The promoter context is a decisive factor in establishing selective responsiveness to nuclear class II receptors

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The vigorous retinoic acid (RA)-dependent activation of the retinoic acid receptor $\beta 2$ (RAR $\beta 2$) gene in embryonal carcinoma (EC) cells is mediated by retinoid receptor heterodimers (RXR–RAR) binding to RAREs that are closely positioned to the TATA box and an EC cell-specific co-factor activity termed E1A-LA. Using a series of direct repeat (DR) elements, we now show that positioning RXR–RAR in close proximity to the basal transcription machinery assembled on the TATA box is decisive in RA responsiveness in EC cells. Notably, a DR1 element functions predominantly as an RAR-responsive element when placed in the context of the RAR $\beta 2$ promoter. Moreover, DR3 and DR4 elements which mediate vitamin D3 and thyroid hormone responses, respectively, in other contexts, are converted to exclusive RAR response elements when placed in the RAR $\beta 2$ promoter and EC cell context. In differentiated cells, the adenovirus E1A_(13S) protein is required to achieve high level RA activation through all of the different DR elements placed in the RAR $\beta 2$ context, suggesting that the molecular bridging function of E1A-LA [E1A_(13S)] is essential to redefining response element specificity. Finally, we show that the arrangement of *cis*-acting elements as present in the RAR $\beta 2$ promoter is not crucial, but rather the close positioning of the RAREs to the TATA. We conclude that the identity of a given *cis*-acting element is defined not only by its affinity for the transactivator, but also by the context in which it is placed, as well as the cell type in which the transactivator is expressed.

Keywords: EC cells/nuclear receptors/retinoic acid/transcription/transfection

Introduction

A major unanswered question concerning control of gene expression is how diverse regulatory proteins bound to their cognate response elements transmit their transactivat-

ing or transrepressing signals to the general transcription machinery. Answering this question includes determining the number and character of factors that are involved in transmission of the signal, as well as the spatial relationships and constraints that operate at the promoter for these interactions. Recent evidence from many laboratories indicates a growing complexity of protein–protein as well as protein–DNA interactions, and that the combinatorial aspects, involving cell type- and/or factor-specific co-activators and co-repressors, are critical determinants of whether a particular transcription factor elicits a cellular response.

Among many model systems, the ligand-activated transcription factors that are members of the nuclear receptor superfamily have been of particular importance in the continuing dissection of the mechanisms responsible for the control of transcriptional initiation (reviewed in Mangelsdorf *et al.*, 1995). The retinoid receptors, RAR (retinoic acid receptor) and RXR (retinoid X receptor), play an important role during development and differentiation, when retinoids lead to sequential induction and/or repression of diverse promoters, mediated by the RXR–RAR heterodimer (Stunnenberg, 1993; Keaveney and Stunnenberg, 1995; Mangelsdorf and Evans, 1995). In embryonal carcinoma (EC) and embryonal stem (ES) cells, the high level activation of the retinoic acid receptor $\beta 2$ (RAR $\beta 2$) promoter is an immediate-early step following induction by retinoic acid (RA) (de Thé *et al.*, 1989, 1990; Zelent *et al.*, 1991). The RAR $\beta 2$ promoter is expressed predominantly in undifferentiated, but not in differentiated cells, and has yielded vital insights into the molecular mechanisms by which RXR–RAR activates transcription. The RAR $\beta 2$ promoter is characterized by two RAREs located in close proximity to the TATA box. Originally, only the consensus RARE 6 bp upstream of the TATA box had been identified (nucleotides –37 to –53) (de Thé *et al.*, 1990; Sucov *et al.*, 1990), which has the typical consensus half-site sequences ^A/GTTCA separated by 5 bp (DR5 element) (Vivanco Ruiz *et al.*, 1991). Later *in vitro* studies revealed the presence of an additional non-consensus DR5-type RARE at position –67 to –83 with respect to transcription initiation (Valcárcel *et al.*, 1994). Our previous studies also have shown that the high level of activation of the RAR $\beta 2$ promoter observed in EC cells requires a pluripotent cell-specific E1A-like activity (E1A-LA) that is down-regulated during differentiation of EC cells (Berkenstam *et al.*, 1992). Further work identified amino acid residues on the surface of the TATA box-binding protein (TBP) involved in E1A-LA co-operativity (Keaveney *et al.*, 1993), underlining the importance of a direct contact between TBP and proteins involved in the mediation of the E1A-like activity. The functional interaction demonstrable between E1A-LA and TBP in this transactivation route can be mimicked by introduction

of the adenovirus E1A_(13S) protein into differentiated cells (Berkenstam *et al.*, 1992; Meyer *et al.*, 1996).

Members of the class II subgroup of the nuclear receptor family, which includes receptors for thyroid hormone (T3R), vitamin D3 (VDR) and peroxisome proliferators (PPAR) in addition to RAR, share the property of forming heterodimers with the RXR receptor (Yu *et al.*, 1991; Bugge *et al.*, 1992; Leid *et al.*, 1992; Marks *et al.*, 1992; Zhang *et al.*, 1992). These receptors typically bind DNA response elements consisting of direct repeats of the consensus half-site AG^G_TTCA spaced by varying numbers of nucleotides, with, for example, RXR–RAR preferring a DR2 or DR5, RXR–VDR a DR3 and RXR–T3R a DR4 element (reviewed in Stunnenberg, 1993; Mangelsdorf and Evans, 1995). The arrangement of, and the spacing between, the half-sites has been considered the major determinant of the receptor selectivity of the response elements, culminating in the formulation of the so-called 3–4–5 rule with respect to the functioning of elements as vitamin D3-, thyroid hormone- or retinoic acid-responsive (Umesono *et al.*, 1991; Mangelsdorf and Evans, 1995). With continued examination, notable exceptions to these rules have arisen (see also Mader *et al.*, 1993a,b), including identification of non-direct repeat half-site responsive elements (Baniahmad *et al.*, 1990; Tini *et al.*, 1993), as well as conflicting reports about the ability of DR1 elements to mediate RA responses (Durand *et al.*, 1992; Nagpal *et al.*, 1992; Kurokawa *et al.*, 1994; La Vista-Picard *et al.*, 1996).

