Nucleic Acids Research, 1993, Vol. 21, No. 5 1231–1237

RXR-dependent and RXR-independent transactivation by retinoic acid receptors

Magdalena Schräder, Adrian Wyss[1], Laurie J.Sturzenbecker[2], Joseph F.Grippo[2], Peter LeMotte[3] and Carsten Carlberg*

Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14, [1]Pharma Research New Technologies, Department of Biology, F.Hoffmann-La Roche Ltd, CH-4002 Basel, Switzerland, [2]Department of Investigative Toxicology, Hoffmann-La Roche Inc., Nutley, NJ 07110, USA and [3]Pharma Preclinical Research, F.Hoffmann-La Roche Ltd, CH-4002 Basel, Switzerland

Received November 16, 1992; Revised and Accepted February 2, 1993

ABSTRACT

The binding affinity of retinoic acid receptors (RARs) to their response elements is strongly enhanced *in vitro* by the formation of heterodimers with retinoid X receptors (RXRs) suggesting that heterodimerization with RXR may be a prerequisite for a RAR-mediated transcriptional response. We found that in *Drosophila* SL-3 cells that are devoid of endogenous RARs and RXRs the presence of RAR is sufficient to confer a response to all-*trans* retinoic acid (RA). The transfection of both RAR and RXR and stimulation with their respective ligands all-*trans* and 9-*cis* RA leads to a synergistic response. On point mutations of the RAR β2 gene promoter RA response element (RARE) the stimulation by RARs showed distinct differences in the absence and presence of RXR. The same differences in transcriptional activity are observed, if mammalian cells containing endogenous RARs and RXRs are stimulated with all-*trans* RA only or additionally with 9-*cis* RA. This establishes an RXR-independent and an RXR-dependent pathway of all-*trans* RA action in *Drosophila* SL-3 cells as well as in mammalian cells. The presence or absence of 9-*cis* RA determines by which of the two pathways a response to all-*trans* RA is mediated.

INTRODUCTION

Retinoic acid, a derivative of vitamin A (retinol), has profound effects on cellular differentiation and embryonic development (1, 2). The effects of all-*trans* RA are mediated by its binding to nuclear RARs which function as transcription factors after activation by the ligand (3). To date, three species of human RARs (α, β and γ), as well as three RAR-related proteins, the retinoid×receptors (RXRs α, β and γ), have been described (4–8). The ligand for the RXRs has recently been shown to be 9-*cis* retinoic acid (9-*cis* RA) (9, 10).

The RARs and RXRs, together with the receptors for steroid hormones, vitamin D and thyroid hormone, constitute a family of proteins with a common modular organization (11–13). Their highly conserved DNA binding domains contain eight invariant cysteines which complex two zinc ions (14). These zinc finger structures are involved in the binding to specific DNA sequences, referred to as hormone response elements (15, 16).

In recent years several RAREs have been characterized. A direct repeat of a (A/G)GTTCAC motif spaced by five nucleotides was identified in the promoters of the human and the mouse RAR β2 genes as well as in the human and mouse RAR α2 genes (17–20). DNase I footprinting of this RARE showed that the *in vitro* binding of RARs is responsible for all-*trans* RA-mediated transactivation (21).

While the steroid hormone receptors bind to their response elements as homodimers (22–24), recent evidence suggested that the retinoid/thyroid hormone/vitamin D subclass of nuclear receptors may only bind to their response elements as heterodimers with a member of the RXR family (25–30) or possibly another accessory protein. Evidence supporting this concept was mainly obtained from band shift experiments that showed enhanced receptor binding in the presence of RXR, thereby suggesting higher binding affinity of heterodimers for the response element as compared to the homodimer. However, whether heterodimerization with RXR is also a prerequisite for RAR-mediated transcriptional response has remained unclear so far. The investigation of this question was hampered by the presence of varying levels of RARs and RXRs in the cell lines.

We found that *Drosophila* SL-3 cells are devoid of RXRs and RARs but were responsive to all-*trans* or 9-*cis* RA upon transfection with the respective receptor expression plasmid. We show that transfection with RAR is sufficient to confer responsivity to all-*trans* RA. Transfection with RAR and RXR as well as addition of both ligands resulted in an enhanced response presumably mediated by RAR/RXR heterodimers. It thus appears that there are two different pathways for all-*trans*

* To whom correspondence should be addressed

RA action, one being RXR-dependent and the other RXR-independent. Point mutational analysis revealed two distinct response element patterns corresponding to the two pathways. This finding was substantiated by the analysis of the panel of response elements in mammalian cells containing endogenous RARs and RXRs. The results suggest that the RXR-dependent pathway of all-*trans* RA action requires the co-presence of 9-*cis* RA, indicating that the action of RAR/RXR heterodimers *in vivo* requires the ligands of both receptors.

MATERIALS AND METHODS

Cell culture

The human breast cancer cell line, MCF-7, was grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The *Drosophila* cell line, SL-3, was grown at room temperature in Schneider's medium (Gibco-BRL) supplemented with 15% FCS.

DNA constructs

The two 42 mer oligonucleotides comprising the wild type RARE (17):

5′ ctagtgctCGGGTAG**GGTTCA**CCGAA**AGTTCA**CTCGactgt 3′
3′ acgaGCCCAT**CCCAAGT**GGCTT**TCAAGT**GAGCtgagcagatc 5′

were synthesized on a Pharmacia Gene Assembler Plus apparatus. The oligonucleotides were purified, phosphorylated and annealed to yield a double-stranded DNA-fragment with Spe I and Xba I ends, called DR5[wt]. This fragment was subcloned into the Xba I site of pBLCAT2 (31) upstream of the *tk* promoter that drives the expression of the chloramphenicol acetyltransferase (CAT) gene. All mutations within the directly repeated motif have the same spacing and flanking sequences and were synthesized and cloned in the same way. All constructs were verified by sequence analysis on alkaline denatured DNA using the chain termination method (32).

