

# 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 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 differences 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-TFII), 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). Umesonon *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 9-*cis*-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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**Table 1.** Nucleotide sequence of response element probes and competitor oligonucleotides and summary of their binding to various receptors *in vitro*

RESPONSE ELEMENTS	RECEPTOR BINDING IN VITRO																
	1	2	3	4	5	6	7	8	9	10	11	12	13	HNF-4	ARP-1	RAR:RXR	PPAR:RXR
RE3			GAGTCA	A	AGGTCA	++	+++		+								
RE3m4A			GAGTCA	A	AGGTCA	+	-		+								
RE3m7C			GAGTCA	C	AGGTCA	-	-		-								
PPRE			AGGACA	A	AGGTCA	++++	++		+								++++
PPREm1G			GGGACA	A	AGGTCA	++++	+		+								++++
PPREm7C			AGGACA	C	AGGTCA	-	-		-								++
apoA-ADR1			AGGGCA	G	GGGTCA	+	++++		++								+
apoA-ADR1m7C			AGGGCA	C	GGGTCA	-	+		-								+
C3P			TGGGCA	A	AGGTCA	++++	+		+								+
C3Pm1A			AGGGCA	A	AGGTCA	+++++	++++		++++								++++
C3Pm1G			GGGCA	A	AGGTCA	+++++	++++		++++								++++
C3Pm7C			TGGGCA	C	AGGTCA	-	-		-								-
RARE2			AGGGCA	G	AGGTCA	+	+++		++								++++
RARE2m7C			AGGGCA	C	AGGTCA	-	+		+								++
DR1G			GGGTCA	G	AGGTCA	++++	++++		++++								++++
DR1Gm7A			GGGTCA	A	AGGTCA	+++++	++++		+++++								+++++
DR1Gm7T			GGGTCA	T	AGGTCA	++++	++++		++++								++++
DR1Gm7C			GGGTCA	C	AGGTCA	+	++		+								+

RG(G/T)TCA motifs are indicated by arrows. Numbers 1–13 correspond to numbers used in the text to describe the position of a base within the repeat. The amount of non-radioactive DR1G competitor required to achieve 50% competition of binding of various receptors to the DR1G probe was calculated and is set arbitrarily as 100 U (++++) for all receptors. The values presented for various response elements correspond to percentage competition achieved with an oligonucleotide concentration equivalent to 100 U DR1G. The higher the number, the greater the competition. The results presented here are a summary of a number of independent experiments, some of which are shown in Figures 2–4.

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 DR1-type elements.

## 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 (13,20; exact sequences are shown in Table 1) into the *Bam*HI site of pBLCAT8+ (14). DR1G/tk-CAT and DR1Gm7A/tk-CAT were constructed by cloning the sequence 5'-GGGTCAAGGTCA-3' or 5'-GGGTCAAAGGTCA-3' (Table 1) into the *Sa*II site of pBLCAT8+. Note that the orientation of repeats in relation to the *tk* promoter was the same in all constructs. Plasmids pACO (-1273/+20)CAT, pACO(-1273/-471)G-CAT, RE3/tk-CAT, mCRBP/II/CAT3 and mCRBP/II/CAT3mut5 have been described previously (13,14). Plasmids mCRBP/II(PPRE)/CAT, mCRBP/II-

(apoA-A)/CAT, mCRBP/II(C3P)/CAT, mCRBP/II(DR1G)/CAT and mCRBP/II(DR1Gm7A)/CAT were constructed by a two step procedure. First, the RE3 sequence of mCRBP/II/CAT3mut5 was deleted and a *Bg*III 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 *Bg*III site by PCR-directed mutagenesis. Note that the orientation of the repeats in relation to the *mCRBP/II* promoter TATA box (14) was the same in all plasmids and is identical to that in *tk*-CAT constructs.

Expression vectors for RAR $\alpha$ , RXR $\alpha$ , 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-14,643 were  $5 \times 10^{-8}$  M,  $5 \times 10^{-8}$  M and  $10^{-5}$  M respectively.