Apart from the spacing between two half-sites, the polarity of the heterodimer bound to its response element is of particular importance. On a DR2 or DR5 element, the RXR partner of a RXR–RAR heterodimer occupies the 5' half-site (Kurokawa *et al.*, 1993; Perlmann *et al.*, 1993; Zechel *et al.*, 1994a,b): this particular arrangement permits RAR, upon ligand binding, to undergo conformational changes necessary to dislodge co-repressors (Kurokawa *et al.*, 1995). Furthermore, the ligand-induced conformational change of RXR–RAR (bound to a DR2 or DR5 element) has been demonstrated not only to permit removal of co-repressors, but also to facilitate positive interactions with co-activators, as revealed by yeast two-hybrid and similar assays (Cavaillès *et al.*, 1994, 1995; Halachmi *et al.*, 1994; Hörlein *et al.*, 1995; Kurokawa *et al.*, 1995; Le Douarin *et al.*, 1995; Lee *et al.*, 1995; Onate *et al.*, 1995; Kamei *et al.*, 1996). In contradistinction to receptor binding to DR2 and DR5 elements, the RXR–RAR heterodimer has been reported to bind a DR1 element with RAR in the 5' position. In this 'reversed' polarity binding of RAR–RXR to DR1 elements, such as found in the rat CRBP_{II} promoter, RAR-specific ligands do not result in dissociation of the co-repressor (Kurokawa *et al.*, 1994, 1995).

The structural analyses of TBP alone (Nikolov *et al.*, 1992; Chasman *et al.*, 1993), or complexed with DNA (Kim *et al.*, 1993a,b) or together with either TFIIB (Nikolov *et al.*, 1995) or TFIIA (Geiger *et al.*, 1996; Tan *et al.*, 1996), emphasize the necessity for understanding the positioning of the basal factors relative to promoter-bound transactivation factors. Particularly in the case of the RAR β 2 promoter, the close apposition of the proximal RARE with respect to the TATA box suggests the necessity for strict spatial alignments to establish transcriptionally

productive interactions of the receptor heterodimer with neighboring component(s) of the basal transcription machinery.

We have now explored those spatial constraints for RA-induced activation of the RAR β 2 promoter, using constructs with varied spacing of the half-site RARE sequences placed within different promoter contexts. Our results show that binding of nuclear receptors to DNA is not the sole regulatory determinant for receptor selectivity and hence responsiveness to a particular ligand; the promoter context is capable of overriding the affinity of transcription factors for their cognate binding sites and, via the interaction with cell-specific factors, allows in selected cell types a response to signals that would remain ineffective in others. Such cell-specific idiosyncracies, as seen in the case of the RA-dependent stimulation of the RAR β 2 promoter in pluripotent but not in differentiated cells, are among those strategies leading to proper differentiation.

Results

Experimental approach

One of the first steps leading to RA-induced differentiation of P19 EC cells is the transactivation of the RAR β 2 promoter through an RARE that is located in very close proximity to the TATA box. We have shown previously that the RAR β 2 promoter is unusual in that it responds differently in pluripotent versus differentiated cells and that EC cells possess an E1A-like activity that mediates a strong co-operativity between RAR and TBP (Berkenstam *et al.*, 1992; Keaveney *et al.*, 1993). We have postulated that the transcription signaling route used in pluripotent cells is distinct from that in differentiated cells in that E1A-LA functions as a molecular bridge to TBP from the RXR–RAR heterodimer bound on the proximal RARE adjacent to the TATA box. In line with this model, we showed that specific amino acids on the surface of TBP are essential for transcriptional signaling in EC cells and most likely are part of a surface on TBP that interacts with an EC cell-specific factor, presumably E1A-LA (Keaveney *et al.*, 1993). The close apposition of the RARE- β 2 to the TATA box, the involvement of an E1A-like bridging factor, the heterodimeric nature of active RXR–RAR receptor complex and the polarity in binding of the receptor heterodimer to its response element all suggest the necessity for a strict spatial alignment in order to obtain maximal transactivation. The asymmetry of the bound heterodimer potentially dictates asymmetry to the interactions with neighboring transcription factor(s) or component(s) of the basal transcription machinery.

To analyze possible constraints *in vivo*, the RARE- β 2 element, a direct repeat element consisting of two half-sites -PuGTTCA- spaced by five nucleotides (a DR5 of the T-type), was replaced by a series of response elements comprising two half-sites of the sequence -PuGGTCA- with variable spacing (DR1–DR5 of the G-type), designated DR1-b2 to DR5-b2 (Figure 1A). DR(G) elements were chosen because they appear to be the archetypal elements for class II receptors. The DR1–DR5 mutations do not alter the spacing between the 3' half-site, the presumed RAR-binding site in DR5 and DR2 elements (Zechel *et al.*, 1994a,b) and the TATA box. Placing binding

Fig. 1. DR1–DR5 response elements support RA activation of the RAR β 2 promoter. The proximal RARE- β 2, DR5(T)-type half-sites were replaced by DR(G)-type half-sites with the indicated spacing. (A) Sequence of the wild-type RAR β 2 (fragment –83 to –23) and DR1–DR5 promoters. (B) P19 EC cells were transfected with 5 μ g of reporter and 0.6 μ g of RSV-CAT control reporter, with or without 1 μ M all-*trans* retinoic acid (AtRA) or the RAR-specific agonist RO13-7410 (RO13). Luciferase assays were as described in Materials and methods; assays are presented as fold induction in the presence of ligand relative to the absence of ligand in all cases normalized for transfection efficiency by CAT activity expressed from control RSV-CAT reporter (Materials and methods). (C) As in (B) using COS-7 cells. These transfections received 2 μ g of psg-hRAR α , with 1 μ g of RSV-E1A_(13S) where indicated.

sites for other closely related class II receptors such as VDR and T3R (DR3-b2 and DR4-b2, respectively) within the context of the RAR β 2 promoter will test whether the signaling pathway is restricted to RXR–RAR or can be utilized by other class II nuclear receptors. Furthermore, because RXR–RAR heterodimers reportedly bind with opposite polarity to DR1 versus DR5 elements (with RAR on the 5' half-site; Kurokawa *et al.*, 1994), these substituted promoters will test the requirement for a specific stereoalignment of the particular receptor domains with respect to bridging factors and components of the basal machinery.

