 20 elements compared respectively; Fig. 1A). Curiously, all four elements with a pyrimidine at position 1 are HNF-4 response elements. To examine the effect of bases at position 1 on specificity and promiscuity of receptor binding a series of oligonucleotide competition experiments were performed. The basic design of the
experiments was as follows. HNF-4, ARP-1, RAR:RXR and PPAR:RXR were incubated with radiolabeled DR1G probe in the presence of a 2-, 6- or 20-fold excess of non-labeled competitor oligonucleotides and the degree of competition was visualized by EMSA. The ability of a C3P element which contains T at position 1 to compete for binding of ARP-1, RAR:RXR and PPAR:RXR to DR1G was at least four times lower than that of DR1G (Fig. 2, compare lanes 2–4 with 14–16; Table 1). However, mutants of C3P modified to contain either A (C3Pm1A) or G (C3Pm1G) at position 1, as in other elements, were as efficient as DR1G in competing for ARP-1, RAR:RXR and PPAR:RXR (Fig. 2, compare lanes 2–4 and 23–28). In contrast, a G instead of an A at position 4 of PPRE was preferred by ARP-1 and RAR:RXR, as C3Pm1A competed more efficiently than PPRE for ARP-1 and RAR:RXR (Fig. 2, compare lanes 8–10 with 23–25). Thus it appears that A at position 4 severely limits binding of ARP-1 and RAR:RXR without any significant effect on binding of PPAR:RXR and HNF-4.

Effect of nature of the spacer base on receptor affinity for DR1 elements

All except two elements with an imperfect 5'-motif contain purines as the spacer base (Fig. 1A), suggesting that a purine residue at this position may increase affinity for receptors. To investigate this further we performed competition assays using DR1G as probe and mutants of RE3, PPRE, apoA-ADR1, C3P and RARE2 containing a C at position 7 as competitors (Fig. 3). In all cases RE3m7C, PPREm7C, apoA-ADRm7C and RARE2m7C were less efficient competitors, irrespective of the receptor tested.

The RARE2 element and C3Pm1A are identical in their sequence except for a G residue at position 7 in RARE2 and an A residue at the same position in C3Pm1A (Table 1). Yet both elements differed greatly in their ability to compete for HNF-4 and, to a lesser extent, for RAR:RXR binding (Fig. 2, compare lanes 17–19 with 23–25). These results suggest that HNF-4 exhibits a greater affinity for elements containing an A residue at position 7. To investigate further the importance of the spacer nucleotide we performed competition assays with a DR1G probe with a G at position 7 and DR1G competitors with either a G, A, T or C residue at position 7 (Fig. 4). DR1Gm7A was more efficient than DR1G in competing for binding of HNF-4, RAR:RXR and PPAR:RXR (Fig. 4, compare lanes 2–4 with 5–7). DR1Gm7T was as efficient as DR1G in competing for binding of all receptor types (compare lanes 2–4 with 8–10; Table 1). DR1Gm7C was the weakest among the DR1G competitors (compare lanes 2–4 with 11–13; Table 1). From these results we conclude that efficient receptor binding, particularly in the case of HNF-4, RAR:RXR and PPAR:RXR, requires DR1 elements with either a purine or a thymidine residue as the spacer nucleotide, among which an A residue resulted in strongest binding. Also, among the receptors tested binding of ARP-1 is least affected by spacer nucleotide.
Ligand-dependent and -independent transcriptional activities of nuclear receptors on promoters containing various DR1 elements

The above in vitro binding studies indicate that single base changes in the core motif and/or in the spacer may be responsible for preferential binding of a given receptor to a particular DR1 element. To investigate whether this in vitro preferential binding may lead to preferential transcriptional activation or repression, transfection experiments were performed in CV1 cells using a reporter construct. The reporter gene was derived from the vector pBLCAT8+ in which the thymidine kinase (tk) promoter was placed under the control of various DR1 elements (14). The transfection experiments were carried out in the presence of either all-trans-RA (T-RA) or 9-cis-RA (9C-RA) [at a concentration at which T-RA does not activate RXR, $5 \times 10^{-8}$ M; 21] or the PPAR ligand WY-14,643 ($10^{-5}$ M).