### Electrophoretic mobility shift assays (EMSA)

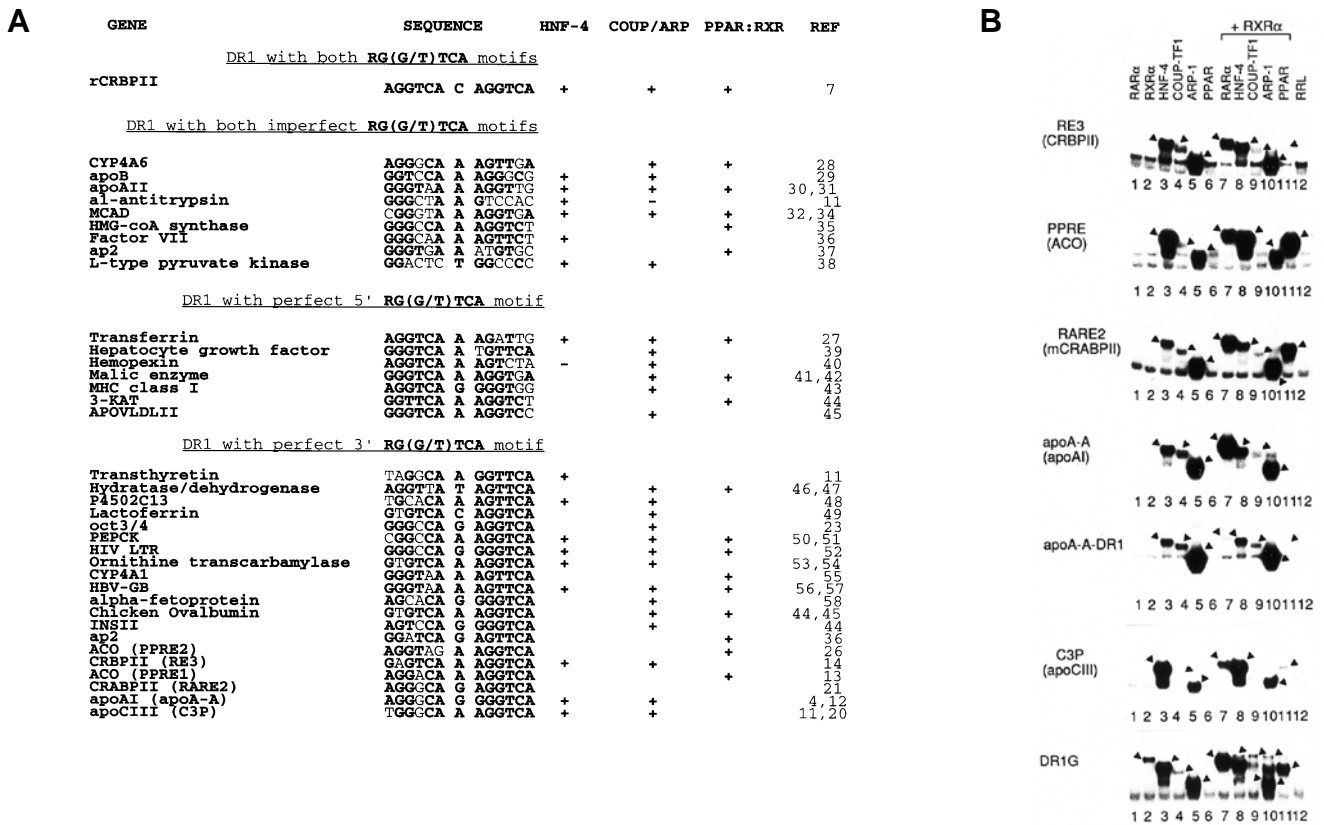
Preparation and incubation of *in vitro* transcribed-translated receptors with  $^{32}$ P-5'-labeled oligonucleotide probes (~50 fmol) and EMSAs were as described previously (14). In competition experiments unlabeled oligonucleotides were added along with  $^{32}$ P-labeled probes. The amount of radioactivity in DNA-protein complexes and free DNA was calculated using a radioanalytical imaging system (Fuji).

## RESULTS

### Binding of RAR $\alpha$ , RXR $\alpha$ , 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 apoA-A element of the *apoAI* gene (12), the RARE2 element of the mouse *CRABP/II* gene (21), the PPRE element of the rat acetyl CoA oxidase gene (13) and the RE3 element of the mouse *CRBP/II* 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 apoA-A 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 RAR $\alpha$  and PPAR on their own was not detected with any of the probes (Fig. 1B, lanes 1 and 6 respectively). RXR $\alpha$  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 RAR $\alpha$ , RXR $\alpha$ , 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 RAR $\alpha$  (lane 1), RXR $\alpha$  (lane 2), HNF-4 (lane 3), COUP-TFI (lane 4), ARP-1 (lane 5) or PPAR (lane 6). In lanes 7–11 RXR $\alpha$  along with either RAR $\alpha$  (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.