DR1–DR5 elements can function as RAREs in the RAR β 2 promoter

The ability of DR1-b2 to DR5-b2 reporters to mediate a retinoid response was compared with the parental RAR β 2 promoter–luciferase construct in the EC cell lines and in differentiated cells (Figure 1). In P19 EC cells, the addition of all-*trans* RA (AtRA), that binds to RAR but not RXR, or addition of the RAR-selective synthetic ligand RO13-7410 (Keidel *et al.*, 1994; also known as TTNPB), resulted in RA inducibility of the DR5-b2 reporters that was very similar to that obtained with the parental RAR β 2 (up to

Fig. 2. Binding of RXR–RAR to the two RAREs of the RAR β 2 promoter. Probes prepared by PCR from promoters tested in Figure 1 were incubated without receptor or with the indicated dilutions of purified RXR–RAR and subjected to DNase I footprinting. Lanes marked G+A indicate chemical cleavage; Dis and Prox indicate the distal RARE (nucleotides –67 to –83) and the proximal RARE (–53 to –37), respectively. (A) Footprinting on the wild-type RAR β 2 promoter and RAR β 2-short [sequences upstream of the *Sma*I site (–59) were deleted]. (B) Footprinting on DR1-b2 to DR5-b2 promoters.

30-fold). The DR2-b2 reporter was about half as potent (15-fold) whereas an ~8-fold enhancement of transcription was obtained with the DR1-, DR3- and DR4-b2 reporters (Figure 1B and data not shown). Mutation of either one or both of the half-sites of the proximal RARE (yielding m3m7) virtually abolished RA responsiveness, indicating that the proximal RARE is crucial in setting up an RA-responsive promoter (Vivanco Ruiz *et al.*, 1991; Berkenstam *et al.*, 1992 and Figure 1B). Addition of the RXR-specific ligands SR11237 or SR11134 (Fanjul *et al.*, 1994) did not result in significant transactivation (data not shown).

In COS-7 cells, the levels of transactivation from the RAR β 2 promoter (Berkenstam *et al.*, 1992) or the DR1-b2 to DR5-b2 reporter series in response to AtRA were low when compared with EC cells, even upon co-transfection with an RAR expression vector (Berkenstam *et al.*, 1992 and Figure 1C). However, co-expression in COS-7 cells of E1A_(13S) and TBP along with RAR resulted in highly elevated levels of transcription, and, more importantly, RAR-dependent activation was obtained irrespective of the spacing between the two half-sites. Thus, the spacing between the half-sites did not affect the functional co-operativity between RAR and TBP via E1A_(13S), following exposure to either AtRA (data not shown) or the RAR-restricted ligand RO13-7410 (Figure 1C). The fact that the differential RA inducibility of the DR1-b2 to DR5-b2 reporters obtained in P19 EC cells (Figure 1B) was not obtained in COS-7 cells (Figure 1C) probably resulted because overexpression of RAR compensated for the differences in the affinity of RXR–RAR for these sites.

Next we performed *in vitro* DNase I footprinting to compare the ability of the RXR–RAR heterodimer to bind the response elements in the DR1-b2 to DR5-b2 reporters, using vaccinia-expressed RXR α and RAR α (Bugge *et al.*, 1992). The wild-type RAR β 2 promoter fragment (Figure 2A) displayed the presence of a RARE at position –83 to –67 (designated distal) in addition to the well-studied RARE- β 2 (designated proximal) at position –53 to –37

(de Thé *et al.*, 1990; Sucov *et al.*, 1990; Vivanco Ruiz *et al.*, 1991). Full protection of the distal site was reached at ~3–5 times the receptor concentration required for full protection of the proximal RARE in the RAR β 2 promoter; binding of RXR–RAR to the proximal and distal RAREs was not, or only weakly, co-operative (M.Meyer and H.G.Stunnenberg, unpublished observations). Deletion of the distal RARE did not significantly affect the affinity of RXR–RAR for the proximal RARE- β 2; that is, occupancy of the proximal site was reached at roughly the same receptor concentration irrespective of the presence of the distal binding site (Figure 2A). Likewise, replacing or mutating the proximal RARE (DR1-b2 to DR4-b2) diminished the occupancy at the proximal site whereas the binding to the distal site was unimpaired (Figure 2B and data not shown). *In vivo*, in EC cells, the distal RARE augments the RA responsiveness of the RAR β 2 promoter, although it is a weak RARE on its own (Berkenstam *et al.*, 1992 and data not shown).

Full protection of the proximal RARE in the DR5-b2 promoter (Figure 2B) was reached at 2- to 5-fold higher concentrations as compared with the wild-type RAR β 2, showing that the affinity of the DR5(G) for RXR–RAR was reduced as compared with the wild-type RARE, a DR5(T) element, and was comparable with that of the distal, non-consensus DR5. Full protection by RXR–RAR of the proximal DR element in the DR1-b2 to DR4-b2 promoters required ~3- to 5-fold higher receptor concentrations than were required to protect the distal RARE. The relative affinity of RXR–RAR for the proximal DR-binding site correlated well with that site's ability to mediate an RA response in P19 cells (Figure 1).

Taken together, the data show that retinoid- and RAR-dependent transactivation can be obtained from DR1 to DR5 elements, when they are placed in the context of the RAR β 2 promoter. Efficient activation in a retinoid-dependent manner can be observed in P19 EC cells with endogenous levels of receptor, or in differentiated cells upon co-expression of RAR along with TBP and E1A_(13S). Two aspects were unexpected: firstly, in EC cells as well

as COS-7 cells, a DR1 element placed in the RAR β 2 context was able to support transactivation in response to RAR-restricted ligands, but not to RXR-restricted ligands; secondly, the DR3 and DR4 elements supported an RA response.