Among several tk-CAT reporters containing DR1-related elements significant activation by HNF-4 (≥4-fold) was obtained only with C3P/tk–CAT and DR1Gm7A/tk–CAT (Fig. 5, compare lane 1 with lane 3). It is interesting that HNF-4 failed to activate PPRE/tk–CAT although HNF-4 bound to PPRE and C3P elements with equal affinity (Table 1).

RAR:RXR, which bound most efficiently to the apoA-A element and with ≈4-fold higher affinity than to RE3, PPRE and C3P, maximally activated apoA-A/tk–CAT (~12-fold), while activation of RE3/tk–CAT and C3P/tk–CAT by these receptors was ≈5-fold (Fig. 5, compare lane 1 with lane 4). Note that activation by RAR:RXR heterodimers was mostly due to RXR, as activation occurred only in the presence of 9C-RA (lane 4). As previously reported by Durand et al. (21), activation by RXR alone (presumably as homodimers) was, in general, higher than that exhibited by RAR:RXR heterodimers (compare lane 2 with lane 4).

The activity of PPAR and RXR in cultured cells was completely different from that of either HNF-4 or RAR:RXR. HNF-4 conferred only ligand-independent activation, whereas RAR:RXR, conferred mostly 9C-RA-dependent activation. In contrast, when PPAR and RXR were co-transfected, PPRE/tk–CAT was activated in the absence of ligand and further stimulation occurred in the presence of the PPAR-specific ligand WY-14,1643 or 9C-RA. PPAR:RXR was less efficient in stimulating the activity of other tk-reporter fusions. It is interesting that only PPRE bound efficiently to PPAR:RXR in vitro.

The reporter genes containing DR1G or DR1Gm7A were not considered in the above comparison since all receptor-bound sufficiently to these synthetic elements in vitro. DR1G/tk–CAT and DR1Gm7A/tk–CAT were activated almost to the same extent by RXR homodimers and PPAR:RXR heterodimers and also by RAR:RXR, albeit at a lower level than that brought about by RXR alone, as previously observed with other DR1 elements (see above and 21; Fig. 5). A noticeable difference between PPRE/tk–CAT, DR1G/tk–CAT and DR1Gm7A/tk–CAT is that PPRE/tk–CAT is activated better by WY-14,1643 whereas the latter two reporters were activated better by 9C-RA when transfected with PPAR and RXR (compare lanes 5). This could be due to binding of RXR homodimers to DR1G and DR1Gm7A.

Promoter context determines the transactivation potential of nuclear receptors

A relatively poor activity of tk–reporter fusions upon co-transfection of HNF-4 and failure of HNF-4 to activate PPRE/tk–CAT and DR1G/tk–CAT prompted us to investigate whether promoter context plays any role in transactivation by nuclear receptors. To
HNF-4, which bound various natural DR1 motifs in the following order, C3P ≥ PPRE > RE3 > apoA-A, activated mCRBPII-(C3P)/CAT, mCRBPII(PPRE)/CAT, mCRBPII(C3P)/CATm7A and mCRBPII-(apoA-A)/CAT ~20, ~20, ~15 and ~4-fold respectively (Fig. 6A). Note that although HNF-4 stimulated mCRBPII-(PPRE)/CAT, it failed to activate pACO(-1273/+20)CAT and pACO(-1273/+47)G-CAT. These results indicate that activation by HNF-4 is dependent not only on its relative affinity for various DR1 elements, but also on the promoter context.

