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Characterization and purification of human retinoic acid receptor-γ1 overexpressed in the baculovirus–insect cell system

Ambati P. REDDY,* Jia-Yang CHEN,† Tim ZACHAREWSKI,† Hinrich GRONEMEYER,† John J. VOORHEES* and Gary J. FISHER*‡

Department of Dermatology, University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A., and †Institute de Chimie Biologie, Faculte de Medicine, Strasbourg, Cedex, France

The full-length cDNA for the human retinoic acid receptor-γ1 (RAR-γ1) has been expressed to high levels in *Spodoptera frugiferda* (Sf9) cells using the baculovirus expression system. Western blot analysis revealed that RAR-γ1 expression increased between 32 and 60 h post-infection. The recombinant receptor was expressed primarily as a nuclear protein and displayed a molecular mass of 50 kDa as determined by SDS/PAGE and gel-filtration chromatography, consistent with its cDNA-deduced size. Based on ligand binding, 2×10^6 RAR-γ1 molecules were expressed per Sf9 cell, a level approx. 2000 times greater than in mammalian cells. The receptor was partially purified 300-fold by sequential anion-exchange, gel-filtration and DNA affinity chromatographies. The overexpressed receptor specifically bound all-*trans*-retinoic acid (RA) and the synthetic retinoid CD367 with high affinity (K_d 0.15 nM and 0.23 nM respectively). The RA metabolites 4-hydroxy-RA and 4-oxo-RA were poor competitors for [^{3}H]CD367 binding to recombinant RAR-γ1 ($K_i > 1$ μM), indicating that 4-oxidation of RA greatly reduces its affinity for RAR-γ1. Gel-retardation analysis demonstrated that RAR-γ1 specifically bound the RA response element of the mouse RAR-β gene. RAR-γ1 species expressed from recombinant baculovirus (in Sf9 cells) and vaccinia virus (in HeLa cells) exhibited similar affinities for RA and CD367 and had comparable DNA-binding properties in gel-retardation experiments. Moreover, a similar requirement for additional DNA-binding stimulatory factor(s) was observed in both cases. These results provide a basis for the use of baculovirus-expressed RAR-γ1 in further functional and structural studies.

INTRODUCTION

Retinoic acid (RA), which is derived from vitamin A, is a key regulator of cellular growth and differentiation. RA and its synthetic analogues (retinoids) are used therapeutically in the prevention and treatment of skin diseases and certain malignancies [1–5]. The molecular mechanisms through which retinoids act are largely unknown. Recently two related families of nuclear receptors for all-*trans*-RA (RARs) [6–11] and its 9-*cis* stereoisomer (RXRs) [12–17] have been identified. Three distinct RAR (RAR-α, -β and -γ) and RXR (RXR-α, -β and -γ) genes have been cloned, and it has been demonstrated that heterodimerization of RARs and RXRs increases the efficiency of binding to certain cognate response elements (RAREs) [13,15]. RARs are members of the superfamily of nuclear receptors (reviewed in [18]) and act as ligand-dependent transcriptional enhancer factors. Differential promoter usage and alternative splicing generate multiple isoforms of each RAR member, which differ in their *N*-terminal sequences [19,20]. The three major RAR gene products (RAR-α1, -β2 and -γ1) display differential tissue distributions, suggesting that they may possess functional specificity [21,22]. Several genes containing a functional RARE have been identified, and are generally composed of direct repeats of the motif 5′-GTTCA or 5′-GGTCA, or closely related sequences [23–28].

Most studies to date on the regulation of RAR expression have described measurements of RAR mRNA levels by Northern blot analysis or *in situ* hybridization [29–31]. In these studies, it has been assumed that relative amounts and changes in mRNA levels reflect similar changes in functional receptor protein. The lack of information concerning the regulation and properties of RARs stems from their low abundance in cells, which express on average 1000 RAR molecules per cell [32]. This small number makes detection of functional RARs by standard ligand-binding assays or immunological methods difficult, and makes purification of RARs from these sources for the purposes of structural and functional characterization not technically feasible.

To obtain sufficient amounts of receptor for functional and structural studies, we have utilized the baculovirus and vaccinia virus expression systems to express large quantities of full-length human RAR-γ1 in *Spodoptera frugiferda* (Sf9) insect cells and HeLa cells, respectively. The recombinant RAR-γ1 was partially purified and its ligand- and DNA-binding properties examined. A comparative analysis revealed no differences between RAR-γ1 overexpressed in mammalian (HeLa) or insect (Sf9) cells. Oxidized RA metabolites, generally believed to have little biological activity [33,34], bound to RAR-γ1, although with significantly lower apparent affinity than RA. The availability of large quantities of RARs, obtained through the baculovirus expression system, will allow in-depth functional and structural studies to be realized.

MATERIALS AND METHODS

Materials

Grace's insect culture medium, yeastolate and lactalbumin hydrolysate were purchased from In Vitrogen (San Diego, CA, U.S.A.). Serum-free growth medium (ExCell-400) was from J.R Scientific (Lenexa, KS, U.S.A).Fetal bovine serum and antibiotics were obtained from Gibco. Cyanogen bromide-activated Sepharose CL-2B was obtained from Sigma. A random-priming

Abbreviations used: AcNPV, *Autographa californica* nuclear polyhedrosis virus; DBSFs, DNA-binding stimulatory factors; PBS, phosphate-buffered saline (2.7 mM-KCl, 1.2 mM-KH_2PO_4, 138 mM-NaCl and 8.1 mM-$Na_2HPO_4,7H_2O$); RA, all-*trans*-retinoic acid; RAR, RA receptor; RARE, RA receptor element; RXR, retinoid receptor; Sf9, *Spodoptera frugiferda*.

‡ To whom correspondence should be addressed: University of Michigan, Department of Dermatology, E. Catherine, Kresge I, R6558, Ann Arbor, MI 48109–0528, U.S.A.

DNA labelling kit and restriction enzymes were obtained from Boehringer Mannheim. Goat anti- chicken antibody was from Fisher Biotech. Oligonucleotides were synthesized at the DNA Core Facility, University of Michigan. [^{3}H]RA and [α-^{32}P]dCTP [3000 Ci/mmol) were from DuPont–New England Nuclear. Sf9 cells were obtained from the American Type Culture Collection. The baculovirus transfer plasmids, pVL1392 and pVL1393, and wild-type virus *Autographa californica* nuclear polyhedrosis (AcNPV) were generously provided by Dr. Max Summers, Texas A&M University (College Station, TX, U.S.A.). [^{3}H]CD367 was kindly provided by Dr. B. Shroot, CIRD, Valbonne, France. 4-Hydroxy-RA and 4-oxo-RA were kindly provided by Hoffman–LaRoche.

