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Pure and Functionally Homogeneous Recombinant Retinoid X Receptor*

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Mouse retinoid X receptor α (RXR α) lacking the amino-terminal region A/B (RXR $\alpha\Delta$ AB) has been purified to more than 98% purity and functional homogeneity from bacterial and baculovirus-based recombinant expression systems with yields of 2–8 mg/liter of culture. The purified protein is soluble, and fluorescence quenching analysis demonstrated that it binds its cognate ligand 9-*cis*-retinoic acid (9-*cis*-RA) stoichiometrically, and with high affinity. Compared with RXR Δ AB expressed in COS-1 cells, bacterially and baculovirus-expressed proteins bind approximately 10 and 5 times less efficiently to direct repeat 1 (DR1) DNA elements, respectively, suggesting that animal cell-specific modification of RXR or interaction with other animal cell-specific factors may modulate DNA binding. 9-*cis*-RA did not stimulate DR1 binding of functional RXR Δ AB expressed in *Escherichia coli*, Sf9 or COS-1 cells. The previously reported ligand effect that can be observed with *in vitro* made receptor may therefore be a consequence of a conformational stabilization of improperly folded *in vitro* synthesized protein.

Retinoic acid (RA)¹ receptors (RARs and RXRs) belong to the superfamily of nuclear receptors which act as ligand-inducible transcription factors by recognizing DNA response elements usually located in the promoter/enhancer regions of responsive genes (for reviews see Evans, 1988; Green and Chambon, 1988; Beato, 1989; Gronemeyer, 1991, 1992; Leid *et al.*, 1992a; Chambon, 1994; Giguère, 1994). With the exception of some "orphan" receptors, which bind to response elements composed of single 5'-PuG(G/T)TCA motifs or derivatives thereof (Wilson *et al.*, 1993; Giguère *et al.*, 1994), the response elements of steroid receptor homodimers are composed exclusively of inverted repeats (or palindromes), whereas the majority of natural RA responsive elements (RAREs) contain a direct repetition (DR) of this motif. RARs and RXRs can bind as either homo-

heterodimers to their cognate response elements, with heterodimerization increasing the efficiency of DNA binding *in vitro* and transactivation in transfected cells *in vivo* (Yu *et al.*, 1991; Bugge *et al.*, 1992; Durand *et al.*, 1992; Hallenbeck *et al.*, 1992; Kliewer *et al.*, 1992; Leid *et al.*, 1992b; Marks *et al.*, 1992; Zhang *et al.*, 1992a; Mader *et al.*, 1993a). We and others have recently determined the parameters that are responsible for receptor homo- or heterodimer-specific DR binding site recognition and the formation of anisotropic receptor-DNA complexes (Kurokawa *et al.*, 1993; Lee *et al.*, 1993; Mader *et al.*, 1993b; Perlmann *et al.*, 1993; Zechel *et al.*, 1994a, 1994b). Reconstitution experiments in yeast have demonstrated that RXR-RAR heterodimers and RXR homodimers can efficiently activate RAREs composed of DR5 and DR1 elements, respectively, whereas RAR homodimers on their own are rather weak transactivators (Hall *et al.*, 1993; Heery *et al.*, 1993, 1994).

In contrast to the impressive amount of data about nuclear receptor function, which are mainly derived from transfection and *in vitro* studies, very little is known concerning the three-dimensional structure of nuclear receptors and the structural consequences of DNA and ligand binding and homo- or heterodimerization. The only structural information available at present is derived from DNA cocrystals of the DNA binding domains of the glucocorticoid and estrogen receptors (Luisi *et al.*, 1991; Schwabe *et al.*, 1993a, 1993b) and the solution structures of glucocorticoid receptor, estrogen receptor, and RXR DNA binding domains determined by NMR (Härd *et al.*, 1990; Schwabe *et al.*, 1990; Lee *et al.*, 1993). Based on our unpublished experience with the overexpression and purification of several nuclear receptors (progesterone, estrogen, RA receptors) in a number of different systems (*i.e.* *Escherichia coli*, yeast, invertebrate (baculovirus), and vertebrate (vaccinia virus) cell-based expression), the main reason for the absence of structural data on intact nuclear receptors resides in the difficulty of obtaining nondegraded, nonaggregated, sufficiently pure, and functionally homogeneous receptor preparations suitable for crystallization. Here we report the first successful purification of an amino-terminally truncated RXR α , comprising the DNA and ligand binding domains (regions C and E), which is pure, nonaggregated, and functionally homogeneous. Initial characterization of the protein purified from *E. coli* and its comparison with RXR α expressed in insect and vertebrate cells reveal that the cognate ligand of RXR, 9-*cis*-RA, does not influence its DNA binding efficiency. Our results also suggest the existence of cell-specific modification of RXR which appears to increase its affinity for DR1 response elements.