While RXR alone or in combination with RAR activated apoA-A/nk-CAT, none of the CRBPII–CAT reporters containing DR1 motifs were activated by RAR and RXR (Fig. 6A, compare lane 1 with lane 4). However, in Cos-1 cells, in which RAR and RXR proteins are made much more efficiently than in CV1 cells, mCRBPII(apoA-A)/CAT and mCRBPII(C3P)/CATm7A were activated 7- and 4-fold respectively, while the activities of the other receptors on these reporters were not significantly influenced by using Cos-1 cells instead of CV1 cells (data not shown). Thus activation by RAR–RXR may be promoter context dependent in cells where RAR and RXRs are expressed at low level. Similar results were also observed with PPAR–RXR, as CRBPII–CAT reporters were activated by PPAR–RXR in Cos-1 cells but not in CV1 cells (Fig. 6A and B, lanes 5).

ARP-1 is a transcription repressor, thus it is difficult to obtain a direct correlation between in vitro binding affinity and in vivo function. However, one can compare its ability to repress activation mediated by other receptors. In this respect it is easier to compare ARP-1 repression of HNF-4 activity on different elements than repression of RAR:RXR or PPAR:RXR activity, because ARP-1 can form heterodimers with RXR but not with HNF-4 (2,14). ARP-1 strongly repressed HNF-4–mediated stimulation of mCRBPII/CATm7A, compared with those of mCRBPII(PPRE)/CAT and mCRBPII(C3P)/CAT (Fig. 6A), in agreement with the in vitro observation that ARP-1 bound more efficiently to RE3 than to PPRE, and C3P and HNF-4 bound more efficiently to PPRE and C3P than RE3 in vitro (Fig. 2 and Table 1).

When the activation patterns of mCRBPII(DR1G)/CAT and mCRBPII(DR1Gm7A)/CAT were compared there was a good correlation between the efficiency of receptor binding in vitro and the extent of activation in vivo in CV1 cells (Fig. 6A). For example, HNF-4 activated mCRBPII(DR1G)/CAT by ~23-fold, whereas mCRBPII(DR1G)/CAT was activated ~13-fold (Fig. 6A, lane 3). Similarly, a modest (~5-fold) ligand-dependent activation by PPAR–RXR was obtained only with mCRBPII(DR1Gm7A)/CAT (lane 5). However, when the receptors were overexpressed, as in Cos-1 cells, both reporters responded equally to transfected receptors. For example, HNF-4, RAR–RXR and PPAR–RXR activated mCRBPII(DR1G)/CAT and mCRBPII(DR1Gm7A)/CAT to almost the same extent in Cos-1 cells (Fig. 6B). As with other reporters, ARP-1 was more efficient in repressing activation by RAR–RXR and PPAR–RXR compared with HNF-4, particularly with respect to mCRBPII(DR1G)/CAT and mCRBPII(DR1Gm7A)/CAT (Fig. 6). In summary, it appears that under limiting receptor concentration (as in CV1 cells) response elements as well as the promoter context determine the extent of transcriptional response by nuclear receptors.

**DISCUSSION**

Although previous studies have shown that DR1-type response elements are promiscuous in vitro binding sites for HNF-4, RXR,
ARP-1, COUP-TFI, PPAR-RXR and RAR-RXR and that these receptors compete with each other for binding to the same element in vitro as well as in vivo, it was not clear whether the degenerate bases within repeated motifs as well as spacer nucleotide influence the efficiency at which an element binds to nuclear receptors (see Introduction for references and Fig. 1). Our results indicate that preferential binding of HNF-4, ARP-1, RAR-RXR and PPAR-RXR to DR1-type elements is determined by the precise sequence of the core motif as well as the spacer nucleotide. Furthermore, our study also suggests that promoter context restricts promiscuous transcriptional regulation by nuclear receptors through DR1-type response elements.