Transfection and CAT assays

For MCF-7 cells, 5×10^5 cells were seeded into 60 mm Petri dishes and grown overnight in phenol red-free RPMI supplemented with 10% charcoal-treated FCS. For SL-3 cells, 2×10^6 cells per 60 mm Petri dish were grown overnight in Schneider's medium supplemented with 15% charcoal-treated FCS.

Liposomes were formed by incubating 5 μg of the reporter plasmid and 3 μg of the reference plasmid pRSVβgal with 30 μg DOTAP (Boehringer Mannheim) for 15 min at room temperature in a total volume of 100 μl. For the transfection of SL-3 cells, 1 μg of a pSG5-(Stratagene) based RAR α-, β-, γ- or RXR α-expression vector was also included. After dilution with 0.9 ml phenol red-free RPMI (or Schneider's medium for SL-3 cells), the liposomes were added to the cells. One ml phenol red-free RPMI, supplemented with 20% charcoal-treated FCS (or 1 ml Schneider's medium supplemented with 30% charcoal-treated FCS for SL-3 cells) was added 8 to 16 h after transfection. At this time all-*trans* RA, 9-*cis* RA, all-*trans* RA plus 9-*cis* RA (final concentrations 1 μM each) or solvent were also added.

The cells were harvested 40 h after initiation of the treatment and were lysed in 100 μl of 250 mM Tris−HCl, pH 7.8 by three cycles of alternate freezing and thawing. For the CAT-assay, 75 μl of the supernatant were incubated with acetyl CoA and [^{14}C] chloramphenicol for 1 to 5 h. The acetylation products were separated on TLC plates and quantitated by liquid scintillation counting. Protein concentrations were determined by the Bradford method (BioRad) and β-galactosidase activity was measured at 420 nm after incubation of 20 μl supernatant with ONPG (o-nitrophenyl-β-D-galactopyranoside). Both the protein concentration and the β-galactosidase activity were used for normalization of the CAT activity and yielded similar results.

Stimulation factors were calculated as the ratio of CAT activity of RA-treated cells to that of mock induced control. In order to normalize the results, a wild type RARE (DR5[wt]) was included in every experiment as an internal standard. The stimulation factor for each mutated element is expressed as a percentage of that of the wild type element within each experiment and for each cell line. Each mutation was analyzed 5−15 times (and at least 3 times in the case of the SL-3 cells) and data are shown as mean +/− standard deviation.

In vitro translation of RARs and RXRs

RAR α, β, γ and RXR α proteins were synthesized *in vitro*. The cDNAs for α, β, γ RAR and RXR α, cloned in pSG5 (Stratagene), were linearized by Bgl II, Xba I, Sal I and Xba I, respectively. Five μg of each of these templates were used for *in vitro* transcription in a total volume of 50 μl containing 0.5 mM dNTPs, 40 U RNasin (Promega), 40 mM Tris−HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT and 20 U T7 RNA polymerase (Promega). The transcription reactions were incubated for 60 min at 37°C and then 10 U of RQ1 RNase-free DNase I (Promega) were added and the mixture further incubated for 5 min at 37°C.

Five μg of each RNA were mixed with 175 μl rabbit reticulocyte lysate, 100 U RNasin and 20 μM complete amino acid mixture (all from Promega) in a total volume of 250 μl. The translation reactions were carried out at 30°C for 90 min. The comparable quantity of the receptor proteins was checked by translations performed in parallel using [^{35}S] methionine. The purity and concentration of labeled receptor proteins within the small scale reactions were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Different relative amounts of respective receptors, which were preincubated for 10 min on ice, were tested for optimal heterodimer formation.

Extracts from SL-3 cells were prepared as described (33).

DNA binding assays

The wild type RARE (DR5[wt]) was prepared by a double digest with Hind III and Bam HI of the response element containing plasmid. The DNA fragment was purified by gel electrophoresis and subsequently labeled by a fill-in reaction using [α-^{32}P]dCTP and T7 DNA polymerase (Pharmacia).

Either 5 μl of *in vitro* translated receptors, 5 μl of SL-3 cell extracts or a mixture of 2.5 μl *in vitro* translated receptor and 2.5 μl SL-3 cell extracts were incubated with about 1 ng of labeled probe (25000 cpm) for 30 min at room temperature in a total volume of 20 μl binding buffer (10 mM Hepes [pH 7.9], 80 mM KCl, 1 mM DTT, 0.2 μg/μl poly(dI/dC) and 5% glycerol). The protein-DNA complexes were resolved on a 5% non-denaturing polyacrylamide (29:1) gel at room temperature in 0.5× TBE (pre-run for 2 h), and the dried gel was exposed to a Kodak XAR film.

RESULTS

Heterodimerization with RXR is not a prerequisite for RAR-mediated transactivation

In order to analyze whether heterodimerization with RXR is a prerequisite for a RAR-mediated transactivation we used a cellular system devoid of endogenous RARs and RXRs. Whereas all mammalian cell lines tested so far were found to contain varying levels of RARs and RXRs, *Drosophila* SL-3 cells do not contain these receptors. In these cells a ligand-mediated response from a reporter plasmid is dependent on the co-transfection of receptor expression plasmids. Transfection of SL-3 cells with the reporter plasmid pBLCAT2 containing a single copy of the RARE from the RAR β2 promoter and the parental receptor expression plasmid did not result in an induction of CAT expression in response to all-*trans* RA, 9-*cis* RA or both ligands (Fig.1a). Co-transfection with RAR α, β or γ expression plasmids and stimulation with all-*trans* RA, or co-transfection with a RXR α expression plasmid and stimulation with 9-*cis* RA yielded a 4- to 10-fold induction of CAT expression (Fig.1a). Thus SL-3 cells are in principle able to respond to these ligands, but only after co-transfection of the respective receptor expression plasmids, indicating the absence of endogenous receptors.