Culture of Sf9 cells

Sf9 cells were grown at 27 °C in monolayer culture, in TNM-FH medium [35,36] supplemented with 10% fetal bovine serum, 100 μg of streptomycin/ml, 100 units of penicillin/ml and 125 ng of amphotericin B/ml. For expression of RAR-γ1, cells were adapted to, and grown in, serum-free growth medium (ExCell-400 medium) supplemented with antibiotics.

Construction of recombinant transfer vectors

A cDNA fragment containing the complete open-reading frame of human RAR-γ1 (nucleotides 43–1800) was excised by restriction enzyme digestion from the parent plasmid pGEM3Z-RAR-γ. The 1767 bp cDNA fragment obtained was purified, and excess 5′ non-coding sequences were deleted by *Bal*31 cleavage. The 5′ ends were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I, and *Eco*RI restriction sites were created with *Eco*RI linkers using standard procedures. The cDNA was then subcloned into the multiple cloning sites in plasmid pVL1392 to form the transfer vector pVL1392/RAR-γ. The orientation of the construct was determined by restriction enzyme analysis and DNA sequencing.

Transfection and screening of recombinant virus

Sf9 cells (2×10^6 cells in a 25 ml T-flask) were co-transfected with 1 μg of AcNPV genomic DNA and 5 μg of the recombinant transfer vector pVL1392/RAR-γ using the modified calcium phosphate co-precipitation method for insect cells [35,36]. Following 6 days of incubation at 27 °C, the cell culture medium was harvested and screened for recombinant virus. Sf9 cells were treated with limiting dilutions of recombinant virus-containing media in microtitre plates. Wells were assayed for recombinant virus by DNA dot-blot hybridization using ^{32}P-labelled RAR-γ1 cDNA as probe, as described by Summers and co-workers [35,36]. Media from positive wells were titrated by plaque assay. Plaques without polyhedra were chosen and recombinant extracellular virus was obtained by four rounds of plaque purification. Three recombinant viral clones, which contained RAR-γ1 cDNA, were identified.

Expression and purification of recombinant RAR-γ1

Sf9 cells (3×10^6 cells/ml) were infected with recombinant RAR-γ1 virus (10^8 plaque-forming units/ml; clone H6/C4) for 2 h at room temperature. After infection, the viral inoculum was replaced with fresh medium. At various times post-infection the cells were harvested by scraping and washed in phosphate-buffered saline (PBS). The washed cells were lysed in buffer containing 20 mM-Tris/HCl (pH 8.0), 130 mM-NaCl, 1 mM-$CaCl_2$, 1 mM-$MgCl_2$, 10% glycerol, 1% Nonidet P40, 2 mM-phenylmethanesulphonyl fluoride, 0.2 units of aprotinin/ml, 0.02 mM-leupeptin and 0.5 μg of pepstatin/ml. The lysate was centrifuged for 15 min at 15000 *g* and the supernatant was utilized for analysis and further purification of RAR-γ1.

Recombinant RAR-γ1 was partially purified by f.p.l.c. The supernatant from recombinant-virus-infected Sf9 cells, containing approx. 60 mg of protein, was applied to a Mono Q column (8 ml) equilibrated with 20 mM-Tris/HCl (pH 7.5), 10% glycerol and 10 mM-thioglycerol. After eluting unbound protein with equilibration buffer, RAR-γ1 was eluted with a 200 ml non-linear gradient of NaCl (0–1 M). Fractions of 2 ml were collected and 0.2 ml of each fraction was analysed for RAR-γ1 by protein immunoblotting using a RAR-γ1-specific antibody, obtained by immunization of chickens with synthetic peptides having the *C*- and *N*-terminal amino acid sequences of human RAR-γ1 (see below). Immunoblots were visualized with alkaline-phosphatase–secondary-antibody conjugates, and the amount of RAR-γ1 was estimated from the intensity of colour on a 1–10 scale, with 10 being most intense. Fractions containing RAR-γ1 were pooled and concentrated by centrifugation in an Amicon Centricon 30, and then applied to a Superdex gel-filtration column (25 ml) equilibrated with 0.3 M-KPO_4, 10% glycerol and 10 mM-thioglycerol (pH 7.5). Fractions (0.5 ml) were collected and assayed as described above. Fractions containing receptors were pooled, diluted 3-fold in 75 mM-Hepes, 0.3 M-KCl, 37.5 mM-$MgCl_2$, 3 mM-dithiothreitol, 50% glycerol and 60 μg of calf thymus DNA/ml, and applied to a RARE affinity column (1 ml). The column was washed with equilibration buffer to remove unbound protein, and bound RAR-γ1 was eluted with equilibration buffer containing 1 M-KCl. The purified receptor was stored at −20 °C, and retained ligand-binding activity for at least 1 month.

Recombinant RAR-γ1 vaccinia virus

Construction of recombinant vaccinia virus expression vectors, infection of HeLa cells with these vectors and preparation of nuclear extracts from these cells has been previously described [37].

Preparation of RARE affinity column

A DNA affinity column was prepared by covalently coupling synthetic concatamers (average size 87 bp) of the 27 bp RARE consensus sequence, found in the promoter of the RAR-β gene, to cyanogen bromide-activated Sepharose CL-2B. The procedure has been described previously [38]. The sequence of the RARE utilized was:

5′-AGGGTTCACCGAAAGTTCACTCGCATA
3′-TCCCAAGTGGCTTTCAAGTGAGCGTAT.

Western blot analysis

Crude or partially purified recombinant RAR-γ1 preparations were resolved by SDS/PAGE on 10% gels [39]. Protein bands were either visualized by Coomassie Blue or silver stain, or electroblotted on to a poly(vinylidene difluoride) membrane. The membrane was blocked with 3% BSA/0.05% Tween 20/Tris-buffered saline for 1 h and incubated with specific anti-RAR-γ1 antibody from chicken egg for 2 h. The membrane was then washed and incubated with alkaline phosphatase-conjugated secondary antibody, and the protein bands were visualized after addition of chromogenic substrate.