Elements that bind preferentially to HNF-4

From various competition assays summarized in Table 1 it is clear that DR1Gm7A, C3Pm1A and C3Pm1G bind more efficiently to HNF-4 than any other elements. However, these elements also bind to other receptors with greater affinity. The element which binds more efficiently to HNF-4 than any other receptor is C3P. This element differs from C3Pm1A and C3Pm1G only at base position 1, where it contains T instead of a purine. It appears that T at this position reduces the affinity for ARP-1, RAR-RXR and PPAR-RXR much more than HNF-4 (Table 1). Thus HNF-4 elements that contain a T at position 4 may be ‘classical’ HNF-4 response elements which bind preferentially to HNF-4. Consistent with this possibility, in Cos-1 cells the mCRBPII promoter containing the C3P element was activated ~90-fold by HNF-4 whereas the same promoter containing DR1G and DRGm7A was activated ~35-fold (despite the same or a higher affinity for HNF-4) (Fig. 6B). Additional base positions that may determine HNF-4 binding preference are base positions 4 and 7. For instance, G instead of T at position 4 may be responsible for reduced binding of RAR-RXR and PPAR-RXR to C3Pm1G compared with DR1Gm7A (Table 1). Similarly, A instead of G at position 7 is beneficial for HNF-4 binding, since C3Pm7A, which is almost identical to RARE2 except for A instead of G at position 7, binds very efficiently to HNF-4 (Table 1). It is interesting to note that among nine naturally occurring HNF-4 response elements with 5′ imperfect repeats four of them contain a pyrimidine at position 1 and seven of them contain A at position 7 (Fig. 1A).

Elements that bind preferentially to ARP-1

DNA binding requirements for ARP-1 appear to be the least stringent, as the majority of naturally occurring DR1-type elements are ARP-1 binding sites (Fig. 1A). Among the elements tested here ARP-1 binds to DR1G, DR1Gm7A, DR1Gm7T, C3Pm1A and C3Pm1G and apoA-ADR1 elements with similar affinity (Table 1). These elements contain degenerate bases at positions 1, 4 and 7, which suggests that degenerate bases at these positions neither selectively increase nor decrease the affinity for ARP-1. However, degenerate bases at these positions may increase the chances of ARP-1 binding by reducing the affinity for other receptors. For example, G instead of A at position 7 may selectively decrease the affinity for HNF-4 and RAR:RXR.
without affecting the affinity for ARP-1 (compare C3Pm1A with apoA-ADR1, Table 1). DR1 elements with C instead of A, G or T at position 7 may bind mostly to ARP-1, as these elements, in general, bind very poorly to other receptors (compare DR1Gm7C with DR1G, DR1Gm7A and DR1Gm7T, Table 1). Nucleotides at position 2 may also influence ARP-1 binding, as replacement of G with A at this position appears to decrease affinity for HNF-4, RAR:RXR and PPAR:RXR much more than that for ARP-1 (compare RE3 with DR1Gm7A, Table 1). Note that four of five elements with degenerate bases at position 2 have been described as ARP-1/COPU-TFI response elements (Fig. 1A).

Elements that bind preferentially to RAR:RXR

Among the elements tested only DR1Gm7A appears to bind RAR:RXR efficiently. However, this element binds all other receptors with greater affinity (Table 1). Unlike HNF-4, none of the degenerate bases within the core motif appear to provide a selective advantage for RAR:RXR binding, either directly or indirectly (by lowering affinity for other receptors; see Table 1). For example, among natural DR1 elements RAR:RXR has a higher affinity for apoA-ADR1 and RARE2. However, ARP-1 and PPAR:RXR also bind to these elements with higher affinity. Thus a DR1 element may function as a RARE only in cells that contain very little PPAR, HNF-4 and ARP-1. Embryonal carcinoma cells such as P19 and F9 may be the cell types in which DR1 elements function as a RARE, as these cells lack HNF-4, PPAR, ARP-1 and COPU-TFI (23). However, after 24 h RA treatment DR1 elements may not function as a RARE in these cells due to RA-mediated induction of ARP-1 and COPU-TFI (23).