Co-transfection with both receptor expression plasmids and stimulation with both ligands showed a synergistic effect for all three RAR subtypes (Fig.1a). While 1 μM is a saturating ligand concentration, the observed synergy is not due to an additive effect of the two ligands (34). The activation with either all-*trans* RA or 9-*cis* RA yielded 70 or 90% of maximal activity, respectively In part this is due to isomerization of 9-*cis* RA into all-*trans* RA and vice versa (34).

The response in the presence of both receptors was likely to be mediated by RAR/RXR heterodimers. The transactivation observed after transfection with only one receptor expression plasmid could be mediated either by RAR or RXR homodimers (35) or by heterodimers of one of these receptors with an endogenous SL-3 cell factor. A likely candidate for such a receptor is the *ultraspiracle* gene product, which is homologous to RXR (36) and is able to form heterodimers with RARs (37).

Therefore, binding of RARs to the RARE of the RAR β2 gene promoter was analyzed by band shift assays in the presence or absence of SL-3 cell extract. A weak retarded complex was observed with RARs alone and was enhanced in the presence of RXR α (Fig.1b), showing that heterodimers of *in vitro* translated RARs and RXR bind more efficiently to the probe than *in vitro* translated RARs alone. Moreover, SL-3 cell extract failed to enhance the binding of *in vitro* translated RARs to the probe, suggesting the absence of an active 'accessory factor' in this cell line. In contrast, extract from SL-3 cells transfected with RXR α expression plasmid enhanced the binding of *in vitro* translated RARs.

Taken together, these data imply that RARs are able to transactivate in the absence of RXR or *Drosophila* 'accessory factors', suggesting that such RXR-independent transactivation may be mediated by RAR homodimers, in spite of the apparently lower affinity binding of *in vitro* translated RARs to the response element. Furthermore, SL-3 cells have proven to be a good cellular system to investigate nuclear hormone receptor-mediated transactivation since they were found to be functionally devoid of relevant receptors (38).

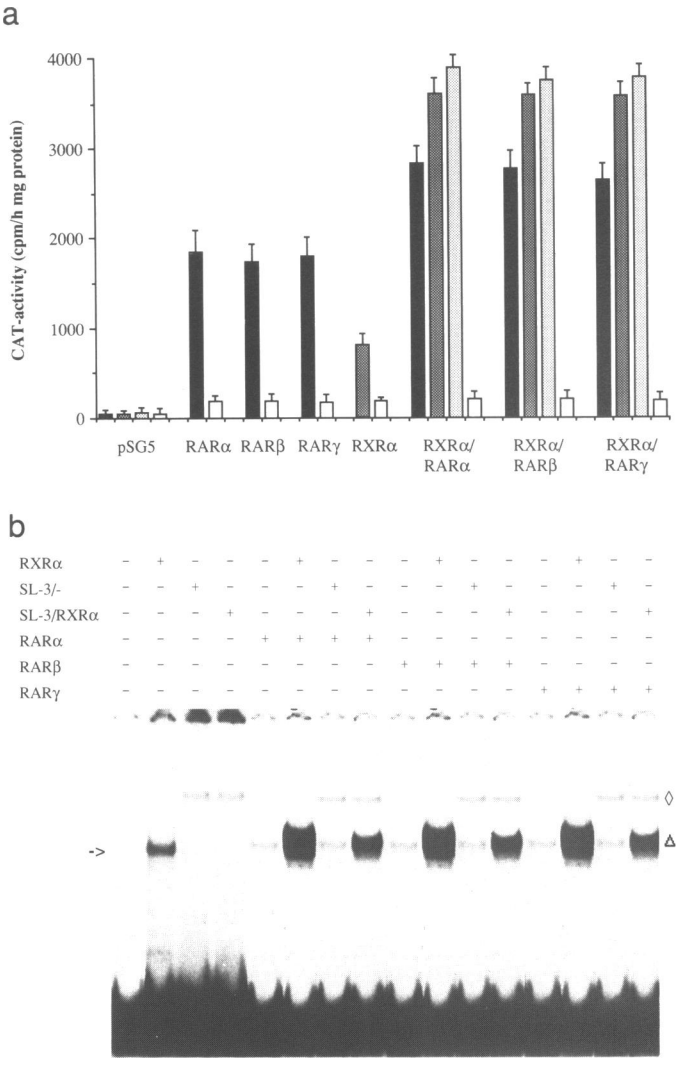

Figure1. RXR-independence versus RXR-dependence on the RAR β2 RARE. **(a)** RAR β2 gene RARE was cloned in front of the *tk* promoter of the CAT reporter plasmid pBLCAT2 to give the wild type construct, DR5[wt]. CAT activities were determined after transient transfection of SL-3 cells with this construct as well as with either the parental expression vector pSG5, the vectors containing cDNAs of RAR α, β, γ and RXR α alone or RARs together with RXR α, as indicated. Transfected cells were treated with either all-*trans* RA (black columns), 9-*cis* RA (dark grey columns), all-*trans* RA and 9-*cis* RA (light grey columns) or solvent (white columns). The columns indicate mean values and the bars standard deviations. **(b)** *In vitro* translated RARs α, β, γ, RXR α and extracts from non-transfected SL-3 cells (SL-3/−) or from SL-3 cells transfected with RXR α expression plasmids (SL-3/RXR α) were incubated as indicated with the [^{32}P]-labeled DR5[wt]-DNA fragment (in the first lane the reaction was carried out with unprogrammed lysate). The respective specific protein-DNA complex was separated from free probe (bottom) on a 5% non-denaturing polyacrylamide gel. The arrow indicates RXR homodimers with slightly higher mobility than RAR homo- and heterodimers (triangle). The rhombus indicates a *Drosophila* protein complexes, binding to the response element that does not interfere with the RAR/RXR binding.