MATERIALS AND METHODS

Bacterial Expression Vectors and Recombinant Baculoviruses

The pSG5-based mRAR α and mRXR α have been described previously (Leid *et al.*, 1992b). All polymerase chain reaction-generated fragments in the subsequently described vectors were resequenced.

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¹ The abbreviations used are: RA, retinoic acid; RARE, RA response element; RAR α , retinoic acid receptor α ; RXR α , retinoid X receptor α ; DR, direct repeat; BV, baculovirus; DTT, dithiothreitol.

mRXR α /pET3 α —*mRXR α /pET3 α* was a gift of J.-Y. Chen and expresses the entire mRXR α with the additional amino-terminal sequence MASMTGGQQMGRGS.

mRXR α Δ AB/pET31—The *KpnI* fragment of mRXR α Δ AB encompassing amino acids 140–467 of mRXR α (Nagpal *et al.*, 1992) was inserted into pET31 yielding mRXR α Δ AB/pET31, which was amplified in XL-1-Blue. pET31 was derived from pET3b and contains an extended polylinker sequence catatgGGTACCTCTAGACCTCGAGCCTGAggattcc (gift of S. Mader; the *KpnI* site is underlined; lower case letters reveal the authentic pET3b sequence). Subsequently, this expression vector was used to transform the *E. coli* BL21(DE3)pLysS; transformed bacteria were grown on LB plates containing 50 μ g/ml ampicillin and 30 μ g/ml chloramphenicol.

mRXR α Δ AB/pET15b—mRXR α Δ AB was prepared by polymerase chain reaction amplification of amino acids 132–467 of mRXR α (Leid *et al.*, 1992b), using primers QS54 (5'-CCTCAGGACATATGGCCTCCTCA-3') and QQ38 (5'-TCTCAGATCTCTAGGTGGCTTGATGTG-3'), and subcloned into the *NdeI*-*Bam*HI sites of pET15b (Novagen). The resulting plasmid was initially transferred into XL-1-Blue and, after verification, into *E. coli* BL21(DE3). The transformed cells were grown on LB plates containing 50 μ g/ml ampicillin.

mRXR α Δ AB/pSG5—mRXR α Δ AB/pET15b was digested with *XbaI* and *EcoRV*, blunt ended with Klenow polymerase, and the cDNA fragment was inserted into the blunt-ended *EcoRI* and *Bam*HI sites of pSG5 (Green *et al.*, 1988).

Recombinant His $_6$ -mRXR α Δ AB Baculovirus—The *NdeI* site at position 9418 of the baculovirus transfer vector pVL1392 (gift of M. Summers; O'Reilly *et al.*, 1992) was eliminated to generate pVL1392/*NdeI*⁻. The histidine tag-encoding sequence of pET15b was subsequently transferred into pVL1392/*NdeI*⁻ as an *XbaI*-*Bam*HI fragment to give pVL1392-His $_6$. This vector was digested with *NdeI* and *Bam*HI, and a polymerase chain reaction fragment encoding amino acids 132–467 of RXR α was inserted such that Met¹³² is the amino-terminal amino acid (giving pVL-His $_6$ -mRXR α Δ AB). Recombinant baculovirus was obtained by complementation of defective virus with the transfer vector during recombination using the BaculoGold system (Pharmingen) according to standard procedures. Briefly, the recombinant transfer vector and linearized baculovirus DNA were cotransfected into Sf9 insect cells using calcium phosphate, and 6 days later the resulting recombinant virus was collected. The recombinant virus was purified by plaque assays and amplified (O'Reilly *et al.*, 1992), and expression was verified by Western blot analysis.