DR1: an 'RARE' as well as an 'RXRE'

The experiments depicted in Figure 1 revealed that a DR1 element placed in the RAR β 2 context supported transactivation in P19 EC cells, and in COS-7 cells upon co-transfection with TBP and E1A₍₁₃₅₎, in response to RAR-restricted ligands. In contrast, a 2 $\times$ DR1-containing chimeric promoter has been reported to be activated only by RXR homodimers in an RXR-restricted ligand-dependent manner, and to be repressed by RXR–RAR heterodimers (Kurokawa *et al.*, 1994). This restricted responsiveness has been attributed to the opposite polarity of RXR–RAR binding to a DR1 versus a DR5 element, resulting in an RXR–RAR–DR1 configuration that is unable to bind RAR-restricted ligands and hence to dissociate the co-repressor, N-CoR (Kurokawa *et al.*, 1995). This apparent ambiguity with respect to transactivation through a DR1 element may be explained in our experiments by (i) the particular *cis*-element, a single DR1 element combined with a non-consensus DR5 element as present in the DR1-b2 reporter as opposed to a 2 $\times$ DR1-thymidine kinase (tk) reporter, (ii) the particular synthetic RXR-selective ligands (SR11234 and SR11237 as opposed to LG69) and (iii) the cell lines, P19 EC and COS-7 as opposed to CV1 cells. Other studies of DR1 element transactivation by RXR–RAR noted influences of receptor isotype as well as synergistic effects of low concentrations of RAR- and RXR-specific ligands (Durand *et al.*, 1992; Roy *et al.*, 1995; La Vista-Picard *et al.*, 1996).

We first reassessed the ability of RXR- and RAR-selective ligands to activate the DR1-b2 reporter in P19 and COS-7 (CV1-derived) and JEG3 cells by co-transfection experiments (Figure 3A). The highest transactivation was obtained with AtRA, whereas 9-*cis* RA (9cRA, capable of binding both RAR and RXR) was about half as potent. The RAR-selective ligand RO13-7410 and the RXR-selective ligand SR11237 elicited 2- to 3-fold activations, whereas the RXR-selective ligand SR11234 did not activate the reporter at all. Although the overall levels of induction in COS-7 cells were lower than those obtained in P19 EC cells, RAR-selective ligands clearly activated a DR1 reporter in COS-7 and JEG3 cells in the presence of co-transfected receptors (Figure 3A and data not shown). The specificity of each selective ligand was confirmed by parallel co-transfer experiments performed using the Gal4-NURRI, Gal4-RXR and Gal4-RAR fusion constructs that are selectively responsive to RXR- and RAR-restricted ligands, respectively (Perlmann and Jansson, 1995; M.V.Sanguedolce and H.G.Stunnenberg, data not shown).

An alternative explanation for our observation of RA transactivation of DR1 could be that the RXR–RAR heterodimer binds with 'reversed' polarity to a DR1 element in the RAR β 2 promoter context as compared with a DR1 element placed in front of the tk promoter. This could be due to interaction of RAR and/or RXR with components of the transcriptional machinery, that compensates for an otherwise unfavorable binding polarity. We therefore tested the RXR–RAR chimeric receptors,

designated T/A and Xho (Kurokawa *et al.*, 1994), chimeras designed to ensure T/A binding to the 5' half-site and the Xho chimera binding to the 3' half-site of a DR1 element (Kurokawa *et al.*, 1994), thus mimicking the RXR–RAR polarity on a DR5 element. Because the level of RAR in P19 cells is highly elevated following RA administration, we performed these experiments in RAC65 cells whose truncated RAR α protein renders them insensitive to differentiation by RA. As anticipated (Figure 3B and data not shown), the DR1-b2 reporter responded to neither RAR- nor RXR-restricted ligands in RAC cells. Co-transfection of RAR and TBP yielded activation of transcription in response to RAR-specific ligands (Figure 3B). Intriguingly, neither T/A nor Xho nor the combination of T/A plus Xho yielded significant levels of RA-induced transcription from the DR1-b2 reporter in RAC65 cells (Figure 3B). The inability of the T/A and Xho chimeras to activate transcription from the DR1-b2 reporter in RAC65 cells was not due to a defect in DNA binding as determined by bandshift assays (data not shown). Furthermore, in COS-7 cells in the presence of TBP and E1A₍₁₃₅₎, the T/A plus Xho chimeras, but not T/A or Xho alone, boosted the level of transcription in the presence of RA to levels very similar to that obtained with wild-type receptors (Figure 3C). Importantly, the RXR-restricted ligand, SR11237, did not elicit a transcriptional response in COS-7 cells, either through the wild-type receptors or through the chimeras. Finally, the T/A and Xho chimeric receptors were unable to activate the wild-type RAR β 2 promoter in COS-7 cells irrespective of whether RAR- or RXR-restricted ligands were used; again the Xho and T/A chimeras bound with almost wild-type affinity to the DR5 in bandshift experiments (Figure 3D and data not shown). This result stresses the fact that a particular alignment of receptor domains with respect to the basal machinery appears to be required to mediate transactivation.

The above results showed that the DR1-b2 reporter was able to mediate transactivation in response to RAR-restricted ligands, and suggested a RXR–RAR binding polarity. To investigate whether the non-consensus DR5 element present in the DR1-b2 promoter influenced (reset) the ligand responsiveness, we tested the ability of RAR- and RXR-restricted ligands to elicit transcriptional activation from the 2 $\times$ DR1-tk reporter (Figure 4). In RAC65, P19 EC and COS-7 cells, 9cRA and the RXR-selective ligand SR11237 effectively boosted the level of transcription from the CRBP_{II}-tk reporter upon co-transfection of RXR α (Figure 4A and B and data not shown). Importantly, RO13-7410 was about half as potent as SR11237. Co-transfection of RAR α resulted in ~8-fold activation in response to AtRA and the RAR-specific ligand RO13-7410, and a 12-fold activation by 9cRA, coupled with a reduction of the SR11237 response as compared with co-transfection of RXR α alone. Co-transfection of RAR α along with RXR α and induction with the RAR-selective ligand RO13-7410 resulted in a 7- to 8-fold activation of transcription (Figure 4A and B and data not shown). Overall, RXR-selective ligands were slightly more potent when RXR α only was co-transfected, whereas RAR-selective ligands were more potent when RAR α or RAR α plus RXR α were co-transfected.