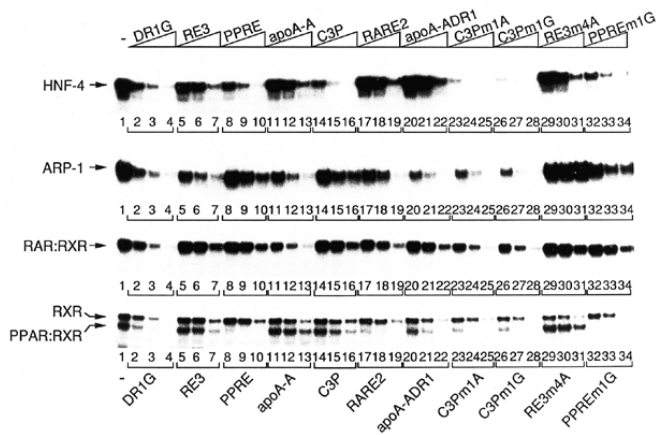
efficiencies to all elements (lanes 3–5 respectively). Note that the elements that strongly bound HNF-4 contain purines as spacer bases (for example compare binding of HNF-4 to PPPE and C3P with its binding to the RARE2 of CRABP/II; Table 1).

RAR $\alpha$ :RXR $\alpha$  heterodimers bound with various efficiency to all elements (Fig. 1B, lane 7 and data not shown; see also 14). Binding of HNF-4 was not modified by the presence of RXR $\alpha$  (compare lane 3 with lane 8). In contrast, COUP-TFI and RXR $\alpha$  formed an additional complex with an intermediate mobility between those of the RXR $\alpha$  homodimer and the COUP-TFI homodimer on the DR1G probe (compare lanes 2, 4 and 9). A similar complex was also formed on the DR1G probe when ARP-1 was incubated with RXR $\alpha$  (compare lanes 2, 5 and 10). These complexes correspond to heterodimers of COUP-TFI:RXR and ARP-1:RXR respectively, as they could be supershifted with an antibody against RXR (data not shown). Efficient binding of PPAR to PPPE, RARE2 and DR1G was observed when incubated along with RXR $\alpha$  (compare lanes 6 and 11). The effect of 9C-RA ( $5 \times 10^{-8}$  M) on binding of RXR, RAR:RXR and PPAR:RXR to various response elements was also examined, since 9C-RA has been shown to induce homodimerization of RXR (22). Although 9C-RA increased binding of RXR homodimers to DR1G probe, it had very little effect on binding of RXR

to other natural DR1 elements. While 9C-RA had no effect on PPAR:RXR binding, it increased the mobility of the RAR:RXR heterodimer, irrespective of the response element tested (data not shown). This could be due to ligand-induced changes in receptor conformation. Taken together, the above results indicate that DR1 elements are promiscuous elements that bind to several nuclear receptors, possibly with different affinities. Since these variations in affinity of a given receptor for different DR1-type elements could be related to base differences in the core motifs and/or in the spacer, the possible contribution of these bases to relative affinity of each of the receptors for the various elements was then investigated.

### 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



**Figure 2.** Competition of DR1G-bound HNF-4, ARP-1, RAR:RXR and PPAR:RXR by various response elements.  $^{32}$ P-Labeled DR1G (50 fmol) probe was incubated with rabbit reticulocyte lysate derived HNF-4, ARP-1, RAR $\alpha$  and RXR $\alpha$  or PPAR and RXR as indicated (lane 1). Reactions in lanes 2–34 in addition contained non-radioactive competitor oligonucleotides corresponding to DR1G (lanes 2–4), RE3 (lanes 5–7), PPRE (lanes 8–10), apoA-A (lanes 11–13), C3P (lanes 14–16), RARE2 (lanes 17–19), apoA-ADR1 (lanes 20–22), C3Pm1A (lanes 23–25), C3Pm1G (lanes 26–28), RE3m4A (lanes 29–31) and PPREm1G (lanes 32–34). The quantity of non-radioactive competitors was 2-fold excess over radioactive probe (100 fmol) in lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29 and 32; 6-fold excess over radioactive probe (300 fmol) in lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30 and 33; 20-fold excess over radioactive probe (1000 fmol) in lanes 4, 7, 10, 13, 16, 19, 22, 25, 28, 31 and 34. Competitor oligonucleotides were added along with the probe. The sequences of the probe and competitor oligonucleotides as well as degree of competition are shown in Table 1. These experiments were performed twice with independently prepared receptors and the results were identical.