Overexpression and Purification of the CDE Regions of Mouse RXR α

A 3-ml LB starter culture containing 100 μ g/ml ampicillin and 30 μ g/ml chloramphenicol was inoculated with mRXR α Δ AB/pET31-transformed *E. coli* BL21(DE3)pLysS (freshly transformed, originating from a single colony) and grown overnight at 37 °C. 5 ml of the starter was used to inoculate 500 ml of LB containing 100 μ g/ml ampicillin. Cultures were grown at 37 °C to an A₆₀₀ of 0.6–0.7, and expression of T7 RNA polymerase was induced by addition of isopropyl-1-thio- β -D-galactopyranoside to 0.5 mM. After continuing the incubation for 2 h at 25 °C, the cells were harvested by centrifugation, the pellets from 5 liters of culture were resuspended in 125 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM KCl), and lysozyme was added to 100 μ g/ml. After placing the culture on ice for 30 min and then at 37 °C for 5 min, the cells were lysed by two cycles of freeze-thawing in liquid nitrogen and cold water. The lysate was sonicated for 1 min (power 60%, Vibracell BioBlock), diluted with 125 ml of ice-cold lysis buffer, and sonicated again. The extract was clarified by centrifugation for 30 min at 30,000 rpm in a Beckman R45-Ti rotor. The supernatant was diluted with 150 ml of lysis buffer without KCl to give the "crude extract." 400 ml of crude extract was loaded onto a 500-ml DEAE-Sepharose CL-6B column (10 \times 15 cm; flow rate 300 ml/h) equilibrated in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM DTT, 10 mM KCl). The column was then washed with 1 liter of buffer A until the A₂₈₀ of the eluent returned to base line. The first third of the flow-through fraction was pooled and loaded onto a 24-ml heparin-Ultrogel column (10 \times 30 cm, flow rate 15 ml/h) equilibrated in buffer A. The column was then washed with 2 column volumes of buffer A to remove unbound protein and eluted with a gradient of 50–1,000 mM KCl in buffer A. mRXR α Δ AB eluted from this column as a sharp peak at 400 mM KCl. Fractions containing mRXR α Δ AB were pooled, dialyzed overnight against buffer B (10 mM potassium phosphate, pH 7.5, 10 μ M CaCl₂, 0.5 mM DTT, and 10% glycerol) or buffer C (for subsequent gel filtration, see below) in Sartorius collodion bags, and loaded onto a HAP-TSK hydroxylapatite column (Toso-Haas, 0.75 \times 7.5 cm, flow rate

0.25 ml/min) equilibrated in buffer B. After washing the column with buffer B and 3 column volumes of buffer B containing 75 mM potassium phosphate, the receptor was eluted with an 18-ml linear gradient from 75 to 250 mM potassium phosphate in buffer B. The peak of mRXR α Δ AB eluted from this column at approximately 170 mM potassium phosphate and was pooled. Protein content, purity, and possible degradations of all chromatographic fractions were determined immediately from silver-stained SDS gels, and parallel immunoblots, pools containing purified protein were collected and reanalyzed. Protein sequencing revealed the expected amino terminus (the amino-terminal methionine was removed), and complete hydrolysis confirmed that the protein was more than 98% pure (data not shown).

Overexpression and Purification of His $_6$ -RXR Δ AB

A 3-ml LB starter culture containing ampicillin (100 μ g/ml) was inoculated with mRXR Δ AB/pET15b-transformed *E. coli* BL21(DE3), grown overnight at 37 °C, and 5 ml was used to inoculate 500 ml of LB containing ampicillin (100 μ g/ml). Cultures were grown at 37 °C to an A₆₀₀ of 0.8, and expression of T7 RNA polymerase was induced by the addition of isopropyl-1-thio- β -D-galactopyranoside to 0.5 mM. After an additional incubation for 2 h at 25 °C, cells were harvested by centrifugation, the pellets from 1 liter of culture were resuspended in 12.5 ml of ice-cold binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0), and lysozyme was added to 100 μ g/ml. After 30 min on ice and 5 min at 37 °C, the cells were lysed by two cycles of freeze-thawing in liquid nitrogen and ice-cold water. The extract was sonicated for 1 min as described above, diluted with 12.5 ml of ice-cold lysis buffer, and sonicated again. The crude extract was obtained after centrifugation for 30 min at 30,000 rpm in a Beckman R45-Ti rotor. A 1-ml Hitrap chelating column (Pharmacia Biotech Inc.) was equilibrated with 10 volumes of sterile deionized water, charge buffer (50 mM NiSO₄), sterile deionized water, and 10 volumes of binding buffer. The crude extract was passed over the column at a flow rate of 1 ml/min, followed by washings with 50 volumes of binding buffer, 30 volumes of 50 mM imidazole in binding buffer, and 30 volumes of binding buffer. The bound protein was eluted with 20 volumes of strip buffer (100 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0).

Sf9 cells were infected with the recombinant His $_6$ -RXR Δ AB baculovirus in 175-cm² Falcon tissue culture flasks at a multiplicity of infection of 1. After 72 h the infected cells were harvested and lysed by two cycles of freeze-thawing in lysis buffer, sonicated two times (2 min on ice), and the extract was clarified by centrifugation for 30 min at 30,000 rpm in a Beckman R45-Ti rotor. The recombinant His $_6$ -RXR Δ AB was purified by using heparin-Ultrogel and Hitrap chelating chromatography as described above.