RXR-independent transactivation by RARs α, β and γ shows similar response element preferences

Having established that the *Drosophila* SL-3 cell system allows the analysis of RXR-dependent and -independent transactivation by RARs, we were interested in determining whether these two

all-*trans* RA signaling pathways have different response motif preferences. First, we assessed the effect of single point mutations at each of the seven positions of the (A/G)GTTCAC repeat on RXR-independent transcription in the presence of each of the three RAR subtypes. To exclude a masking of the effect of a mutated motif by a wild type motif present in the element, symmetrical mutations were introduced into both copies of the repeat. For each RAR subtype, the stimulation factor (all-*trans* RA treated/vehicle treated) obtained with a mutated element was expressed as percentage of the response obtained with the wild type RARE, DR5[wt]. Interestingly, all three RARs showed very similar response element preference patterns (Fig.2). The first position analyzed is a G in the first half site and an A in the second half site within the DR5[wt] element. Changing both positions to A (DR5), to C (DR5[1C]) or to G (DR5[1G]) did not significantly alter the transcriptional activity of the element, whereas a change to T (DR5[1T]) reduced the activity by 50%. Even though mutations at this position did not drastically alter the transcriptional activity, this is the first nucleotide of the response motif according to the published literature (39). In addition, this position also seems to be important for binding of RAR/RXR heterodimers (see Fig.3). The next nucleotide in the wild type motif consists of a G. Changing it to A (DR5[2A]), to C (DR5[2C]) or to T (DR5[2T]) essentially abolished the transcriptional response of this element. Changing the T at position 3 to G (DR5[3G]) reduces the activity by half, whereas a change to A (DR5[3A]) or C (DR5[3C]) reduces the activity of the element even further. The T at position 4 is as crucial as position 2 since all substitutions eliminated the activity of the element. An exchange of the C at position 5 of the wild type sequence to G (DR5[5G]) or T (DR5[5T]) leaves 20–30% of the wild type activity, whereas a change to A (DR5[5A]) reduced the transcriptional activity even more. Changing the A at position 6 of the wild type motif to any other nucleotide reduces the activity of the element by more than 70%. However, at the seventh position any nucleotide is tolerated.

This mutational analysis shows that for the RXR-independent activity of any of the three RARs the preferred response motif sequence is (A,C,G)GTTCA when arranged as a direct repeat with a spacing of five nucleotides. Furthermore, the similar absolute transactivation potency of the three RAR subtypes on the DR5[wt] (Fig.1a), as well as their similar sensitivity to point mutations indicate that there are, at least for the response elements tested, no differences in the activity of the three RAR subtypes. Interestingly, the motif PuGGTCA, which often occurs in natural RAREs (40–43), was found to have only half of the activity of the DR5[wt].

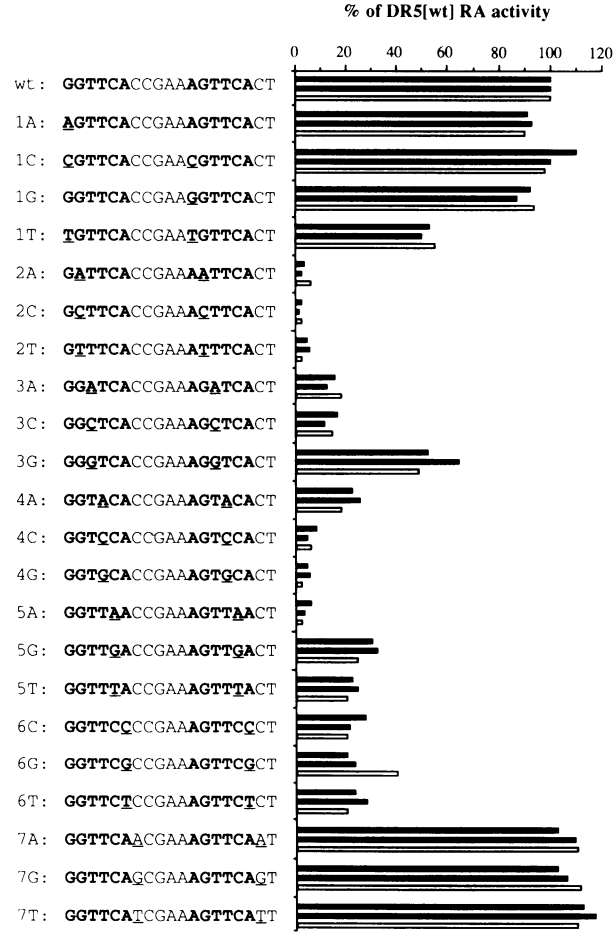

Figure 2. RXR-independent activity of RARs α, β and γ on RARE point mutations. *Drosophila* SL-3 cells were transfected with pBLCAT reporter plasmids containing a RARE with the indicated point mutations. The core sequence of the respective response elements is given to the left of the columns. Each of these reporter plasmids was separately co-transfected with the RAR α- (black columns), β- (grey columns) or γ- (white columns) expression vectors. The stimulation factor was determined from the ratio of all-*trans* RA-induced to uninduced cells relative to the activity of DR5[wt], which was determined in each experiment (for details, see materials and methods). Each column indicates the mean value from 5 or more independent experiments, the standard deviation was below 6% in all cases (error bars were omitted to avoid overloading of the figure).