These results showed a differential output, with the RAR- or RXR-selective ligand 'preference' dependent on

Fig. 3. RAR-specific activation of DR1-b2. The DR1-b2 reporter was transfected along with the indicated receptor expression plasmids into the EC cell lines P19 and RAC65, as well as in COS-7 cells in the presence or absence of specific ligands. Fold induction is presented as described in the legend to Figure 1. (A) P19 cells transfected with the DR1-b2 construct and the indicated ligands at 1 μ M. (B) RAC65 and (C) COS-7 cells transfected with the DR1-b2 reporter along with expression vectors for RAR α , RXR α and the chimeric receptors T/A and Xho and ligands at 1 μ M RO13-7410 (white boxes) and SR11237 (filled boxes). (D) COS-7 cells transfected with the parental RAR β 2 reporter along with the chimeric receptors T/A and Xho and ligands at 1 μ M as indicated.

the co-transfected receptor(s). To test whether the relative concentrations of RAR and RXR determine the ligand response mediated by a DR1 element, co-transfections were performed using a constant amount of RXR α and increasing concentrations of RAR α . A strong, RXR-selective response was obtained in P19 EC, COS-7 and JEG3 cells upon co-transfection of RXR α (Figure 4C and data not shown). Co-transfection of increasing amounts of RAR α expression vector blunted the SR11237-induced transactivation and boosted the RO13-7410 response. At equimolar concentrations of RAR α and RXR α (determined by Western blotting), the CRBP2 reporter was

transactivated equally well by RXR- and RAR-selective ligands (Figure 4C and data not shown). Thus, the data demonstrate that the 2 $\times$ DR1-tk reporter can mediate an RAR- as well as an RXR-selective response. Co-transfection of the chimeric T/A and Xho receptors yielded the reported results, that is potent activation by the RAR-selective ligand, RO13-7410 and much less activation by the RXR-selective ligand, SR11237 (Kurokawa *et al.*, 1994).

Taken together, the co-transfection data suggest that ligand selectivity is not determined solely by features inherent in a DR1 element, that is the configuration of the

Fig. 4. RAR-specific activation of 2×DR1-tk. Activation of 2×DR1-tk reporter (two tandem DR1 elements derived from the CRBP1 promoter) in RAC65 and COS-7 cells. (A) RAC65 cells and (B) COS-7 cells transfected with the 2×DR1-tk reporter and expression vectors for RAR α , RXR α and the chimeras T/A and Xho. Ligands were added at 1 μ M as indicated. Fold induction was determined as described in the legend to Figure 1. (C) 2×DR1-tk reporter was transfected with a constant amount of RXR α expression plasmid and increasing amounts of RAR α expression plasmid (μ g as indicated). The RAR-specific agonist RO13-7410 or the antagonist SR11237 was added at 1 μ M as indicated.

binding site and a fixed polarity in receptor binding. The differential response to RAR- and RXR-restricted ligands *in vivo* seems to be due, at least in part, to the relative concentrations of RAR and RXR present in the cells. Additionally, the level of the co-repressor N-CoR in a particular cell type might shift the balance between RAR and RXR ligand inducibility. It is also evident that protein-protein interactions between RAR and/or RXR, with neighboring factors and cell type-specific co-factors such as E1A-LA, might influence, or even determine, the polarity of receptor binding and, consequently, the ability of receptor to elicit a transcriptional response.

DR3 and DR4 in the RAR β 2 promoter context function as RAREs

The experiments depicted in Figure 1 showed that DR3-b2 and DR4-b2 reporters were readily activated in response to RA by the endogenous level of RAR and RXR, when tested in EC cells. Furthermore, the DR3-b2 and DR4-b2 reporters were avidly activated in non-pluripotent cells such as COS-7 or JEG-3 upon co-transfection of RAR, if TBP and the adenoviral E1A_(13S) were included. DR3- and DR4-type elements are generally associated with VD3 and T3 responses, respectively (Stunnenberg, 1993; Mangelsdorf *et al.*, 1995) rather than with RA responses (Figure 1). It was surprising, therefore, to find that the DR3-b2 and DR4-b2 reporters were not activated by VD3 and T3, respectively, even if the cognate receptors were overexpressed (Figures 5A and 6A). Moreover, co-transfection of TBP and/or E1A_(13S) did not yield or restore VD3 or T3 responsiveness (Figure 5A and data not shown). The same DR3 and DR4 elements functioned as genuine VD3 and T3 response elements, respectively, when placed upstream of the tk promoter, yielding DR3-tk and DR4-tk, respectively (Figures 5B and 6B). Interestingly, co-transfection of TBP alone, or along with, adenoviral E1A_(13S), blunted, rather than boosted, transactivation from the DR3-tk and DR4-tk reporters (Figure 5B and data not shown). Similar results were obtained with a naturally occurring T3RE from the Moloney murine leukemia virus (MoMLV) long terminal repeat. Although MoMLV T3RE (a non-consensus DR4 element) is a strong thyroid hormone response element when placed upstream of the tk promoter (Sap *et al.*, 1990; Vivanco Ruiz *et al.*, 1991), it was unable to mediate a T3 response when placed in the context of the RAR β 2 promoter (data not shown). Two conclusions can be drawn from these experiments. Firstly, VDR and T3R cannot activate transcription from their 'cognate' response elements when these are placed in the context of the RAR β 2 promoter.