Retinoid binding assay

Binding of [^{3}H]RA or [^{3}H]CD367 to recombinant RAR-γ1 was measured by incubating receptor and ligand (40 nM-retinoid; 200000 c.p.m.) in 10 mM-Tris/HCl (pH 7.5), 10% glycerol and 10 mM-thioglycerol in a final volume of 100 μl, for 4 h at 4 °C. ^{3}H-labelled ligand bound to receptor was separated from free ligand by passage of the incubation mixture (50 μl) through a GF-250 size-exclusion column equilibrated with 0.3 M-KPO_4 (pH 7.8) [40]. The column was eluted with equilibrium buffer at a flow rate of 0.5 ml/min. Fractions (0.3 ml) were collected and

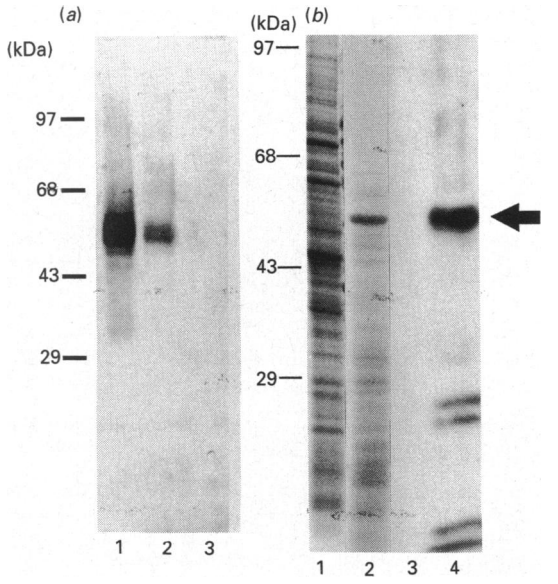

Fig. 1. Time course of RAR-γ1 expression, detected by Western analysis, in Sf9 cells

(*a*) Lysates were prepared from Sf9 cells infected with recombinant baculovirus, containing the open-reading frame of human RAR-γ1, for 60 h (lane 1) and 32 h (lane 2), and from non-infected Sf9 cells (lane 3). Each sample (20 μg) was subjected to SDS/PAGE and immunoblotted with anti-peptide antibody to RAR-γ1. The migration of molecular mass standard proteins is shown. (*b*) The lysates from non-infected (lanes 1 and 3) and infected (at 60 h post-infection) (lanes 2 and 4) Sf9 cells were centrifuged, and the supernatants were subjected to SDS/PAGE. Lanes 1 and 2 were stained with Coomassie Blue for protein, and lanes 3 and 4 were transferred to nitrocellulose and immunoblotted with anti-RAR-γ1 antibody. The arrow indicates the position of RAR-γ1.

radioactivity was counted by liquid scintillation spectrometry. For determination of equilibrium binding dissociation constants (K_d) and of the ability of unlabelled RA metabolites to compete for binding of [^{3}H]CD367, dextran-coated charcoal was employed to separate bound from free ligand [41]. Binding data were analysed by the method of Scatchard [42].

Gel-retardation assay

The DNA-binding properties of recombinant RAR-γ1 were studied by a gel-retardation assay as described by Nicholson *et al.* [37]. Antibodies utilized in these studies (see Fig. 7) have been described previously [43].

Preparation of chicken anti-RAR-γ1 antibodies

Chicken egg antibodies to RAR-γ1 were prepared by immunization of single-comb White Leghorn laying hens (shaver stockcross 288) (16–17 months old) with 100 μg of synthetic peptide containing the *N*-terminal (RGLGQPDLPKEMASC) or *C*-terminal (CSSEDEVPGGQGKGGLKSPA) sequence of human RAR-γ1, conjugated to keyhole limpet haemocyanin. The antigen was dissolved in 0.75 ml of PBS, mixed with an equal volume of complete Freund's adjuvant, and injected intramuscularly into two sites in the breast. Two groups of three chickens each were injected with the two different peptide antigens. Booster immunizations were given on days 12 and 20. Eggs were collected each day and antibodies were prepared from the yolks by precipitation with poly(ethylene glycol) 8000, as described in [44]. The antibodies were titred by e.l.i.s.a. using the appropriate unconjugated peptide as antigen and a horseradish peroxidase-conjugated goat anti-chicken second antibody. All six chickens produced antibodies of similar titres, with maximal production between days 30 and 35. None of the three antibodies raised to the *N*-terminal peptide recognized the *C*-terminal peptide, and none of the three anti-*C*-terminal antibodies recognized the *N*-terminal peptide. For Western blot and immunodot blot analysis the antibodies were used at a dilution of 1:100, and *C*- and *N*-terminal antibodies yielded similar results.

RESULTS

Expression of RAR-γ1 in Sf9 insect cells

Recombinant baculovirus containing the coding sequences for RAR-γ1 was generated according to standard procedures described by Summers & Smith [35]. Briefly, a transfer vector (pVL1392/RAR-γ1) was constructed (see the Materials and methods section) such that a non-fusion full-length RAR-γ1 protein was expressed. Homologous recombination was achieved by co-transfecting wild-type AcNPV genomic DNA and pVL1392/RAR-γ1 genomic DNA into Sf9 cells. Occlusion-negative plaques were identified by visual inspection or plaque hybridization and purified from contaminating wild-type virus by four rounds of plaque purification. The resulting pure recombinant virus, which was free of occlusion bodies, was used for protein production.

In order to determine the time course of RAR-γ1 protein expression, Sf9 cells were infected with RAR-γ1-containing baculovirus and total cell lysates were analysed for RAR-γ1 by Western blotting using chicken anti-RAR-γ1 antibody (see the Materials and methods section), 32 and 60 h post-infection. A major band of 50 kDa, which was not seen in lysates from uninfected cells, was detected 32 h post-infection (Fig. 1*a*). This 50 kDa band, which is the expected size of RAR-γ1, increased in abundance between 32 and 60 h post-infection, and the level of RAR-γ1 remained constant between 60 and 90 h after infection. At times greater than 90 h post-infection, numerous bands of lower molecular mass were observed, presumably reflecting proteolysis of RAR-γ1 (results not shown). This degradation of RAR-γ1 coincided with significant viral lysis of the cells. In order to minimize protein degradation associated with cell lysis, infected cells were routinely harvested between 60 and 90 h post-infection.

To estimate the level of RAR-γ1 expression, the protein patterns of supernatants from uninfected and RAR-γ1-infected Sf9 cells were analysed by SDS/PAGE and Western blot. A prominent protein band with apparent molecular mass of 50 kDa, which was absent from uninfected cells, was present in the supernatant from RAR-γ1-infected cells (Fig. 1*b*, lanes 1 and 2). Western blot analysis indicated that this band was RAR-γ1 (Fig. 1*b*, lanes 3 and 4).

The intracellular localization of recombinant RAR-γ1 protein in the insect cells was analysed by Western blot analysis of nuclear and cytoplasmic fractions from infected Sf9 cells. The results indicated that RAR-γ1 was predominantly present in the nuclear fraction (results not shown).