Response motif preferences for RXR-dependent transactivation by RAR α

Having established the response motif preferences for RXR-independent RAR-mediated transactivation, we were interested in determining whether these were different for RAR/RXR heterodimer-mediated transactivation. Therefore, the same panel of response element mutants was analyzed in SL-3 cells cotransfected with RAR α and RXR α and stimulated with all-*trans* RA plus 9-*cis* RA. Interestingly, the nucleotides at positions one and seven of the motif are more important for RXR-dependent than for RXR-independent transactivation by RAR. An A at position one (DR5) is the preferred nucleotide, resulting in an activity that is 50% higher than that of the DR5[wt] element (Fig.3). All other nucleotide-variations at this position implicate activities lower than that of the DR5[wt] element. At position seven, which is a C in DR5[wt], an A shows an equal activity, whereas a change to G or T reduces the activity of the element by approximately half. The activity pattern of mutations at positions two to six of the element is similar to that observed for the RXR-independent transactivation by RAR although there are some noteworthy differences. A change of the C at position five of the motif to A (DR5[5A]) leaves less than 10% of the DR5[wt] activity when transfecting only RARs (Fig.2), whereas upon transfection of both RAR α and RXR α this mutant shows 50% of wild type activity (Fig.3). Mutations of the C at position five of DR5[wt] to G (DR5[5G]) or T (DR5[5T]) or mutations at position six to C, G or T show activities in the range of 20–30% of the DR5[wt] in presence of just RARs, whereas the activities are only 10–15% in the presence of both receptors.

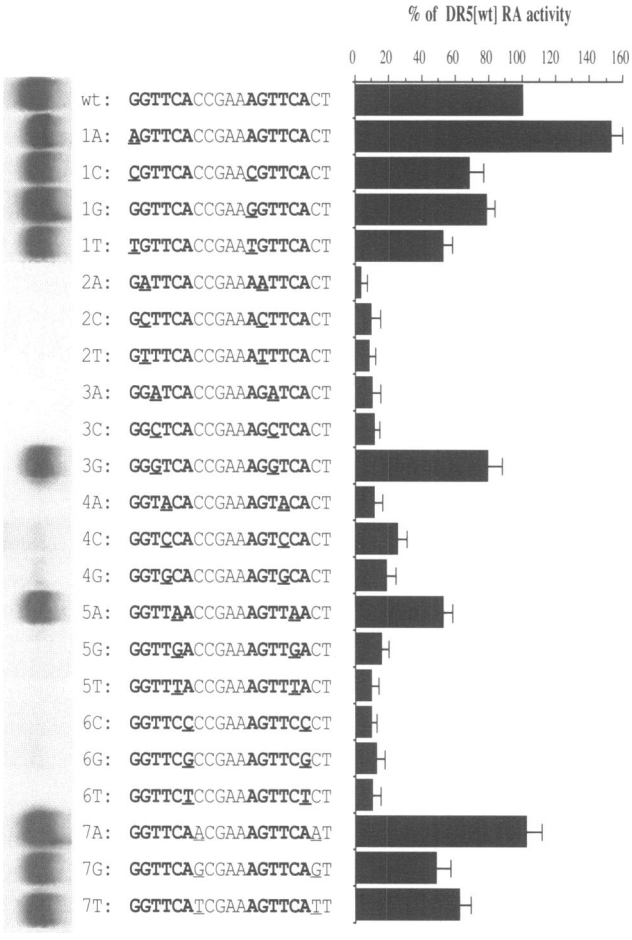

Figure 3. RXR-dependent activity of RAR α on RARE point mutations. *Drosophila* SL-3 cells were transfected with pBLCAT reporter plasmids containing a RARE with the indicated point mutations. The core sequence of the respective response elements is given below the columns. Each of these reporter plasmids was co-transfected together with RAR α and RXR α expression vectors. The stimulation factor was determined from the ratio of all-*trans* RA plus 9-*cis* RA-induced to uninduced cells relatively to the activity of DR5[wt], which was determined in each experiment (for details, see materials and methods). The bars indicate standard deviation. As control, equal amounts of the respective [^{32}P]-labeled DNA fragments were shifted with a preincubated mixture of *in vitro* translated RAR α and RXR α receptor protein. The specific protein−DNA complexes are shown below the core sequences.

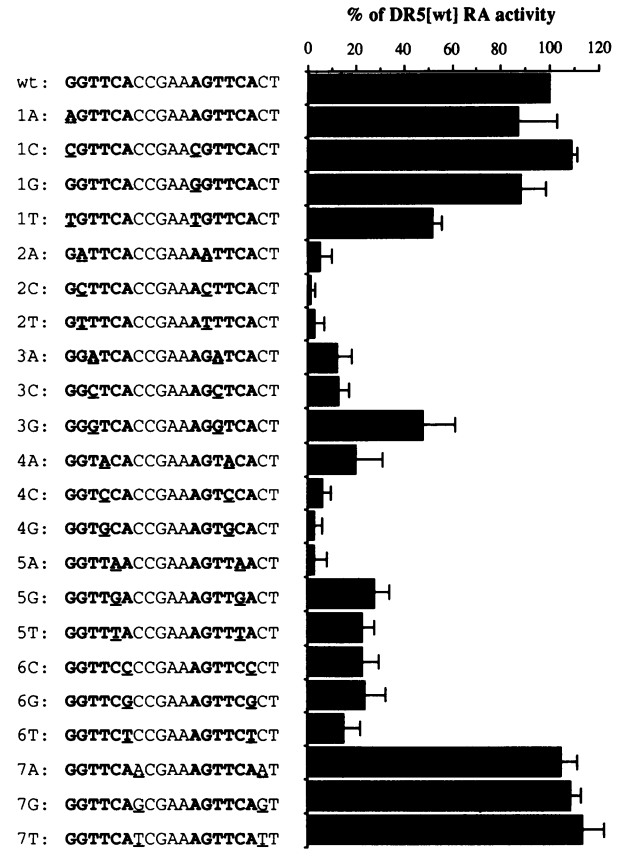

Figure 4. All-*trans* RA-dependent activity of endogenous RARs and RXRs in MCF-7 cells. Human breast cancer cells, MCF-7, were transfected with the pBLCAT reporter plasmids containing a RARE with point mutations as indicated by the core sequences given on the left to the columns. The stimulation factor was determined from the ratio of all-*trans* RA-induced to uninduced cells relatively to the activity of DR5[wt], which was determined in each experiment (for details, see materials and methods). The bars indicate standard deviation.