DNA Binding Assays

The indicated amounts (see figure legends) of protein and 10 fmol of ³²P-labeled oligonucleotide probe were incubated for 15 min in a final volume of 20 μ l of binding buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.4 mM DTT, 5% glycerol) containing 2 μ g of poly(dI-dC), as well as 50 mM KCl for homodimer binding and 100 mM for heterodimer binding. When required, 9-*cis*-RA (1 μ M) was added, and incubation was continued at room temperature for another 30 min. The receptor-DNA complexes were resolved by electrophoresis through 6% polyacrylamide gels (0.5 \times TBE buffer; prerun for 3 h) at 13 volts/cm at 4 °C.

In Vitro Transcription/Translation and Transient Transfections

In vitro transcription/translation was carried out as described (Leid *et al.*, 1992b). Ligand was added for 15 min on ice in the dark before performing the electrophoretic mobility shift assays. Transient transfections were done as described by Bocquel *et al.* (1989). In cases in which the effect of 9-*cis*-RA was to be assayed, the ligand was added during transient expression and to all of the buffers used throughout the experiment.

Ligand Binding Assays

Quenching of tryptophan fluorescence of purified receptor by 9-*cis*-RA was monitored by excitation at 280 nm and measuring the fluorescence emission at 340 nm using a SLM 48000 (Aminco) fluorescence spectrometer or a modified Hitachi F2000. Note that the only two tryptophan residues of RXR α Δ AB (Trp²⁸⁷ and Trp³¹⁰) are located in the ligand binding domain; it is not known whether ligand binding quenches the fluorescence originating from only one or both residues. The emission maximum was occasionally verified by recording of the 220–450 nm emission spectrum with a separate sample. At least 10 different 9-*cis*-RA concentrations were used to construct Cogan plots (Cogan *et al.*, 1976) for the determination of the K_d and functional

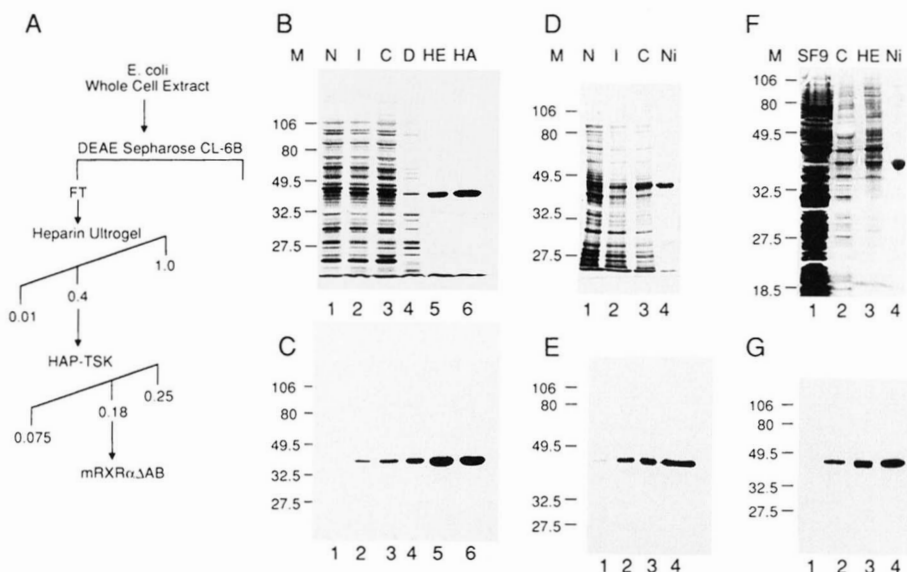


FIG. 1. Purification of RXR α ΔAB. Panel A, RXR α ΔAB was expressed from pET31 in *E. coli* BL21(DE3)pLysS as described under "Materials and Methods" and purified by chromatography on DEAE, heparin, and hydroxylapatite, as illustrated. Panel B, representative silver-stained SDS-polyacrylamide gel of noninduced (*N*; lane 1) and isopropyl-1-thio- β -D-galactopyranoside-induced bacteria (*I*; lane 2), crude extract (*C*; lane 3), and the protein pools obtained at different steps (*D*, DEAE flow-through, lane 4; *HE*, heparin, lane 5; *HA*, hydroxylapatite, lane 6) of the purification procedure. Panel C, corresponding immunoblot of the identical fractions. Panel D, histidine-tagged RXR α ΔAB, expressed from pET15b in *E. coli* BL21(DE3), was purified by single-step chromatography on a Ni²⁺-charged Hitrap chelating column. Shown is a representative silver-stained SDS gel of the fractions. *Ni*, pool of the receptor protein eluted from the column. For details, see "Materials and Methods." Panel E, corresponding immunoblot of the identical fractions. Panel F, purification of histidine-tagged RXR α ΔAB, expressed from recombinant baculovirus in Sf9 cells, by heparin and Ni²⁺-chelating chromatography. A representative silver-stained SDS gel is shown. Panel G, corresponding immunoblot of the identical fractions. *M*, position of marker proteins.