Purification of recombinant RAR-γ1

Recombinant RAR-γ1 was partially purified from Sf9 cell lysates by sequential ion-exchange, gel-filtration and affinity chromatographies. The cell lysate was centrifuged and the supernatant was applied to a Mono Q anion-exchange column connected to an f.p.l.c. system. After washing the column to remove unbound protein, RAR-γ1 was eluted by a gradient of NaCl (Fig. 2). RAR-γ1 in the column fractions was measured by Western dot-blot. RAR-γ1 eluted as a single symmetrical peak between 0.25 and 0.35 M-NaCl. Fractions containing RAR-γ1 were combined, concentrated and applied to a Superdex gel-

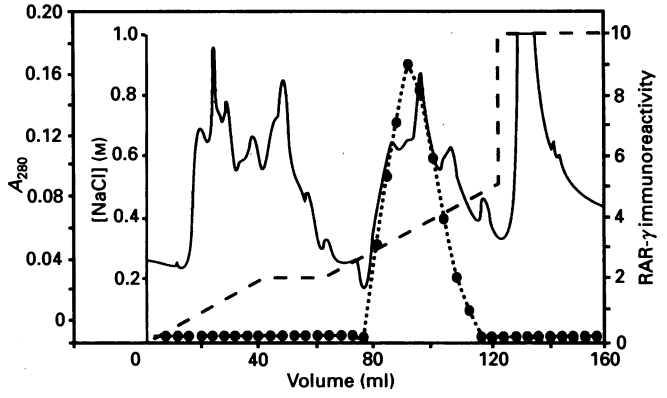

Fig. 2. Elution profile of recombinant RAR-γ1 on a Mono Q ion-exchange column

The lysate from Sf9 cells infected with RAR-γ1 was centrifuged, and the supernatant was applied to a Mono Q column (8 ml). Elution of RAR-γ1 was assayed by immunodot-blot (●---●). The dashed line indicates the NaCl concentration gradient, and the solid line indicates the elution of protein measured by absorbance at 280 nm.

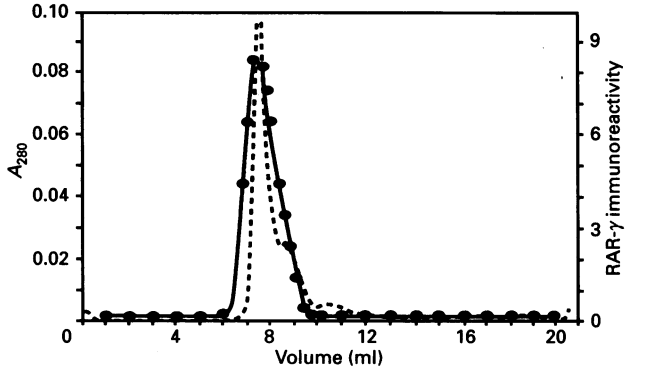

Fig. 3. Elution profile of recombinant RAR-γ1 on Superdex gel-filtration column

Fractions containing RAR-γ1, obtained from Mono Q chromatography (see Fig. 2), were pooled, concentrated and applied to a Superdex gel-filtration column. ●, Estimation of RAR-γ1 by immunodot-blot; ---, absorbance at 280 nm.

Table 1. Purification of recombinant RAR-γ1 from Sf9 insect cells

RAR-γ1 was determined by binding of [^{3}H]CD367, as described in the Materials and methods section.

Fraction	Total protein (mg)	Total RAR-γ1 (pmol)	Yield (%)	Specific binding (pmol/mg)	Purification (fold)
Lysate	60	366	100	6.1	1
Mono Q	6	180	50	30	5
Superdex	2	150	38	75	12
RARE affinity	0.02	37	10	1850	308

filtration column. RAR-γ1 eluted as the major protein peak with an apparent molecular mass of 50 kDa (Fig. 3). Fractions containing RAR-γ1 were pooled and passed through a Sepharose CL-2B column to which a double-stranded DNA fragment containing a RARE had been covalently coupled. After washing through the unbound protein, RAR-γ1 was eluted with 1M-KCl.

This three-column procedure resulted in a 300-fold increase in

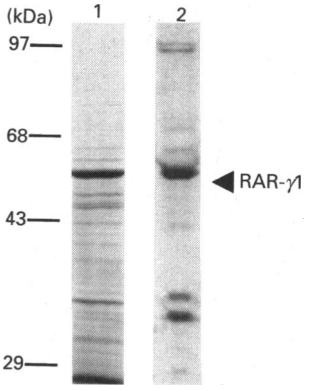

Fig. 4. SDS/PAGE of recombinant RAR-γ1 following purification by DNA affinity chromatography

Fractions containing RAR-γ1 from Superdex gel-filtration chromatography (see Fig. 3) were pooled and passed through a Sepharose 2B-CL column to which had been covalently coupled 81 bp DNA fragments, containing three 27 bp repeats of a RARE (see the legend to Fig. 7). Protein bands in the gel were stained with Coomassie Blue. Comparison of the total lysate (lane 1) with the preparation following DNA affinity chromatography (lane 2) shows substantial purification of RAR-γ1. Molecular mass protein standards are shown on the left.

specific RA binding relative to the crude lysate (Table 1). The final specific ligand binding achieved (approx. 2 nmol/mg of protein) indicated a level of purity of 10% (20 nmol of pure RAR-γ1 is approx. 1 mg of protein). Estimation of RAR-γ1 purity by Coomassie Blue protein staining following SDS/PAGE yielded a higher value of approx. 50% (Fig. 4). This difference may have been due to instability of RAR-γ1 ligand binding during purification, and/or preferential staining of RAR-γ1 by Coomassie Blue, thus making it appear more prominent than it actually was.

Ligand binding of recombinant RAR-γ1

Recombinant RAR-γ1 was incubated with [^{3}H]RA or [^{3}H]-CD367, a stable RA analogue known to have RAR binding characteristics similar to those of RA [28]. The mixture was applied to a size-exclusion column to separate bound from free ligand. Fig. 5(*a*) shows the elution profile of RAR-γ1 incubated with [^{3}H]RA or [^{3}H]CD367. A single peak of radioactivity was observed with either ligand which eluted with an apparent molecular mass of 50 kDa, the expected size of RAR-γ1. No detectable retinoid binding was observed in cell lysates from uninfected Sf9 cells. Addition of 100-fold excess unlabelled RA or CD367 reduced the 50 kDa peak by over 90% (Fig. 5*b*), indicating that binding was specific. Based on the specific radioactivity of the [^{3}H]RA, and assuming a stoichiometry of 1 mol of RA bound/mol of RAR-γ1, the level of expression of RAR-γ1 in baculovirus-infected Sf9 was approx. 2×10^6 receptor molecules/cell. This is approx. 2000 times greater than the level of endogenous RARs in mammalian cells.