The receptor-binding properties of the panel of response elements were also analyzed by band shift assays, using equal amounts of *in vitro* translated RAR α and RXR α. The specific protein-DNA complexes for each element are shown below their respective transactivation activity (Fig.3). There is a good correlation between DNA binding affinity and transactivation potency for each of the mutated response elements. This result supports the observation that transactivation upon transfection of both receptors is mediated by RAR α/RXR α heterodimers.

Activity of the mutant response elements in mammalian cells containing endogenous RARs and RXRs

Having established the existence of RXR-dependent and RXR-independent transactivation by RAR that differ in their response element preferences, we were interested in investigating the response element preferences of a response to all-*trans* RA in mammalian cells which contain a mixture of endogenous RARs and RXRs. We found by Northern blot analysis that the human breast cancer cell line MCF-7 expresses relatively high levels of RAR α, RAR γ and RXR α (data not shown). Therefore, MCF-7 cells were transfected with the panel of mutant response elements (without any co-transfection of receptor expression plasmids) and stimulated with either all-*trans* RA or with all-*trans* RA plus 9-*cis* RA. Upon stimulation with all-*trans* RA, the transactivation activity pattern obtained in MCF-7 cells (Fig.4) resembles that observed in SL-3 cells transfected with RARs only and stimulated with all-*trans* RA (Fig.2). This resemblance is especially evident at positions one and seven, where (with the exception of a T at position one) all substitutions have a similar activity. This pattern is different from that observed for RAR/RXR-mediated transactivation in SL-3 cells (Fig.3) suggesting that the observed response in MCF-7 cells is of the RXR-independent type in spite of the presence of RXR α (and, potentially, β and γ).

Transfection of the panel of response elements into MCF-7 cells and stimulation with all-*trans* RA plus 9-*cis* RA, however, shows an activity pattern (Fig.5) reminiscent of that observed

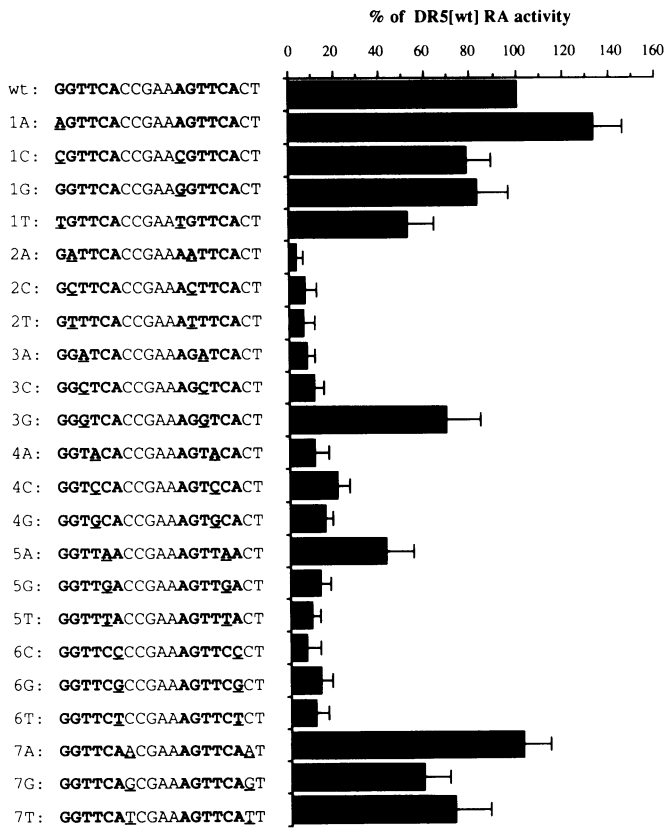

Figure 5. All-*trans* RA plus 9-*cis* RA-dependent activity of endogenous RARs and RXRs in MCF-7 cells. MCF-7 cells were transfected with the pBLCAT reporter plasmids containing a RARE with point mutations as indicated by the core sequences given on the left to the columns. The stimulation factor was determined from the ratio of all-*trans* RA plus 9-*cis* RA-induced to uninduced cells relative to the activity of DR5[wt], which was determined in each experiment (for details, see materials and methods). The bars indicate standard deviation.

in SL-3 cells cotransfected with both receptors and stimulated with both ligands (Fig.3). This result suggests that the response to the combined presence of both ligands is mediated by RAR/RXR heterodimers and it implies that the recruitment of RXR for a RAR/RXR mediated response requires its ligand, 9-*cis* RA.

The results show that mammalian cells containing both RARs and RXRs also posses RXR-dependent and RXR-independent signaling pathways for RA. Importantly, the availability of 9-*cis* RA, the ligand for RXRs, determines by which of the two pathways a response to RA is mediated.

DISCUSSION

Drosophila SL-3 cells, in contrast to mammalian cells that contain various levels of RARs and RXRs, are an appropriate cellular system to test functionally whether RARs can transactivate only as heterodimers with RXR (25–30). Co-transfection of any of the three RARs or of RXR α was sufficient to confer a response to all-*trans* RA or 9-*cis* RA, respectively, whereas co-transfection of RARs and RXR α stimulated with both ligands resulted in a synergistic response (Fig.1a). RXR α and 9-*cis* RA were not prerequisites for a RAR-mediated response. This established the existence of a RXR-dependent and RXR-independent pathway of RAR-mediated transcription.