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 23–38). In contrast, in the case of HNF-4, C3P was as efficient as DR1G, indicating that efficient binding of HNF-4 does not require a purine at position 1, even though C3Pm1A and C3Pm1G were somewhat more efficient competitors (Fig. 2, compare lanes 2–4, 14–16 and 23–28; Table 1). Apart from bases at position 1, a degenerate base at position 4 is well tolerated by HNF-4, since A instead of G (compare PPRE with C3Pm1A) or T (compare PPREm1G with DR1G; Table 1) at position 4 had a less dramatic effect on HNF-4 compared with ARP-1 and RAR:RXR binding (Fig. 2, compare lanes 2–4, 8–10, 23–25 and 32–34; Table 1).

The RE3 element of *CRBP2* was nearly as efficient as DR1G in competing for ARP-1 binding, but not for HNF-4, RAR:RXR or PPAR:RXR, suggesting that A instead of G at position 2 has limited effect on ARP-1 binding (Fig. 2, compare lanes 2–4 with 5–7; Table 1). In fact, RE3, which is identical to DR1Gm7A

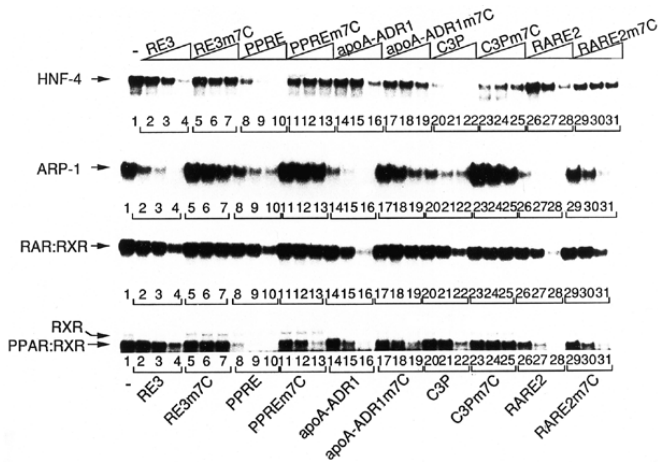
except for an A instead of G at position 2, competed nearly as efficiently as DR1Gm7A for binding of ARP-1, although DR1Gm7A is much more efficient than RE3 as well as DR1G in competing for HNF-4, RAR:RXR and PPAR:RXR binding (data not shown; see Fig. 4 below). However, RE3 binds efficiently to ARP-1 only when bases at positions 1 and 4 are not degenerate, as RE3m4A, which has A instead of T at position 4 (as in PPREm1G), failed to compete for ARP-1 binding (Fig. 2, compare lanes 5–7 with lanes 29–31). G or T instead of A is preferred by ARP-1 at position 4, as C3Pm1A binds ARP-1 much better than PPRE (Fig. 2, compare lanes 2–4, 8–10 and 23–25; Table 1).

The PPRE of the *ACO* gene contains an A at position 4. A G residue at this position did not enhance binding of PPAR:RXR, as the mutant C3Pm1A, which is identical to PPRE except for a G at position 4, was as efficient as PPRE at competing for binding to PPAR:RXR (Fig. 2, compare lanes 2–4, 8–10 and 23–25; Table 1). 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.