homogeneity of a preparation. Protein (1 μ M) was placed in a cuvette in buffer including 10 mM Tris-HCl, pH 8.0, 1 mM β -mercaptoethanol, 100 mM KCl. 9-*cis*-RA was added directly into the cuvette from a concentrated solution in ethanol. The ethanol concentration did not exceed 0.1%. The exposure of samples to the exciting light was minimized by closing the shutter between measurements and reducing the incident light (Hitachi). Data were corrected for inner filter effect and analyzed by the linearization method of Cogan *et al.* (1976). Inner filtering and any quenching due to residual ethanol was determined with a non-ligand-binding peptide (NH₂-NAWVAVRNRC-COOH) which was used in an amount giving the same fluorescence as non-liganded RXR α ΔAB (see Fig. 4A, inset a). The fraction of free binding sites of RXR (α) was determined from $\alpha = F - F_{\max}/F_0 - /\max$, where F_{\max} represents the fluorescence at saturation, F the fluorescence measured at each retinoid concentration, and F_0 the initial fluorescence intensity. A plot of $P_0\alpha$ versus $R_0(\alpha/1 - \alpha)$ (see Fig. 4A, inset b), where P_0 is the total receptor concentration and R_0 the retinoid concentration, yields a straight line with a slope of $1/n$ (n , number of binding sites per receptor) and a y intercept of $-K_d/n$. If a receptor has a single binding site, it follows that a preparation which is functionally homogeneous should give a slope of 1.

For gel filtration, mRXR α ΔAB was dialyzed against buffer C (10 mM Tris-HCl, pH 8.0, 1 mM DTT, 500 mM KCl), incubated with or without an excess 9-*cis*-RA overnight and loaded onto a Superdex 200 gel filtration column (1.6 \times 60 cm, flow rate 1 ml/min; Pharmacia). The UV spectra shown in Fig. 4B were taken at equivalent protein concentrations for the apo and holo forms of the truncated receptor. If the ϵ_{340} used to calculate the concentration of protein-bound RA was taken from the ϵ_{340} of RA in methanol ($\epsilon_{340} = 37,000 \text{ M}^{-1} \text{ cm}^{-1}$), the percentage of bound ligand was determined to be 67%. If one assumes that the ϵ_{340} of RA bound to the receptor is more accurately approximated by the ϵ_{340} of RA in 20% aqueous methanol, then the fractional saturation is virtually 100%. However, because of the inaccuracy in the estimation of the extinction coefficient of protein-bound RA, gel filtration could not be used to determine the degree of receptor saturation.

RESULTS

Purification of Recombinant RXR—Immunoblot analysis of recombinant full-length mouse RXR α in both pET3a-*E. coli* and baculovirus systems, revealed extensive proteolysis which apparently occurred in the intact cells during the time of induction/infection (data not shown). Purification and subse-

quent amino-terminal sequencing of the proteolytic fragments indicated a cluster of proteolytic cleavage sites between amino acids 60 and 100 (data not shown). Similar proteolysis was noticed in regions A/B of RXR β and γ , as well as of RAR α , β , and γ . Consequently, we expressed the amino-terminally truncated RXR α ΔAB with or without a histidine tag (hereafter called His₆-RXR α ΔAB) in bacteria using the T7 system of Studier and Moffatt (1986). The same histidine-tagged RXR α ΔAB was also expressed from recombinant baculovirus (rBV)-infected Sf9 cells (for details see "Materials and Methods"). Using sequential chromatography on DEAE-Sepharose, heparin-Ultrogel, and hydroxylapatite (Fig. 1A) non-tagged RXR α ΔAB was purified in three steps to apparent homogeneity as judged from silver-stained SDS gels (Fig. 1B, lane 6) and immunoblotting (Fig. 1C). Most importantly, no degradation of RXR α ΔAB was seen on overloaded SDS gels (not shown) or immunoblots using antibodies directed against the RXR ligand binding domain (Fig. 1C, lane 6). With this procedure, 2–3 mg of purified RXR α ΔAB/liter of culture could be obtained. Essentially the same result was obtained in a one-step purification for the His₆-RXR α ΔAB by nickel chelate affinity chromatography (compare Fig. 1, D and E, lanes 4 with lanes 6 in Fig. 1, B and C, respectively). In the latter case, up to 5–8 mg of purified receptor could be obtained from 1 liter of bacterial culture. Using the same purification technology, His₆-RXR α ΔAB was purified from rBV-infected Sf9 cells in a two-step procedure to more than 95% apparent homogeneity (Fig. 1, F and G, lanes 4; for details see "Materials and Methods") with yields of about 4 mg/liter of culture.