Baculovirus-expressed RAR-γ1 bound both [^{3}H]RA and [^{3}H]CD367 with similar high affinity. Using the dextran-coated charcoal method described by Dokoh *et al.* [41] the equilibrium dissociation constants for crude and partially purified receptors were found to be identical and varied between 0.05 and 0.3 nM for RA (Fig. 5*c*) and between 0.2 and 1.0 nM for the synthetic analogue CD367 (Fig. 5*d*). We also compared the above results with those obtained for vaccinia-virus-expressed RAR-γ1 without detecting any significant differences (results not shown). Thus we exclude the possibility that cell-specific modifications of

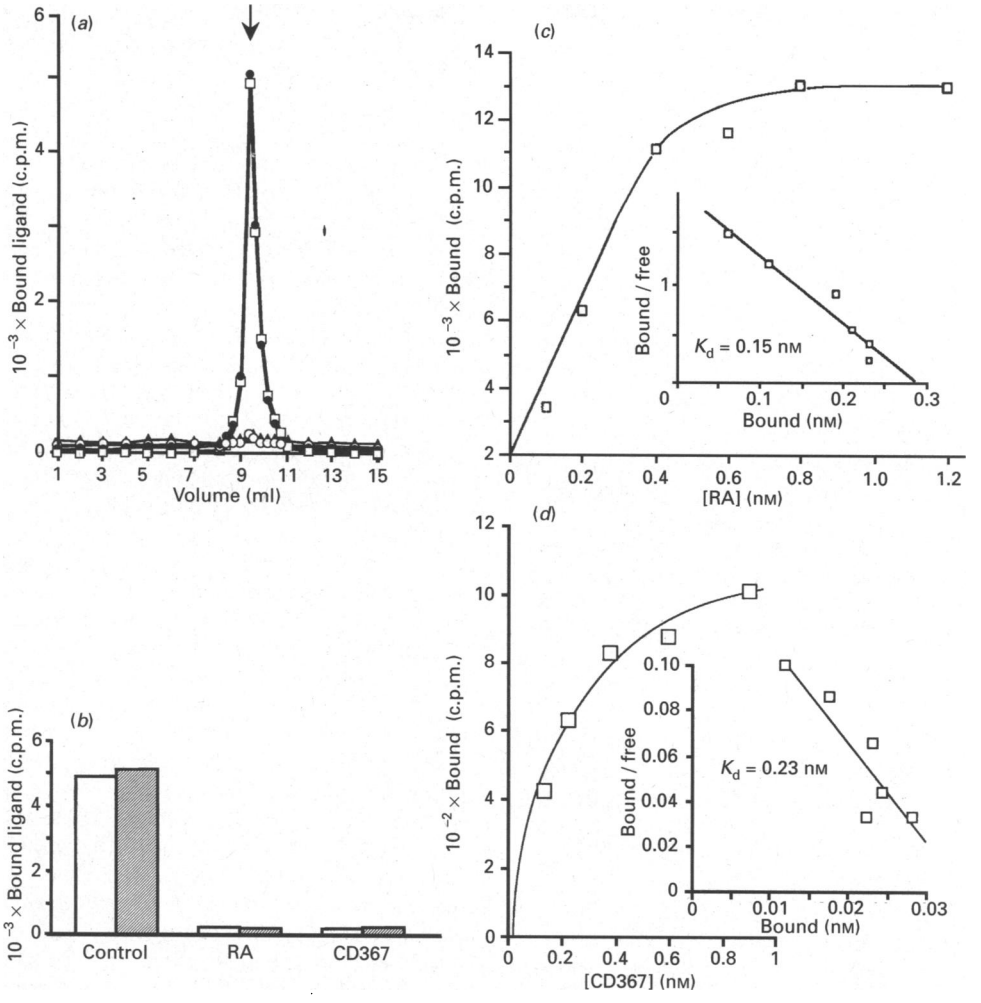

Fig. 5. Binding of RA and the RA analogue CD367 to recombinant RAR-γ1

(*a*) Lysates from non-infected (○,▲) and baculovirus RAR-γ1-infected (●,□) Sf9 cells were centrifuged to remove insoluble debris and incubated with 40 nM-[^{3}H]RA (●,○) or [^{3}H]CD367 (□,▲) for 4 h at 4 °C. Bound and free ligand were separated by passage of the incubation mixtures through a GF-250 size-exclusion column. Peaks of bound [^{3}H]RA and [^{3}H]CD367 were observed with apparent molecular masses of 50 kDa, the expected size of RAR-γ1. (*b*) Recombinant RAR-γ1 was incubated with 40 nM-[^{3}H]RA (□) or [^{3}H]CD367 (■) in the presence and absence of 5 μM unlabelled RA or CD367. Binding of ligand to RAR-γ1 was determined as in (*a*). (*c*) Concentration-dependence of [^{3}H]RA binding to recombinant RAR-γ1. Scatchard analysis of the data is shown in the inset. RA binding was determined by separation of bound and free ligand using charcoal adsorption as described in the Materials and methods section. (*d*) Concentration-dependence of [^{3}H]CD367 binding to recombinant RAR-γ1. Scatchard analysis of the data is shown in the inset.

RAR-γ1 made in mammalian cells, but not in insect cells, play a role in ligand binding.

To further examine the ligand-binding properties of baculovirus-produced RAR-γ1, two naturally occurring metabolites of RA, 4-hydroxy-RA and 4-oxo-RA, were tested for their abilities to decrease the binding of [^{3}H]CD367 to RAR-γ1. Competition of [^{3}H]CD367 binding by these metabolites, rather than their direct binding to RAR-γ1, was measured because of the unavailability of labelled metabolites. 4-Hydroxy-RA and 4-oxo-RA were found to be poor competitors, compared with unlabelled CD367, for [^{3}H]CD367 binding to RAR-γ1 (Fig. 6). Whereas 10 mM unlabelled CD367 reduced binding of [^{3}H]CD367 by one half, 100-fold higher concentrations (1 μM) of 4-hydroxy-RA and 4-oxo-RA reduced binding only 25%. These data indicate that oxidation of RA at the 4- position greatly decreases its ability to bind to RAR-γ1.

DNA binding of recombinant RAR-γ1

In gel-retardation experiments baculovirus-expressed RAR-γ1 bound specifically to synthetic DNA encompassing the RARE of the RAR-β gene (Fig. 7*b*, lane 14, complex a). In the presence of the anti-RAR-γ1 specific antibody mAb4g this complex was supershifted (lane 15, complex b). No specifically retarded band was observed with mock-infected Sf9 cells (lane 9; note that a very weak band close to the position of band "a" was not supershifted by the monoclonal antibody, as shown in lane 10). An excess of non-radioactive RARE abrogated the signal and no retarded band was seen with a mutant RARE (results not shown). Addition of CD367 to the incubation mixture containing either unpurified or purified RAR-γ1 and labelled RARE, prior to electrophoretic separation of the mixture, increased the mobility of the RAR-γ1/RARE complex on long-run retardation gels, while RA had a very small, if any, effect (results not shown).