Although the data imply that the distal DR5 element is not critical in setting up RA responsiveness, contributions from other hitherto unidentified *cis*-acting elements in the RAR-b2 promoter fragment remain possible. We assessed this possibility by placing a fragment of the RAR β 2 promoter (and the mutants derived thereof) in front of the heterologous tk promoter. In this manner, the arrangement of presumed *trans*-acting factors binding to the proximal RAR β 2 promoter would be preserved. However, the close apposition of the RXR-RAR heterodimer to the TATA box and, therefore, the functional interaction between RAR and TBP via E1A-LA, is abrogated (Berkenstam *et al.*, 1992; M.Feigenbutz and H.G.Stunnenberg, unpublished observations). Figure 6B reveals that a promoter fragment lacking the TATA box and downstream sequences but comprising the DR5DR5 arrangement placed in front of herpes simplex virus (HSV)-tk (DR5DR5-tk) was strongly activated in response to RA and, as expected, did not convey T3 responsiveness. Conversion of the distal DR5 into a DR4 (DR4DR5-tk) had no effect on RA-dependent transactivation. Conversion of the proximal DR5 into a DR4 (DR5DR4-tk) reduced RA responsiveness (Figure 6B), but mediated a 4- to 5-fold induction by T3 in the presence of exogenous T3R. Most importantly, conversion of both DR5 into DR4 elements resulted in a DR4DR4-tk reporter that responded avidly to T3 induction in P19 EC, COS-7 and JEG3 cells upon co-transfection of a T3R expression vector (Figure 6B and data not shown). The same reporter responded very poorly if at all to RA; co-transfection of E1A_(13S) and TBP did not boost the RA-induced level of transcription (not shown). Note that the DR4DR4 arrangement of binding sites when positioned next to the TATA box (DR4DR4-b2) yielded the opposite result: robust induction by RA but refractive to T3 induction (Figure 6A). Along these same lines, a moderate but significant T3 induction was achieved with a single DR4 element joined to the tk promoter; as expected, the proximal consensus DR4 element was more

Fig. 6. The DR4DR4-b2 functions exclusively as a RARE in the context of the RAR β 2 promoter, although as a T3RE in the context of the tk promoter. Fold induction of luciferase activity was calculated as before following transfection of P19 cells with the indicated reporter plasmids and ligands. **(A)** RAR β 2 promoter constructs containing substitutions of a DR4(G) element (dark triangles) for either the proximal, distal or both DR5 elements (white triangles); m3m7 is a double mutation of the DR5 sequence which abolishes receptor binding. **(B)** Tk promoter containing a fragment from the RAR β 2 (–57 to –124) constructs inserted at –109.

potent than the distal non-consensus DR4 element in mediating T3 induction (Figure 6B and not shown).

Discussion

It has been a commonly accepted practice to establish or corroborate the identity of a given *cis*-acting element by testing it in front of 'naïve' promoters (often derived from the HSV-tk gene). This procedure has allowed the identification of an impressive array of distinct DNA response elements and their corresponding *trans*-acting factors. In the particular case of the class II nuclear receptor family, these co-transfection analyses have revealed that the arrangement of the half-sites into inverted, everted or direct repeats affects the receptor selectivity (Näär *et al.*, 1991; Umesono *et al.*, 1991; Vivanco Ruiz *et al.*, 1991), which culminated in the postulation of an underlying rule (Umesono *et al.*, 1991; Mangelsdorf and Evans, 1995), according to which the spacing between the half-sites is the determining factor in specifying the identity of a response element for a receptor. However, the procedure has also yielded ambiguous results, and the established identity of certain elements remains debatable. For

example, when assayed in CV-1 cells or COS-1 cells, respectively, a consensus DR4 element is reportedly either non-responsive (Umesono *et al.*, 1991) or, on the contrary, responsive (Mader *et al.*, 1993b; Song *et al.*, 1994) to transcription stimulation by RA (rather than T3). Similarly, DR1 elements have given conflicting results about RA inducibility (Durand *et al.*, 1992; Nagpal *et al.*, 1992; Kurokawa *et al.*, 1994; Roy *et al.*, 1995; La Vista-Picard *et al.*, 1996).

Using a heterologous transfection system, we have shown here that prototypic response elements generally associated with VDR and T3R (DR3 and DR4, respectively), while behaving as expected in the tk promoter context, behaved only as RAREs when placed in the context of the RAR β 2 promoter. In fact, all tested DR1–DR5 elements when placed in the context of the RAR β 2 supported RA-dependent activation when tested in pluripotent cells with endogenous receptors (P19 EC). In non-EC cells, vigorous RA activation of the various promoters depended on simultaneous co-expression of adenoviral E1A_(13S) and TBP. In particular, a DR1 element in the RAR β 2 context permitted RAR-selective but very poor RXR-selective responsiveness when stimulated by RXR-

restricted ligands. These findings are surprising in view of previous results indicating that RXR–RAR binds to a DR1 element with polarity opposite to that observed for a DR5 (Kurokawa *et al.*, 1994). We have obtained preliminary results that reveal no preferred orientation of RXR–RAR on a DR1 element (J.L.Betz and H.G. Stunnenberg). Although the proposed ‘altered’ polarity of receptor binding to DR1 elements provided an attractive explanation for the observed lack of activation seen in transfections with a DR1 element (Kurokawa *et al.*, 1995), our results suggest a greater complexity. The ability of RXR–RAR to mediate RAR-selective ligand-dependent transactivation via DR1 elements was not restricted to EC cells (which have been reported to contain low levels of N-CoR, Kurokawa *et al.*, 1995). Furthermore, we also observed that RAR ligand-dependent activation via a DR1 element (either a single or a multimerized DR1 element) depends on the relative concentrations of RXR and RAR monomers. We conclude that the promoter context in which a particular element is placed can be the determining factors for receptor selectivity. The term ‘promoter context’ is often used to indicate the importance of particular receptor isotypes and/or functional domains, as well as the differential ligand responsiveness in activation in very complex natural promoters or large regulatory regions (Nagpal *et al.*, 1992; Roy *et al.*, 1996; Taneja *et al.*, 1996). Our data highlight the importance of the promoter context by showing the differential ability of biologically distinct members of the same class II subfamily (TR, VDR and RAR) to transactivate through ‘prototypic’ *cis*-acting elements.

How can differential responsiveness be brought about?

Several explanations can be put forward to account for the influence of the promoter context on the ligand responsiveness of a receptor. Firstly, particular combinations and arrangements of *cis*-acting elements might generate specificity. This type of specificity may be particularly relevant to enhancers where a combination of mutually interacting *trans*-acting factors determine the final impetus on transcription. An example is the *hoxb1* enhancer; restriction of *hoxb1* expression in rhombomere 4 is dependent on an RARE that closely resembles a DR2-type element. Mutation of this RARE results in spreading of *hoxb1* expression into neighboring rhombomeres (Studer *et al.*, 1994; Ogura and Evans, 1995). Although this element is crucial for repression of *hoxb1* expression in chick and mouse, it comprises a consensus DR2 element and hence probably acts as a ‘normal’ positive element when placed upstream of the tk promoter.