Bacterially Produced RXR α ΔAB Has the Same DR Binding Site Repertoire as RXR Produced in Animal Cells but Binds with Reduced Affinity to Its Cognate Element—To analyze the purified protein, its DNA binding characteristics were determined first. As far as DR response elements, which constitute the majority of the known natural RAREs (Leid *et al.*, 1992a; Giguère, 1994) are concerned, we have shown previously that RXR binds with a strong homocooperativity to DR1, followed by

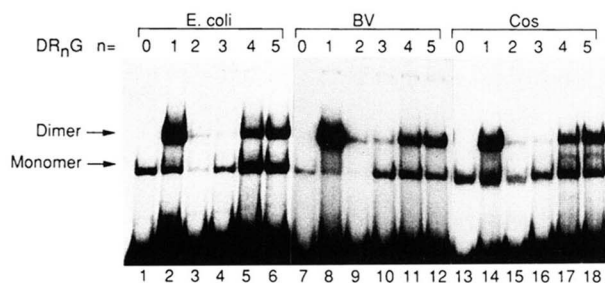


FIG. 2. Specificity of DNA binding of histidine-tagged RXR α ΔAB. Electrophoretic mobility shift assays carried out with purified His₆-RXR α ΔAB (60 ng) expressed in *E. coli* (lanes 1–6), purified His₆-RXR α ΔAB (50 ng) expressed in Sf9 cells (BV; lanes 7–12) or crude extract of COS-1 cells transiently expressing His₆-RXR α ΔAB (10 ng; lanes 13–18) using as probes radioactively labeled oligonucleotides containing DRs of the PuGGTCA motif spaced by 0–5 base pairs. DR spacing is indicated at the top.

much less efficient binding to DR5 and DR4 (Mader *et al.*, 1993b). The same binding site repertoire was observed for purified RXRΔAB (data not shown) and His₆-RXRΔAB produced in bacteria (Fig. 2, lanes 1–6) or rBV-infected Sf9 cells (lanes 7–12). Indeed, no qualitative difference in the binding pattern was observed when COS-1 cell extracts transiently expressing His₆-RXRΔAB were analyzed in parallel (lanes 13–18). As reported previously, purified *E. coli*-produced RXRΔAB heterocooperated efficiently with RAR for binding to DR2 and DR5, exhibiting cooperativity factors between 24 and 40 (Mader *et al.*, 1993b). Similar results were obtained with His₆-RXRΔAB purified from *E. coli* or rBV-infected Sf9 cells, and a systematic variation of the RAR:RXR ratio in DNA affinity chromatography revealed that all of the *E. coli*-produced RXRΔAB was capable of binding to the DR5-DNA matrix as a heterodimer with RAR (data not shown). We conclude that neither the expression in a bacterial or invertebrate host cell nor the purification procedure has affected the specificity of DNA binding of RXRΔAB.

Although the specificity of DNA binding was not affected, the affinity of DNA binding was clearly reduced when RXRΔAB was expressed in *E. coli* or Sf9 cells as compared with animal cells (Fig. 3). To quantitate DNA binding we used immunoblotting to calibrate the amount of His₆-RXRΔAB, either purified from Sf9 cells or in crude extracts from COS-1 cells, relative to purified *E. coli*-produced protein (Fig. 3, B and C). Based on this calibration, about 10-fold more bacterially and 5-fold more Sf9 cell-expressed His₆-RXRΔAB was required than COS-1 cell-produced receptor to obtain a similar efficiency in DR1 binding (Fig. 3A; the amounts indicated were directly measured by protein determination of the purified preparation (*E. coli* and BV) and confirmed by immunoblotting (Fig. 3B); His₆-RXRΔAB present in COS-1 cell extracts was determined from comparative immunoblots (Fig. 3C)).

Fluorescence Quenching by 9-*cis*-RA Binding as a Novel and Accurate Measure of the Functional Homogeneity of Purified RXR—One of the most important aspects of a purification, in particular in view of the preparation of protein for three-dimensional structural analysis, is to obtain a protein that is not only homogeneous as can be judged from SDS gels (purity relative to other proteins) and immunoblots (degradation) but also “functionally” homogeneous. Thus, a purified receptor should be able to bind the cognate ligand quantitatively, ensuring that all of the molecules have a common structure in solution. Functional heterogeneity indicates structural variations or aggregation and is likely to inhibit crystallogenesis.