RAR-γ1 expressed in mammalian and insect cells binds similarly to a RARE

To investigate whether RAR-γ1 expressed in Sf9 cells binds to its cognate enhancer with similar potency as the receptor made in mammalian cell, we calibrated by Western blot extracts of baculovirus- and vaccinia virus-produced RAR-γ1 (Fig. 7*a*), and compared extracts containing equal amounts of receptor in gel-shift assays. As shown in Fig. 7(*b*), lanes 5, 6, 13 and 14, both

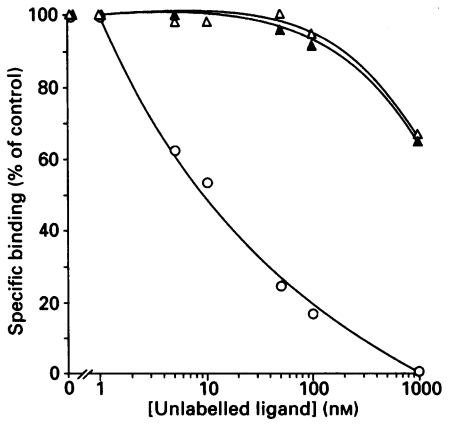

Fig. 6. Competition of [^{3}H]CD367 binding to recombinant RAR-γ1 by unlabelled CD367 (○), 4-hydroxy-RA (△) and 4-oxo-RA (▲)

Recombinant RAR-γ1 was incubated with [^{3}H]CD367 (1 nM) alone or with the indicated concentrations of unlabelled metabolites. The RAR-γ1/ligand complex was separated from free ligand by charcoal adsorption as described in the Materials and methods section. Results are the means of duplicate measurements from two experiments.

receptor preparations bound with similar efficiencies to the RARE (note that the 2-fold difference seen in this particular experiment was not seen in three other independent experiments).

DNA-binding stimulatory factors increase the efficiency of interaction of RAR-γ1 with the RAR-β/RARE

Several nuclear receptors, including the RAR-α1, have been reported to bind with increased efficiency to their cognate response elements in the presence of so-called DNA-binding stimulatory factors (DBSFs) [45–50]. We tested whether DBSFs, commonly added as crude HeLa cell extracts, had an effect on the DNA binding of vaccinia virus- and baculovirus-expressed RAR-γ1 (Fig. 7*c*). In fact, when the DNA binding of similar Western blot-calibrated amounts of the receptor (Fig. 7*a*) expressed in both systems was compared in the presence (Fig. 7*c*) and absence (Fig. 7*b*) of HeLa cell extract, approx. 20–50 times more RAR/RARE complexes were seen (compare Figs. 7*b* and 7*c*, lanes 4, 5, 12 and 13). We conclude that RAR-γ1 expressed using either system binds equally to the RAR-β gene RARE, and that DBSFs present in HeLa cells strongly increase DNA complex formation and/or stability.

DISCUSSION

Emerging evidence is revealing the importance of RA in the regulation of morphogenesis during mammalian development, and in postnatal regulation of cellular growth and differentiation. This has led to a keen interest in understanding the molecular basis of the mechanism of action of RA. The molecular cloning of RARs and the demonstration of their ability to function *in vitro* as RA-inducible transcription enhancers has focused attention on the RARs as potential primary mediators of the biological effects of RA. However, the low level of expression of RARs in mammalian tissues has hindered detailed characterization of their functional and structural properties. Successful expression of the human RAR-γ1 cDNA, using the baculovirus expression system, has overcome this difficulty and provided sufficient amounts of the protein for initial characterization.

Full-length recombinant RAR-γ1 displayed a molecular mass of 50 kDa by SDS/PAGE and gel-filtration chromatography, consistent with its expected size based on the deduced amino acid sequence [11]. RAR-γ1 was a prominent protein, as detected by SDS/PAGE, in the soluble fraction of RAR-γ1-infected Sf9 cells, indicating a high degree of overexpression. Based on ligand binding, approx. 2×10^6 receptors were expressed per Sf9 cell, a level similar to that reported for other members of the nuclear receptor family expressed in Sf9 cells, including rat [51] and human [52] glucocorticoid receptors, human oestrogen receptor [53] and mineralocorticoid receptor [54]. This level is, however, approx. 5-fold lower than the level of expression of human 1,25-dihydroxyvitamin D_3 [55] and thyroid hormone b1 receptors [56].

Recombinant RAR-γ1 specifically bound RA and the RA analogue CD367 with similar high affinity. The K_d values for these ligands are consistent with those reported for binding by recombinant RAR-α expressed in COS cells [40] and in bacteria [57]. We observed that use of high-performance size-exclusion chromatography to separate bound from free ligand yielded apparent K_d values 5–10-fold higher than those obtained using the charcoal adsorption method. This difference may be due to loss of RAR-γ1 binding activity during chromatography and/or competition for ligand binding to RAR-γ1 by the column matrix, since free ligand is retained by the column.

Although the metabolites 4-hydroxy-RA and 4-oxo-RA have been reported to be essentially inactive [33,34], high concentrations (100-fold excess) of these compounds decreased binding of [^{3}H]CD367 by recombinant RAR-γ1. These data suggest that these compounds retain some residual binding capacity, and therefore may be able to activate RARs. In support of this notion, Duell *et al.* [58] recently found that at relatively high concentrations (ED_{50} values 10-fold higher than for RA), 4-hydroxy-RA and 4-oxo-RA were able to stimulate RAR-dependent reporter gene transcription. A previous study, however, failed to detect binding of 4-hydroxy- and 4-oxo-RA to truncated forms of RAR-α and -β, containing the ligand-binding domain, expressed in bacteria [59]. This may have been due to the low concentration of 4-oxidized metabolites employed, as well as a decreased affinity of the truncated receptors for ligand. Alternatively, the data may reflect differences in ligand-binding properties among RAR-α, -β and -γ. Differences in the ability of the three RARs to stimulate reporter gene transcription in response to a variety of retinoids have been previously observed [60].

Although there is little information on the physiological concentrations of 4-oxidized RA metabolites in tissues, it appears that they are present in small amounts relative to RA. In human skin, which expresses high levels of RAR-γ1 mRNA [61], the level of 4-hydroxy-RA was 100 times lower than that of RA, and 4-oxo-RA was undetectable, following 4 days of treatment with 0.1 % RA cream [58]. These data, taken together with the results of the binding studies, suggest that in human skin *in vivo*, 4-oxidized RA metabolites contribute little, if anything, to activation of RAR-γ1 under physiological conditions. We have observed, however, that at high pharmacological doses 4-hydroxy-RA and 4-oxo-RA induce responses similar to RA in mouse skin (N. J. Reynolds & G. J. Fisher, unpublished work).