Secondly, the presence of cell type-specific co-factors (co-repressors or co-activators) can direct differential responsiveness. An example is the recently cloned B cell-specific co-factor, termed OBF1, OCA-B or BOB1, that stimulates immunoglobulin promoter activity (Luo *et al.*, 1992; Gstaiger *et al.*, 1995; Luo and Roeder, 1995; Strubin *et al.*, 1995). Expression of OBF-1 in HeLa cells selectively stimulates the activity of a natural immunoglobulin promoter that otherwise is not or very poorly expressed in non-B cells. In pluripotent cells, we have characterized an activity, E1A-LA, that is present in pluripotent cells and which behaves as a cell-specific co-activator (Berkenstam

et al., 1992; Keaveney *et al.*, 1993). We have shown in the present study that E1A-LA and the adenoviral E1A_(13S) specifically mediate transcription elicited by RAR and do not function in conjunction with the closely related T3R and VDR receptors. Particularly relevant to our investigations is the possibility that the recently described co-activator CBP [cAMP-response element binding protein (CREB)-binding protein], which plays a central role in different signaling pathways (Kamei *et al.*, 1996), might function as a molecular link between receptors, E1A_(13S) and TBP. The N-terminal region of CBP contacts the AF-2 region of RAR, acting as a bridging factor between RAR and the basal transcription machinery (Kamei *et al.*, 1996), whereas the C-terminal region of CBP can contact E1A (Arany *et al.*, 1995; Lundblad *et al.*, 1995), which in turn has been shown to contact TBP (Lee *et al.*, 1991). A number of potential nuclear co-factors have been identified (SRC-1, TIF1, RIP 140 among others) (Le Douarin *et al.*, 1995; Onate *et al.*, 1995). It remains to be determined, however, if they play a role in selectivity of responsiveness.

Finally, the composition of components of the basal transcription machinery assembled on a promoter may determine selectivity. In this regard, different subsets of TFIID complexes are reported to be involved in transactivation. If the transcription activation factor (TAF) compositions of these distinct forms of TFIID serve as molecular adaptors for different transcription factors (Pugh and Tjian, 1990; Chen *et al.*, 1994) then such ‘adapted’ TFIID complexes may mediate cell type- or promoter-specific transactivation. For example, a distinct TFIID complex containing TAF_{II}30 is required for estrogen receptor-mediated transcription activation (Jacq *et al.*, 1994), and TAF_{II}28 has been shown to be involved in transactivation by the ligand-dependent activation function of RXR (May *et al.*, 1996). With respect to the RAR β 2 promoter and pluripotent cells, the cell-specific co-activator, E1A-LA, that plays a important role in RA-dependent transactivation, may be considered to be a specific TAF (Meyer *et al.*, 1996).

What are the decisive features of the RAR β 2 promoter?

We have shown previously that the RAR β 2 promoter is unusual in that it responds differently in pluripotent versus differentiated cells (Berkenstam *et al.*, 1992; Keaveney *et al.*, 1993). We have shown that EC cells possess an E1A-like activity that mediates a strong co-operativity between RAR and TBP. The E1A-LA effect could be observed with the RAR β 2 promoter but not if the RARE is placed upstream of the tk promoter (Berkenstam *et al.*, 1992; M.Feigenbutz and H.G.Stunnenberg, unpublished observations). We have postulated that the transcription signaling route used in pluripotent cells is distinct from that in differentiated cells in that E1A-LA functions as a molecular bridge to TBP from the RXR–RAR heterodimer bound on the proximal RARE adjacent to the TATA box. Our present observations corroborate and extend our model of an EC cell-specific bridging activity: promoters in which the proximal RARE- β 2 is replaced by DR1–DR5 elements remain highly RA responsive in pluripotent cells but can only be poorly activated in differentiated cells, unless the adenoviral E1A_(13S) protein is co-transfected. It

is plausible that appropriate protein interactions between RAR, E1A-LA and TBP could well compensate for the reduced affinity of RXR-RAR for DR1, DR3 and DR4 elements, thereby yielding the relatively high levels of RA-dependent transcription. This putative RAR-E1A-TBP bridging complex most likely requires a precise positioning of the different partners, particularly in the context of a promoter such as the RAR β 2 promoter, where the RARE is located in close proximity to the TATA box. The asymmetry of the bound heterodimer so near the TATA box necessitates a strict spatial alignment of the factors to obtain maximal transactivation and dictates asymmetry to the interactions with neighboring transcription factor(s) or component(s) of the basal transcription machinery. The spatial positioning of RAR and the basal machinery is probably required for interaction with bridging factors such as the E1A-like activity and the adenoviral E1A_(13S). In line with this model is our previous discovery that specific amino acids on the surface of TBP are essential for transcriptional signaling in EC cells and most likely are part of a surface on TBP that interacts with an EC cell-specific factor, presumably the E1A-LA (Keaveney *et al.*, 1993). Furthermore, a direct contact between RAR and the adenoviral E1A_(13S) protein reportedly occurs *in vitro* (Folkers and van der Saag, 1995). It seems plausible that the complex containing the E1A-LA [or E1A_(13S)] actually requires the proximity of all partners, explaining why the E1A-enhancing effect is not observed at a distance (M. Feigenbutz and H.G. Stunnenberg, unpublished observations).

Do other *cis*-acting elements influence the activation from our test promoters? In the case of the RAR β 2 promoter, a putative TPA-responsive element (TRE) and cAMP-responsive element (CRE) have been described (Shen *et al.*, 1991). Our data (Figures 5 and 6) indicate, however, that the mere presence and arrangement of upstream binding sites, including the presumed TRE that overlaps the distal RARE, CRE or other unidentified binding sites for *trans*-acting factors, are not the decisive factors in determining receptor selectivity. Relocation of RAR β 2 proximal promoter fragments, including the putative CRE, TRE and RAREs but excluding the TATA box, to position -109 of the tk promoter did not sustain the receptor selectivity observed in the original RAR β 2 context. For example, the DR4DR4-tk reporter mediated exclusively a T3 response, whereas the same arrangement of binding sites in the RAR β 2 context, that is placed next to the TATA box and tested in EC cells, acted as an RA-responsive promoter and had lost its ability to respond to T3. This strongly suggests that the close apposition of the RARE to the TATA box is one, if not the, decisive factor.