Presently, all methods used to determine receptor-ligand interaction involve a physical separation of free and bound ligand by, for example, charcoal adsorption or gel filtration. We ob-

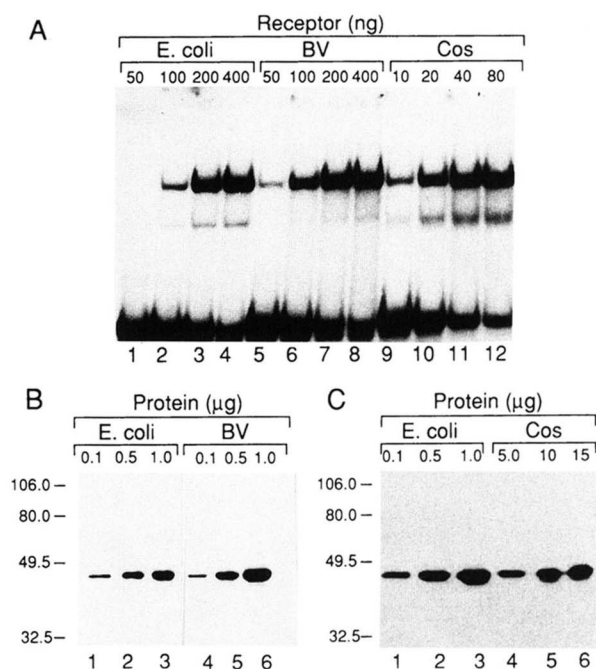


FIG. 3. Lower DNA binding efficiency of *E. coli* and Sf9-produced His₆-RXR α ΔAB. Panel A, gel retardation assays were performed with purified His₆-RXR α ΔAB expressed in *E. coli* (lanes 1–4) or Sf9 cells (lanes 5–8) or crude extract from transiently transfected in COS-1 cells (lanes 9–12) using as a probe a DR1G oligonucleotide. The amounts of receptor used for each lane are indicated at the top and were calibrated from (i) the measurements of the purified protein using the Bradford method and (ii) the comparative immunoblots shown in panels B and C. Panels B and C, immunoblots were used to calibrate the amounts of crude (COS-1) relative to the purified (*E. coli*, Sf9) His₆-RXR α ΔAB for gel retardation. Panel B, increasing amounts of purified protein expressed in *E. coli* and Sf9 cells were used for immunoblotting confirming similar purity of the fractions. Panel C, purified receptor expressed in *E. coli* and crude extract of COS-1 cells transiently expressing the same receptor were compared on immunoblots. Note that receptor purified from *E. coli* is about 50 times purer than that present in the COS-1 cell extract (compare lanes 1 and 4 where the signals are not at saturation).

served that these methods do not allow to accurately quantitate the amount of 9-*cis*-RA-binding to RXRΔAB in purified preparations, since each method yielded different values, even for the same preparation. For example, charcoal assays suggested that only up to 10% of the purified receptor could bind 9-*cis*-RA (data not shown), whereas gel filtration indicated that at least 67% of purified RXRΔAB bound 9-*cis*-RA. These latter data were obtained by incubating freshly purified RXRΔAB with an excess of ligand and chromatographing it on a gel filtration column to separate holoreceptor from free ligand. In parallel, an identical amount of aporeceptor was chromatographed under otherwise identical conditions and UV spectra of apo- (dashed line) and holo-RXRΔAB (solid line) were compared (Fig. 4B). The additional peak at 340 nm in the spectrum of the holoreceptor is indicative of bound 9-*cis*-RA, and, using the extinction coefficient of $\epsilon_{340} = 37,000 \text{ M}^{-1} \text{ cm}^{-1}$ determined for pure 9-*cis*-RA in methanol, we calculated that around 67% of the purified protein bound its cognate ligand. The different values obtained are most likely due to the conditions of the assays which alter the equilibrium of ligand binding and/or denaturation during the assay (e.g. on the highly active charcoal surface). In addition, we observed that the extinction coefficient of 9-*cis*-RA in aqueous solutions was significantly different from that in methanol (see “Materials and Methods”), such that it is impossible to determine bound ligand from the UV spectrum of the holoreceptor, and concluded that none of the above methods can be used to quantitate ligand binding accurately.