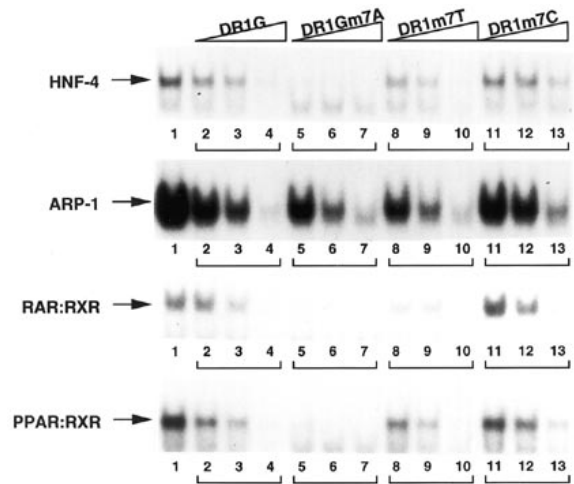
**Figure 3.** Competition of DR1G-bound HNF-4, ARP-1, RAR:RXR and PPAR:RXR by various elements and the same elements with C as the seventh base.  $^{32}$ P-Labeled DR1G (50 fmol) probe was incubated with rabbit reticulocyte lysate derived HNF-4, ARP-1, RAR $\alpha$  and RXR $\alpha$  or RXR $\alpha$  and PPAR as indicated (lane 1). Reactions in lanes 2–31 in addition contained non-radioactive competitor oligonucleotides corresponding to RE3 (lanes 2–4), RE3 with C instead of A as the seventh base (RE3m7C, lanes 5–7), PPRE (lanes 8–10), PPRE with C instead of A as the seventh base (PPREm7C, lanes 11–13), apoA-ADR1 (lanes 14–16), apoA-ADR1 with C instead of G as the seventh base (apoA-ADR1m7C, lanes 17–19), C3P (lanes 20–22), C3P with C instead of A as the seventh base (C3Pm7C, lanes 23–25), RARE2 (lanes 26–28) and RARE2 with C instead of G as the seventh base (RARE2m7C, lanes 29–31). The concentration of oligonucleotide competitors was as in Figure 2.

### 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 ( $\geq 4$ -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  $\sim 4$ -fold higher affinity than to RE3, PPRE and C3P, maximally activated apoA-A/*tk*-CAT ( $\sim 12$ -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



**Figure 4.** Competition of DR1G-bound HNF-4, ARP-1, RAR:RXR and PPAR:RXR by PuGGTCA direct repeats with G or A or T or C as the seventh base.  $^{32}$ P-Labeled DR1G (50 fmol) probe was incubated with rabbit reticulocyte lysate derived HNF-4, ARP-1, RAR $\alpha$  and RXR $\alpha$  or PPAR and RXR $\alpha$  as indicated (lane 1). Reactions in lanes 2–13 in addition contained non-radioactive competitor oligonucleotides corresponding to DR1G, which has G as the seventh base (lanes 2–4), DR1G with A as the seventh base (DR1Gm7A, lanes 5–7), DR1G with T as the seventh base (DR1Gm7T, lanes 8–10) and DR1G with C as the seventh base (DR1Gm7C, lanes 11–13). The concentration of oligonucleotide competitors was as in Figure 2.

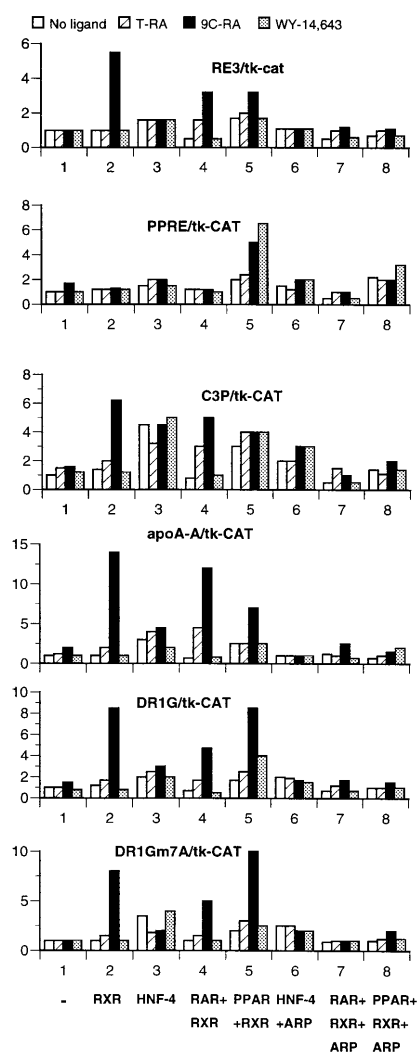
(presumably as homodimers) was, in general, higher than that exhibited by RAR:RXR heterodimers (compare lane 2 with lane 4).