Recombinant RAR-γ1 was also shown to directly bind a synthetic 27 bp DNA fragment containing the perfect direct repeat, 5′GTTCAC, which defines the RARE in the RAR-β gene. This sequence has been shown to mediate RA-induced transcription of reporter plasmid constructs when transfected into cells [62]. Binding of the RARE by recombinant RAR-γ1 was, however, independent of RA. Binding of recombinant RAR-α, expressed in bacteria, to a synthetic RARE was also found to be independent of RA [57]. Note, however, that we observed an increased mobility of RAR/RARE complexes when the receptor was exposed to CD367, suggesting a change in

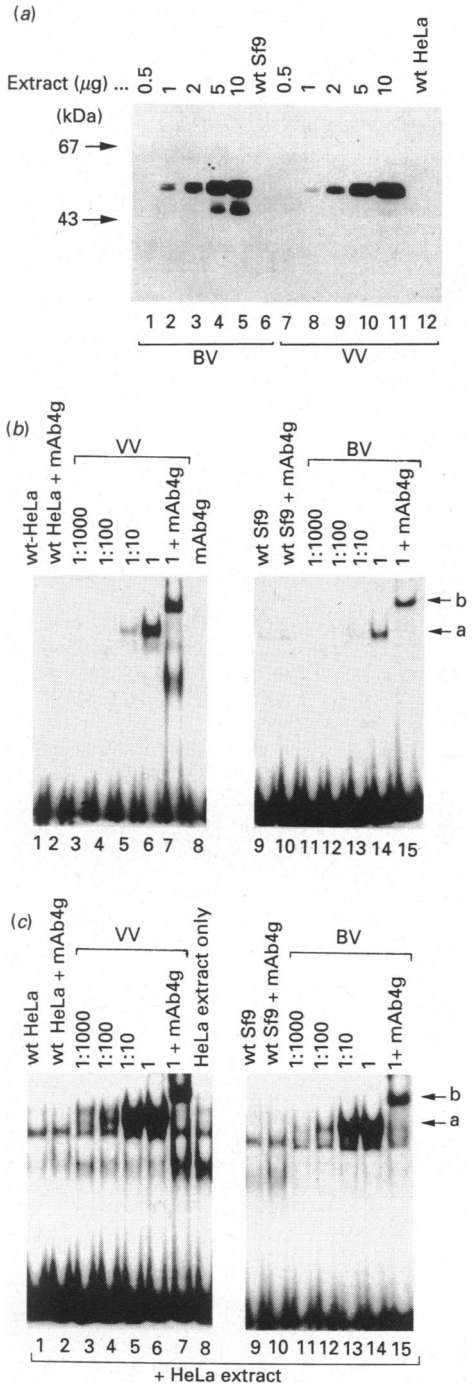

Fig. 7. Gel-retardation analysis comparing vaccinia virus (VV) and baculovirus (BV) overexpressed RAR-γ1

(*a*) Comparison of BV (lanes 1–5) and VV (lanes 7–11) RAR-γ1 levels by Western blot analysis. Lanes 6 and 12 represent Sf9 and HeLa cells respectively infected with wild-type (wt) virus. (*b*) Gel-retardation assays of BV and VV RAR-γ1. Cell extracts (5 μg of total protein) were serially diluted as indicated (VV, lanes 3–7; BV, lanes 11–14). The position of the specific RAR-γ1/DNA complexes is indicated by a. No complex is observed in cells infected with wild-type virus (lanes 1 and 9). The position of the specific complexes supershifted by the monoclonal antibody mAb4g is indicated by b (lanes 7 and 15). No complex is observed in lanes 2, 8 or 10 containing extracts prepared from wild-type virus-infected cells incubated with the antibody, or containing the antibody alone. (*c*) Gel-retardation assays in which BV- and VV-expressed RAR-γ1-containing extracts were incubated with HeLa cell extracts (2 μg). Lanes 1–15 are as indicated in (*b*), except that lane 8 contains 2 μg of HeLa cell extract alone. HeLa cell extracts were prepared as described [13].

receptor structure upon ligand binding. It is not known whether RARs are bound constitutively to DNA *in vivo*, as could be deduced from *in vitro* gel-retardation experiments. A modulatory role of heat shock protein 90 (hsp90), hypothesized in the case of steroid receptors [63,64], can apparently be excluded, since RAR/hsp90 complexes have not been observed *in vitro* [62]. It is thus possible that the RAR is associated with its response elements even *in vivo*, perhaps acting as a repressor in the absence of ligand, and that exposure to RA results in the formation (or, alternatively, unmasking) of its transcription activation function.

Recently it has been demonstrated that the DBSF present in HeLa cells, which stimulates the DNA binding of RARs, is RXR-β, and that RAR/RXR heterodimers bind more efficiently to certain RAREs than the corresponding homodimers [13,15]. Here we observe, in accordance with that study, that both baculovirus- and vaccinia virus-produced RAR-γ1 require an additional factor, present in the HeLa cells extract, for optimal binding to DNA, which in view of the study by Leid *et al.* [13] is most likely a member of the RXR family.

In summary, we have demonstrated the feasibility of employing the baculovirus expression system for producing RAR-γ1 protein. Moreover, by comparing vaccinia virus- and baculovirus-produced RAR-γ1, we have shown that RARs expressed in insect and mammalian cells are functionally (DNA and ligand binding properties) equivalent. The availability of recombinant RAR-γ1 will allow further detailed characterization of its role in the regulation of gene transcription. It is likely that the baculovirus expression system will be useful for expression of full-length and mutated forms of the other RARs, facilitating functional and structural comparisons of the different RARs.

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REFERENCES

1. Bollag, W. (1979) Cancer Chemother. Pharmacol. **3**, 207–215
2. Bollag, W. (1983) Lancet **i**, 860–863
3. Lippman, S. M., Kessler, J. F., & Meyskens, F. L. (1987) Cancer Treat. Rep. **71**, 391–405
4. Orfanos, C. E., Ehlert, R. & Gollnick, H. (1987) Drugs **34**, 459–503
5. Roberts, A. B. & Sporn, M. B. (1984) in The Retinoids (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), vol. 2, pp. 209–286, Academic Press, New York
6. Sherman, M. I. (1986) in Retinoids and Cell Differentiation (Sherman, M. I., ed.), pp. 161–186, CRC Press, New York
7. Giguere, V., Ong, E. S., Sequie, P. & Evans, R. M. (1987) Nature (London) **330**, 624–629
8. Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. (1987) Nature (London) **330**, 444–450
9. Benbrook, D., Lernhardt, E. & Pfahl, M. (1988) Nature (London) **333**, 669–672
10. Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, D. & Dejean, A. (1988) Nature (London) **332**, 850–853
11. Krust, A., Kastner, P. H., Petkovich, M., Zelent, A. & Chambon, P. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5310–5314
12. Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. & Evans, R. M. (1990) Nature (London) **345**, 224–229
13. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. & Chambon, P. (1992) Cell **68**, 377–392
14. Rowe, A., Eager, N. S. & Brickell, P. M. (1991) Development **111**, 771–778

15. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devaaru, O. V., Naar, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K. & Rosenfeld, M. G. (1991) Cell **76**, 1251–1266
16. Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C. I., Rosenberger, M., Lovey, A. & Grippo, J. F. (1992) Nature (London) **355**, 359–361
17. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. & Thaller C. (1992) Cell **68**, 397–406
18. Gronemeyer, H. (1991) Annu. Rev. Genet. **25**, 89–123
19. Zelent, A., Mendelsohn, C., Kastner, P., Krust, A., Garnier, J. M., Ruffenach, F., Leroy, P. & Chambon, P. (1991) EMBO J. **10**, 71–81
20. Leroy, P., Krust, A., Zelent, A., Mendelsohn, C., Garnier, J. M., Kastner, P., Dierich, A. & Chambon, P. (1991) EMBO J. **10**, 59–69
21. Kastner, P., Krust, A., Mendelsohn, C., Garnier, J. M., Zelent, A., Leroy, P., Staub, A. & Chambon, P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2700–2704
22. Dolle, P., Ruberte, E., Leroy, P., Morriss-Kay, G., & Chambon, P. (1990) Development **110**, 1133–1151
23. de The, H., Vivanco-Ruiz, M. M., Tiollais, P., Stunnenberg, H. & Dejean, A. (1990) Nature (London) **343**, 177–180
24. Duester, G., Shean, M. L., McBride, M. S. & Stewart, M. J. (1991) Mol. Cell. Biol. **11**, 1638–1646
25. Vasios, G., Gold, J. H., Petkovich, M., Chambon, P. & Gudas, L. J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9099–9103
26. Lucas, P. C., O'Brien, R. M., Mitchell, J. A., Davis, C. M., Imai, E., Forman, B. M., Samuels, H. H. & Granner, D. K. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2184–2188
27. Smith, W. C., Nakshatri, H., Leroy, P., Rees, J. & Chambon, P. (1991) EMBO J. **10**, 2223–2230
28. Leroy, P., Nakshatri, H. & Chambon, P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10138–10142
29. Zelent, A., Krust, A., Petkovitch, M., Kastner, P. & Chambon, P. (1989) Nature (London) **336**, 714–716
30. de The, H., Marchio, A., Tiollais, P. & Dejean, A. (1989) EMBO J. **8**, 429
31. Giguere, V., Ong, E. S., Evans, R. M. & Tabin, C. (1989) Nature (London) **337**, 566–569
32. Nervi, C., Grippo, J., Sherman, M. I., George, M. A. & Jetten, A. M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5854–5858
33. Silva, D. P., Valliere, C. R. & DeLuca, H. (1987) Arch. Biochem. Biophys. **259**, 391–401
34. Frolik, C. A., Roberts, A. B., Tavela, T. E., Roller, P. P., Newton, D. L. & Sporn, M. B. (1979) Biochemistry **28**, 2092–2097
35. Summers, M. D. & Smith, G. E. (1987) Texas Agriculture Experiment Station, Bulletin 1555
36. Luckow, V. A. & Summers, M. D. (1989) Virology **170**, 31–39
37. Nicholson, R. C., Mader, S., Nagpal, S., Leid, M., Rochette-Egly, C. & Chambon, P. (1990) EMBO J. **9**, 4443–4454
38. Kodanga, J. T. & Tjian, R. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5889–5893
39. Laemmli, U.K. (1970) Nature (London) **227**, 680–685
40. Cavey, M. T., Martin, B., Carlavan, I. & Shroot, B. (1990) Anal. Biochem. **186**, 19–23
41. Dokoh, S., Pike, J. W., Chandler, J. S., Mancini, J. M. & Haussler, M. R. (1981) Anal. Biochem. **116**, 221–232
42. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. **51**, 660–672
43. Rochette-Egly, C., Lutz, Y., Saunders, M., Scheuer, I., Gaub, M. P. & Chambon, P. (1991) J. Cell Biol. **115**, 535–545
44. Gassmann, M., Thommes, P., Weiser, T. & Hubscher, U. (1990) FASEB J. **4**, 2528–2532
45. Glass, C. K., Devary, O. V. & Rosenfeld, M. G. (1990) Cell **63**, 729–738
46. Mukherjee, R. & Chambon, P. (1990) Nucleic Acids Res. **18**, 5713–5716
47. Edwards, D. P., Kuehnel, B., Estes, P. A. & Nordeen, S. K. (1989) Mol. Endocrinol. **3**, 381–391
48. Burnside, J., Darling, D. S. & Chin, W. W. (1990) J. Biol. Chem. **265**, 2500–2504
49. Murray, M. B. & Towel, H. C. (1989) Mol. Endocrinol. **9**, 1434–1442
50. Vasios, G., Mader, S., Gold, J. D., Leid, M., Lutz, Y., Gaub, M. P., Chambon, P. & Gudas, L. (1991) EMBO. J. **10**, 1149–1158
51. Alnemri, E. S., Maksymowych, B., Robertson, N. M. & Litwack, G. (1991) J. Biol. Chem. **266**, 3925–3936
52. Srinivasan, G. & Thompson, E. B. (1990) Mol. Endocrinol. **4**, 209–216
53. Brown, M. & Sharp, P. A. (1990) J. Biol. Chem. **265**, 11238–11243
54. Alnemri, E. S., Maksymowych, A. B., Robertson, N. M. & Litwack, G. (1991) J. Biol. Chem. **266**, 18072–18081
55. Ross, T. K., Prahl, J. M. & DeLuca, H. F. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6555–6559
56. Barkhem, T., Carlsson, B., Simons, J., Moller, B., Berkenstam, A., Gustafsson, J. A. & Nilsson, S. (1991) J. Steroid Biochem. **38**, 667–675
57. Yang, N., Schüle, R., Manglesdorf, D. J. & Evans, R. M. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3559–3563
58. Duell, E. A., Aström, A., Griffiths, C. E. M., Chambon, P. & Voorhees, J. J. (1992) J. Clin. Invest., in the press
59. Crettaz, M., Baron, A., Siegenthaler, G. & Hunziker, W. (1990) Biochem. J. **272**, 391–397
60. Aström, A., Pettersson, U., Krust, A., Chambon, P. & Voorhees, J. J. (1990) Biochem. Biophys. Res. Commun. **173**, 339–345
61. Elder, J. T., Fisher, G. J., Zhang, Q. Y., Eisen, D., Krust, A., Kastner, P., Chambon, P. & Voorhees, J. J. (1991) J. Invest. Dermatol. **96**, 425–433
62. Sucov, H. M., Murakami, K. K. & Evans, R. M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5392–5396
63. Baulieu, E. E. (1991) Ann. N.Y. Acad. Sci. **626**, 545–560
64. Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C., Dalman, F. C. & Welsh, M. J. (1989) Cancer Res. **49**, 2222–2229

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