The response motif preferences of the RXR-dependent and -independent transactivation by RARs in SL-3 cells showed some striking differences at nucleotide positions one and seven of the response motif (Figs.2 and 3). This suggests that two different complexes are formed on the response element depending on the presence or absence of RXR α and its ligand. Since a synergistic effect was observed when RARs, RXR and both ligands were present, and since the combined presence of both RAR and RXR induced an intense complex in band shift assays (Fig.1b), it can be assumed that this response was mediated by RAR/RXR heterodimers.

In the case of RXR-independent transactivation, RARs could either form a transcriptionally active homodimeric complex, or they could heterodimerize with an endogenous *Drosophila* SL-3 cell protein, such as the *ultraspiracle* gene product (36, 37). Gel shift analysis of *in vitro* translated RARs showed weak retarded complexes that were not enhanced by addition of *Drosophila* SL-3 cell extract. This counter indicates the presence of a SL-3 cell-derived protein heterodimerizing with RARs (Fig.1b). This suggests that the RXR-independent pathway of transactivational activity of RARs in SL-3 cells is most likely mediated by RAR homodimers.

Interestingly, the functional analysis of the panel of mutated response elements in mammalian cells is comparable to the RXR-dependent or RXR-independent response motif preference patterns of *Drosophila* SL-3 cells (compare Figs. 2 and 4 and Figs. 3 and 5). This similarity depends on whether the mammalian cells were stimulated with all-*trans* RA plus 9-*cis* RA, or all-*trans* RA alone (Figs.4 and 5). This result suggests that RXR-dependent and -independent pathways of all-*trans* RA signaling exists also in mammalian cells. In the absence of exogenous 9-*cis* RA, the response to all-*trans* RA seems to be mediated by the RXR-independent pathway, despite the presence of RXRs in the cell. MCF-7 cells apparently do not convert all-*trans* RA efficiently enough to 9-*cis* RA.

This implies that both ligands are required either for the formation of a RAR/RXR heterodimeric complex on the response element *in vivo* or for the stimulation of transcriptional activity by the ternary complex. This concept is supported by recent findings that the vitamin D receptor/RXR- and thyroid hormone/RXR-mediated responses require the ligands of both receptors (30, 33).

There is, however, an apparent discrepancy when comparing the functional properties of the receptors *in vivo* with their *in vitro* binding to the response element in a band shift assay. In spite of the similar transactivation activity of RARs in the presence and the absence of RXR and its ligand (Fig.1a), *in vitro* translated RARs alone show only weak binding to the response element (Fig.1b).

The response motif mutational analysis revealed distinct differences in RXR-dependent and RXR-independent transcription. For RXR-independent transcription, A, C and G at the first position resulted in the same activity, whereas T is associated with a two-fold lower activity. All other single point mutations within the two motifs in the DR5[wt] element excepting T→G at position 3 reduced the activity by more than 50%.

Interestingly, all three RAR subtypes showed very similar transactivation potencies on the DR5[wt] and similar activities on all mutant response elements when analyzed in the context of the *tk* promoter in SL-3 cells. The transcriptional activity of

the three receptors is likely to depend on the promoter context and the cell line used (18, 44–46), whereas these parameters are unlikely to influence the response motif preferences. We therefore conclude that for a spacing of 5 nucleotides the preferred response element for a RXR-independent transactivation by RARs is composed of two copies of the (A/C/G)GTTCA motif. Similar conclusions were reached by other groups (19, 21).

For RXR-dependent transactivation by RARs, the positions one and seven additionally influenced the activity of the RARE. Changing position one in the first half-site of the wild type RARE from G to A increased the activity by 50%. Mutations at position seven to G or T reduced the activity by approximately half. Other mutations that reduced the activity by less than 50% were T→G at the third and C→A at the fifth position. All these point mutations show distinct differences from the response motif preferences of the RXR-independent pathway.

In sum, our results provide evidence for a RXR-dependent and an RXR-independent mode of action of all-*trans* RA. Based on these and other data (40) our working model implies that the nature of the response element and the presence of the respective ligands determine whether a given gene is regulated by receptor homo- or heterodimers. Certain response elements favour the binding of receptor homodimers (33, 35) whereas others can bind either homodimers or heterodimers. Whether the latter class of elements respond to homodimers or heterodimers depends not only on the presence of the respective receptors within the cell but also on the presence of all-*trans* RA or 9-*cis* RA. The model further implies that the ternary complex is stabilized by ligand, a fact that with the exception of RXR homodimers (35), was not so far been reproduced in *in vitro* binding assays. An alternative possibility is that receptor dimers can bind to DNA in absence of ligand but that the ligand is necessary for efficient transactivation. However, the ligand plays a pivotal role in triggering the biological response notwithstanding the presence of other receptors in the cell.

ACKNOWLEDGEMENTS

We would like to thank Prof. G.Schütz (DKFZ, Heidelberg) for the gift of the pBLCAT2 plasmid, Drs M.Follo and W.Hunziker (Roche Basel) for discussions, A.Goswamy (Roche Basel), E.Silverman, Drs K.M.Müller and C.Meier (Université Genève) for critical reading of the manuscript and Prof. W.Wahli (Université Lausanne) for the gift of SL-3 cells.