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-14,643 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 because all receptors bound efficiently 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-14,643 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



**Figure 5.** Effect of RAR $\alpha$ , RXR $\alpha$ , HNF-4, PPAR and ARP-1 on activity of the *tk* promoter containing various DR1-type response elements. Five micrograms of various reporters were co-transfected into CV1 cells along with 0.5  $\mu$ g indicated expression vectors (or parental vector pSG5).  $5 \times 10^{-8}$  M T-RA or 9C-RA was added 24 h after transfection, whereas WY-14,643 was added at the time of transfection and replaced again after 24 h. Cell extracts were prepared 40 h after transfection. Transfection efficiency was standardized by co-transfection of 2  $\mu$ g  $\beta$ -galactosidase expression vector pCH110. Total amount of DNA in each transfection was standardized to 20  $\mu$ g using carrier DNA (Bluescript). In all transfections the amount of expression vector was kept constant by substituting with pSG5. Receptor-mediated stimulation is presented as fold stimulation where the CAT activity of a reporter when co-transfected alone in the absence of ligand is considered as one. Experiments were repeated three times with similar results.

this end the RE3 element of the mouse *CRBP*II promoter was replaced by various DR1 elements and cloned with the pBLCAT3+ reporter (14; see Materials and Methods). In addition, we also used the reporter pACO(-1273/+20)CAT, which contains the PPRE of the *ACO* gene within its natural context, and the reporter pACO(-1273/-471)G-CAT, in which the *ACO* gene proximal promoter sequence has been replaced by  $\beta$ -globin promoter sequences (13). Interestingly, when *CRBP*II-CAT reporters were tested there was not only strong transactivation by HNF-4, but also a correlation with relative *in vitro* binding affinity (Fig. 6A, compare lane 1 with lane 3). For example,

HNF-4, which bound various natural DR1 motifs in the following order, C3P  $\geq$  PPRE  $>$  RE3  $>$  apoA-A, activated mCRBP $\text{II}$ -(C3P)/CAT, mCRBP $\text{II}$ -(PPRE)/CAT, mCRBP $\text{II}$ /CAT3mut5 and mCRBP $\text{II}$ -(apoA-A)/CAT  $\sim$ 20,  $\sim$ 20,  $\sim$ 15 and  $\sim$ 4-fold respectively (Fig. 6A). Note that although HNF-4 stimulated mCRBP $\text{II}$ -(PPRE)/CAT, it failed to activate pACO(-1273/+20)CAT and pACO(-1273/-471)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.