Taken together, the specificity of the RAR β 2 promoter for RAR appears to result from the close apposition of the nuclear receptor response element to the TATA box and the presence of E1A-LA, a cell type-specific co-factor and a molecular bridge between RAR and TBP. These two features appear to override the spacing rule that defines receptor selectivity based on the affinity of a given receptor pair for a particular DR element. The importance of the E1A-LA in 'redefining' the receptor selectivity raises the possibility that other cell type-specific co-factors exist that, in particular cases, can also override the 1-5 rule, which include SRC-1, RIP140 and others. Our

study also emphasizes the need to address experiments to dissection of naturally occurring promoter-enhancer configurations rather than reliance on idealized model systems.

Materials and methods

Plasmids

Reporter plasmids R140-Luc (RAR β 2 -124 to +14) and T3REM_oMLV-TK-Luc have been described previously (Vivanco Ruiz *et al.*, 1991). Mutants of the RAR β 2 promoter with altered spacing of the proximal RARE half-site were constructed by site-directed mutagenesis of plasmid pBSLUCM1 which contains the luciferase gene under control of a RAR β 2 promoter and with an *Xho*I site in place of a RARE, just upstream of the TATA box to permit insertion of altered sequences. The oligos used [DR(G)-type] were as follows: DR5, TCGACAGGTCATTTTCAGGTCA; DR4, TCGACAGGTCATTTTCAGGTCA; DR3, TCGACAGGTCATTCAGGTCA; DR2, TCGACAGGTCATTCAGGTCA; DR1, TCGACAGGTCACAGGTCA; with appropriate lower strand oligos producing *Xho*I-compatible ends.

The DR4DR4-, DR4DR5- and DR5DR4-b2 constructs were built by a PCR-mediated megaprimer mutagenesis approach (Baretino *et al.*, 1994). The DR-tk, as well as the DR-DR-tk constructs were obtained by inserting, respectively, the appropriate DR oligos, or fragments of the corresponding b2 promoter constructs, into the *Xho*I site of plasmid TK109Luc (Nordeen, 1988).

Plasmids expressing human RXR α , human RAR α , human VDR or chicken T3R α had been constructed by cloning the appropriate cDNAs into the mammalian expression vector pSG-5 (Green *et al.*, 1988) as previously detailed. Expression plasmids for hTBP and for adenovirus E1A_(13S) have been described (Berkenstam *et al.*, 1992). Truncated derivatives of RXR and RAR have been described (Valcárcel *et al.*, 1994). Construction of recombinant vaccinia viruses expressing 6-His derivatives of full-length or truncated receptors was as previously described (Janknecht *et al.*, 1991).

Cell culture and transient transfection assays

P19 and RAC65 EC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, non-essential amino acids and penicillin-streptomycin on plates treated with 0.1% gelatin. COS-7 and 293 cells were cultured in DMEM likewise supplemented. JEG cells were cultured in DMEM supplemented with 10% bovine calf serum. Cells were seeded at 5×10^5 cells per 6 cm Petri dish and transfected by the calcium phosphate method before the cells could attach. Each dish received 5 μ g of luciferase test reporter, 0.6 μ g of RSV-CAT control reporter and, when indicated, 0.5 μ g of reporter-expressing plasmid, 1 μ g of E1A_(13S) expression plasmid, 2 μ g of TBP expression plasmid, with the amount of DNA per dish made constant with addition of empty pSG5 vector. After 12 h, the cells were incubated in fresh medium with or without ligands [1 μ M AtRA or 9cRA, 1 μ M agonist RO13-7410 (TTNPB) or antagonists SR11234 or SR11237, 0.2 μ M T3, 1 μ M VD3]. Cells were harvested after 24 h for determination of luciferase and CAT activities; all luciferase activities were normalized for RSV-CAT expression as detailed previously (Berkenstam *et al.*, 1992).

Protein extracts, DNA binding and DNase I footprinting

COS-7 cells transiently transfected with receptor expression plasmids were harvested and lysed by freeze-thawing, as detailed (Berkenstam *et al.*, 1992); aliquots were tested for equivalence of receptors by Western blotting as described previously (Valcárcel *et al.*, 1994). Purification of receptors from vaccinia-infected HeLa cells using Ni²⁺-NTA chromatography was performed as detailed (Schmitt *et al.*, 1993).

Receptor-DNA binding assays were performed essentially as detailed (Bugge *et al.*, 1992), using extracts from transiently transfected cells and synthetic oligonucleotide probes. Briefly, receptors were incubated for 10 min on ice in reaction buffer [20 mM HEPES pH 7.9, 50 mM NaCl, 4 mM MgCl₂, 8% (v/v) glycerol, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, 0.1 μ g/ μ l bovine serum albumin (BSA), 0.1 μ g/ μ l poly(dIdC)], followed by addition of ³²P-labeled oligonucleotides and an additional 10 min incubation. Samples were electrophoresed for 3-4 h (150 V, 15 mA) on pre-run 6% polyacrylamide, 0.25 $\times$ TBE gels, dried and autoradiographed.

Footprinting was performed as previously described (Leblanc and Moss, 1994) using probes of ~200 bp prepared by PCR from the

appropriate reporter plasmids with one end-labeled oligo and one non-labeled oligo.

Acknowledgements

We thank Herbert Holz and Vera Sonntag-Buck for excellent technical assistance, and C.Garcia Jimenez for the gift of recombinant RAR and RXR proteins. We thank Chris Glass and Riki Kurokawa for gifts of plasmids 2×DR1-tk, and plasmids expressing the receptor chimeras T/A and Xho. We are grateful to Francis Stewart and members of the Stunnenberg laboratory for critical comments on the manuscript. We thank Lew Pizer, Department of Microbiology, University of Colorado Health Sciences Center, for sharing his laboratory facilities. B.P.L. was supported by a postdoctoral fellowship from MRC Canada; M.V.S. was supported by an EMBO fellowship, and J.L.B. was supported by a National Institutes of Health grant (USA).

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Received on October 21, 1996; revised on January 31, 1997