REFERENCES

1. Tabin, C.J. (1991) Cell *66*, 199–217.
2. Leid, M., Kastner, P. and Chambon, P. (1992) Trends Biochem. *17*, 427–433.
3. Glass, C.K., DiRenzo, J., Kurokawa, R. and Han, Z. (1991) DNA & Cell Biol. *10*, 623–638.
4. Giguère, V., Ong, E.S., Segui, P. and Evans, R.M. (1987) Nature *330*, 624–629.
5. Brand, N., Petkovich, M., Krust, A., Chambon, P., de Thé, H., Marchio, A., Tiollais, P. and Dejean, A. (1988) Nature *332*, 850–853.
6. Krust, A., Kastner, P., Petkovich, M., Zelent, A. and Chambon, P. (1989) Proc. Natl. Acad. Sci. U.S.A. *86*, 5310–5314.
7. Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. (1990) Nature *345*, 224–229.
8. Mangelsdorf, D.J., Borgmeyer, U., Heyman, R.A., Zhou, J.Y., Ong, E.S., Oro, A.E., Kakizuka, A. and Evans, R.M. (1992) Gen. Dev. *6*, 329–344.
9. Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M. and Thaller, C. (1992) Cell *68*, 397–406.
10. Levin, A.A., Sturzenbecker, L.J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A. and Grippo, J.F. (1992) Nature *355*, 359- 361.
11. Evans, R.M. (1988) Science *240*, 889–895.
12. Green, S. and Chambon, P. (1988) Trends Genet. *4*, 309–314.
13. Beato, M. (1989) Cell *56*, 335–334.
14. Green, S. and Chambon, P. (1987) Nature *325*, 75–78.
15. Berg, J.M. (1989) Cell *57*, 1065–1068.
16. Umesono, K. and Evans, R.M. (1989) Cell *57*, 1139–1146.
17. de Thé, H., Vivanco-Ruiz, M.M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990) Nature *343*, 177–180.
18. Hoffmann, B., Lehmann, J.M., Zhang, X., Hermann, T., Husmann, M., Graupner, G. and Pfahl, M. (1990) Mol. Endo. *4*, 1727–1736.
19. Sucov, H.M., Murakami, K.K. and Evans, R.M. (1990) Proc. Natl. Acad. Sci. U.S.A. *87*, 5392- 5396.
20. Leroy, P., Nakshatri, H. and Chambon, P. (1991) Proc. Natl. Acad. Sci. U.S.A. *88*, 10138- 10142.
21. Vivanco Ruiz, M.M., Bugge, T.H., Hirschmann, P. and Stunnenberg, H.G. (1991) EMBO J. *10*, 3829–3838.
22. Kumar, V.J. and Chambon, P. (1988) Cell *55*, 145–156.
23. Tsai, S.Y., Carlstedt-Duke, J., Weigel, N.I., Dahlman, K., Gustafsson, J.A., Tsai, M.J. and O'Malley, B.W. (1988) Cell *55*, 361–369.
24. Fawell, S.E., Lees, J.A., White, R. and Parker, M.G. (1990) Cell *60*, 953–962.
25. Yu, V.C., Delsert, C., Andersen, B., Holloway, J.M., Devary, O.V., Näär, A.M., Kim, S.Y., Boutin, J.-M., Glass, C.K. and Rosenfeld, M.G. (1991) Cell *67*, 1251–1266.
26. Bugge, T.H., Pohl, J., Lonnoy, O. and Stunnenberg, H.G. (1992) EMBO J. *11*, 1409–1418.
27. Kliewer, S.A., Umesono, K., Mangelsdorf, D.J. and Evans, R.M. (1992) Nature *355*, 446–449.
28. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P. (1992) Cell *68*, 377–395.
29. Marks, M.S., Hallenbeck, P.L., Nagata, T., Segars, J.H., Appella, E., Nikodem, V.M. and Ozato, K. (1992) EMBO J. *11*, 1419–1435.
30. Zhang, X.-K., Hoffmann, B., Tran, P.B.-V., Graupner, G. and Pfahl, M. (1992) Nature *355*, 441- 446.
31. Luckow, B. and Schütz, G. (1987) Nucl. Acids Res. *15*, 5490.
32. Chen, E.Y. and Seeburg, P.H. (1985) DNA & Cell Biol. *4*, 165–170.
33. Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L.J., Grippo, J.F. and Hunziker, W. (1992) Nature in press.
34. Carlberg, C. (1992) in preparation.
35. Zhang, X.-K., Lehmann, J., Hoffmann, B., Dawson, M.I., Cameron, J., Graupner, G., Herrmann, T., Tran, P. and Pfahl, M. (1992) Nature *358*, 587–591.
36. Oro, A.E., McKeown, M. and Evans, R.M. (1990) Nature *347*, 298–301.
37. Yao, T.-P., Segraves, W.A., Oro, A.E., McKeown, M. and Evans, R.M. (1992) Cell *71*, 63–72.
38. Martinez, E., Givel, F. and Wahli, W. (1991) EMBO J. *10*, 263–268.
39. Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991) Cell *65*, 1255–1266.
40. Vasios, G.W., Gold, J.D., Petkovich, P., Chambon, P. and Gudas, L.J. (1989) Proc. Natl. Acad. Sci. U.S.A. *86*, 9099–9103.
41. Duester, G., Shean, M.L., McBride, M.S. and Stewart, M.J. (1991) Mol. Cell. Biol. *11*, 1638- 1646.
42. Mangelsdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E. and Evans, R.M. (1991) Cell *66*, 555–561.
43. Smith, W.C., Nakshatri, H., Leroy, P., Rees, J. and Chambon, P. (1991) EMBO J. *10*, 2223- 2230.
44. Husmann, M., Lehmann, J., Hoffmann, B., Hermann, T., Tzukerman, M. and Pfahl, M. (1991) Mol. Cell. Biol. *11*, 4097–4103.
45. Lehmann, J.M., Zhang, X.-K. and Pfahl, M. (1992) Mol. Cell. Biol. *12*, 2976–2985.
46. Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H. and Chambon, P. (1992) Cell *70*, 1007–1019.