Elements that bind preferentially to PPAR:RXR

As with HNF-4 and RAR:RXR, PPAR:RXR appears to bind DR1Gm7A very efficiently (Table 1). Although no individual element binds preferentially to PPAR:RXR alone, the PPRE of the ACO gene and the RARE2 of CRABP II appear to be the least promiscuous PPAR:RXR binding elements (Table 1). Bases at positions 4 and 7 likely determine which other receptors compete with PPAR:RXR for binding to PPREs. For example, the PPRE of the ACO gene, which contains A instead of G or T at position 4, also binds HNF-4 with high affinity, but not RAR:RXR and ARP-1 (compare PPREm1G with DR1Gm7A; Table 1). In contrast, the RARE2 of CRABP II, which contains G instead of A at position 7, efficiently binds to ARP-1 but not to HNF-4 and RAR:RXR (Table 1). Thus the PPAR-mediated transactivation response from elements with A as the seventh base and a degenerate base at position 4 (particularly G) is likely to be modulated by HNF-4, whereas elements with G at position 4 and a degenerate base at position 7 will be modulated by ARP-1. Two recent studies have revealed that the sequences that flank the 5′ half-site of DR1 elements determine affinity for the PPAR:RXR heterodimer (24,25). Consistent with our results, it was also suggested that adenine as the spacing nucleotide is preferred by PPAR (24). It remains to be determined whether the sequences that flank the 5′ half-site influence binding of other receptors to PPRE and play a role in restricting a promiscuous response.

Transactivation by HNF-4 and PPAR:RXR is promoter context dependent

During our attempts to find a correlation between in vitro binding and in vivo activation function we observed that transactivation by a given receptor is not only dependent upon relative affinity for the element but also on the promoter context. A striking example is promoter context-dependent transcriptional regulation by HNF-4 and PPAR:RXR through the PPRE sequence of the ACO gene. While PPRE behaved as a PPRE within the context of the ACO and tk promoters, it behaved as an HNF-4 response element within the context of the CRBP II promoter. Furthermore, while ARP-1 could efficiently inhibit the RAR:RXR-mediated transactivation response through all elements within the context of the tk and CRBP II promoters, the HNF-4- and PPAR-mediated responses within the context of the CRBP II and ACO promoters respectively were only partially repressed by ARP-1 (except in the case of weak HNF-4 response elements such as RE3 and apoA-ADR1; Figs 5 and 6). The mechanism(s) responsible for the promoter-specific difference in HNF-4- and PPAR-mediated activation and ARP-1-mediated repression remains to be investigated and could involve interactions with other promoter-bound transcription factors (26,27). Thus while degenerate bases within the DR1 motifs determine which of the nuclear receptors bind efficiently, additional transcription factors that synergize with nuclear receptors ultimately determine whether a given promoter is activated or repressed by nuclear receptors. These transcription factors may confer additional control over promiscuous activation of DR1-containing promoters, particularly in organs such as liver which contains a number of nuclear receptors including HNF-4, ARP-1, RARs, RXRs and PPARs.

ACKNOWLEDGEMENTS

Most of the work in this manuscript was performed when the authors were in Prof. P. Chambon’s laboratory (IGBMC, Strasbourg, France). We thank Prof. P. Chambon for his generous support and critical reading of the manuscript. We are grateful to Drs J. Darnell, B. O’Mally and S. Green for various plasmids. We also thank members of the receptor group for plasmids, oligonucleotide probes and advice, A. Staub and F. Ruffenach for oligonucleotide synthesis, C. Werlé and S. Metz for their help in preparing the figures and secretarial staff in preparing the manuscript. Studies in the IGBMC were supported by funds from the Institut National de la Science et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, the Collège de France, the Association pour la Recherche sur le Cancer, the Human Frontier Science Program and the Foundation pour la Recherche Médicale.

REFERENCES