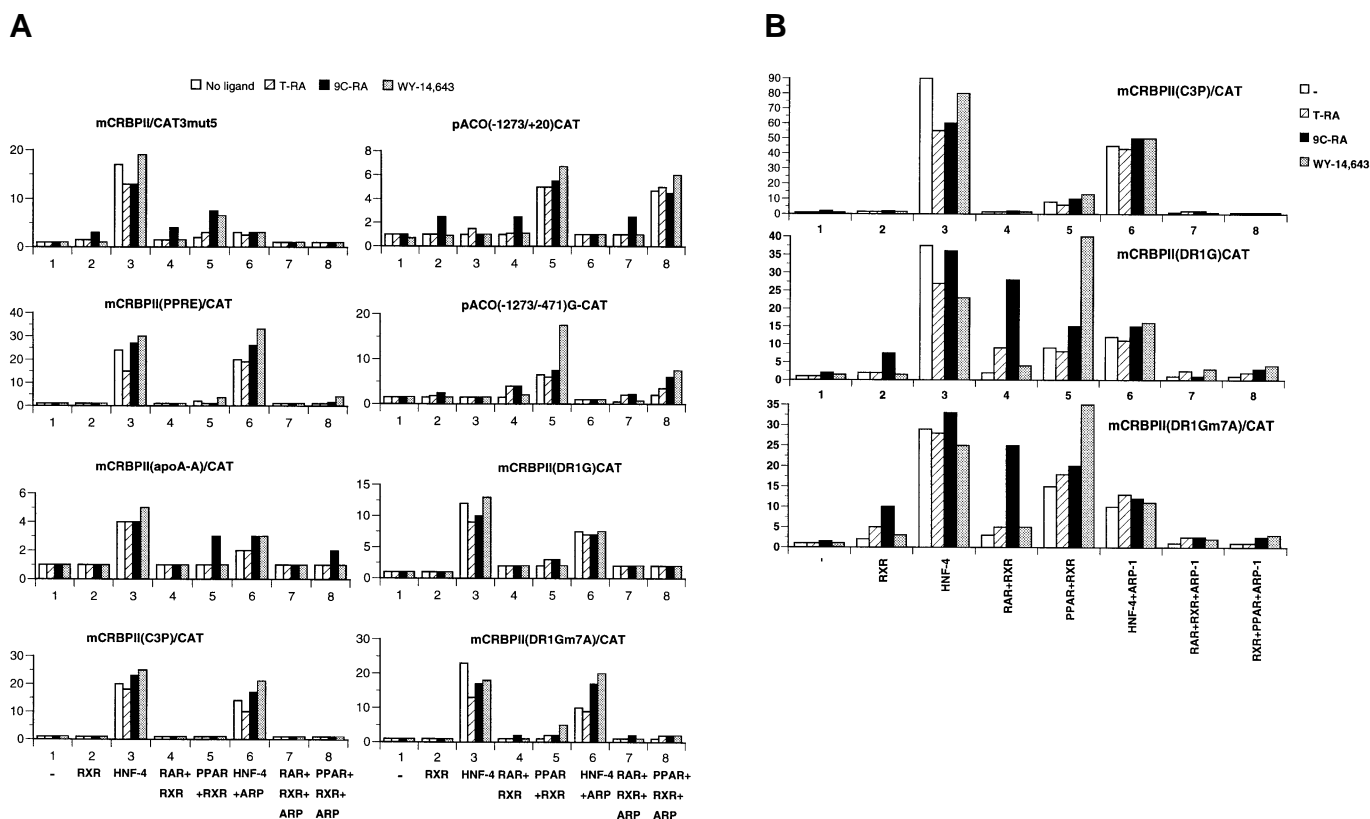
While RXR alone or in combination with RAR activated apoA-A/*tk*-CAT, none of the *CRBP*II-CAT reporters containing DR1 motifs were activated by RAR and RXR (Fig. 6A, compare lane 1 with lane 4). However, in Cos-1 cells, in which RAR and RXR proteins are made much more efficiently than in CV1 cells, mCRBP $\text{II}$ -(apoA-A)/CAT and mCRBP $\text{II}$ /CAT3mut5 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 *CRBP*II-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 mCRBP $\text{II}$ /CAT3mut5, compared with those of mCRBP $\text{II}$ -(PPRE)/CAT and mCRBP $\text{II}$ -(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 mCRBP $\text{II}$ -(DR1G)/CAT and mCRBP $\text{II}$ -(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 mCRBP $\text{II}$ -(DR1Gm7A)/CAT by  $\sim$ 23-fold, whereas mCRBP $\text{II}$ -(DR1G)/CAT was activated  $\sim$ 13-fold (Fig. 6A, lane 3). Similarly, a modest ( $\sim$ 5-fold) ligand-dependent activation by PPAR:RXR was obtained only with mCRBP $\text{II}$ -(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 mCRBP $\text{II}$ -(DR1G)/CAT and mCRBP $\text{II}$ -(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 mCRBP $\text{II}$ -(DR1G)/CAT and mCRBP $\text{II}$ -(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,



**Figure 6.** Effect of nuclear receptors on activity of the *mCRBP II* promoter containing various DR1-type response elements. Five micrograms of indicated reporters (see Materials and Methods for constructs) were transfected into CV1 cells (A) or Cos-1 cells (B) along with 0.5  $\mu$ g indicated expression vector. Experiments were performed as in Figure 5.

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 1 may be 'classical' HNF-4 response elements which bind preferentially to HNF-4. Consistent with this possibility, in Cos-1 cells the *mCRBP II* promoter containing the C3P element was activated ~90-fold by HNF-4

whereas the same promoter containing DR1G and DR1Gm7A 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 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/COUP-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 COUP-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 COUP-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 *CRABP2* 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 *CRABP2*, 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 transcriptional 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 *CRBP2* promoter (Figs 5 and 6). In fact, all elements that functioned as an RXRE (RE3, apoA-A, C3P, DR1G and DR1Gm7A) in the context of the *tk* promoter functioned only as a HNF-4 response element within the context of the *CRBP2* promoter. Furthermore, while ARP-1 could efficiently inhibit the RAR:RXR-mediated transcriptional response through all elements within the context of the *tk* and *CRBP2* promoters, the HNF-4- and PPAR-mediated responses within the context of the *CRBP2* 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.Stuab 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.

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