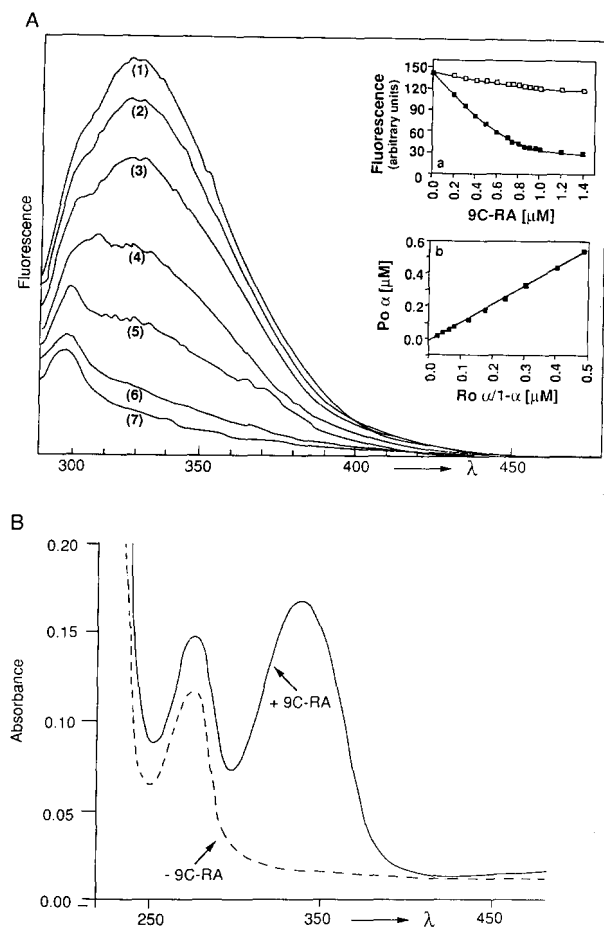


Fig. 4. RXR α AB expressed in *E. coli* bind quantitatively its cognate ligand, 9-*cis*-RA. *Panel A*, fluorescence spectra of purified RXR α AB (purity as shown in Fig. 1, *B* and *C*, lanes 6) in the absence or presence of increasing amounts of 9-*cis*-RA (curves 1–7 correspond to 9-*cis*-RA concentrations of 0, 50, 100, 200, 300, 600, and 900 nM 9-*cis*-RA). *Inset a*, plot of the fluorescence measured for RXR α AB (1 μ M; filled symbols) or an unrelated peptide (open symbols; for details see “Results”) giving the same initial fluorescence, in the presence of increasing amounts of 9-*cis*-RA. *Inset b*, analysis of fluorescence quenching shown in *inset a* according to Cogan (Cogan *et al.*, 1976). Note that Cogan plots from several different preparations of the purified receptor revealed dissociation constants between 8 and 20 nM and ligand binding homogeneities between 90 and 98%. No significant differences were observed for the ligand binding characteristics of the purified histidine-tagged receptor. *Panel B*, UV absorption spectra of the protein fraction obtained by gel filtration of identical samples of purified RXR α AB (5 μ M) before (dashed line) and after (solid line) incubation with 9-*cis*-RA. From the different absorptions of the holo- and apo-receptor and the extinction coefficient of RA (in methanol) about 67% of the receptor were still ligand-bound after gel filtration (for details see “Materials and Methods”).

To avoid the above problems, we have established a fluorescence-based method to monitor binding of 9-*cis*-RA to the receptor which involves neither a separation of bound and free ligand nor exposure of the protein to active surfaces. The absorption spectra of retinoids, which are centered around 350 nm, significantly overlap with the fluorescence emission spectra of tryptophans, typically centered at 330–340 nm. Consequently, the intrinsic fluorescence of a retinoid-binding protein will be quenched upon ligand binding if a tryptophan residue(s) is located in the vicinity of the binding site. Hence, changes in protein fluorescence upon binding of ligand can be used to follow the interactions of a retinoid with a binding protein without perturbing the equilibrium distribution. This method was employed previously to obtain the equilibrium dissociation constant as well as the number of binding sites within ligand-

protein complexes of a variety of retinoid-binding proteins (Cogan *et al.*, 1976; Noy and Xu, 1990; Noy and Blaner, 1991; Fiorella and Napoli, 1991; Chen *et al.*, 1993). Since the only tryptophan residues of RXR α AB (Trp²⁸⁷ and Trp³¹⁰) are located in the ligand binding domain, it was reasonable to assume that the fluorescence of either one or both of these residues will be affected by binding of 9-*cis*-RA. Indeed, the fluorescence of purified RXR α AB (excitation, 280 nm; emission, 340 nm) was progressively quenched upon addition of increasing amounts of 9-*cis*-RA (Fig. 4A). The fluorescence intensity approached a plateau as saturation of the protein with 9-*cis*-RA was achieved (Fig. 4A, *inset a*, filled symbols), indicating that quenching was directly related to ligand binding. To provide further evidence that this quenching is indeed related to 9-*cis*-RA binding rather than an inner filter effect, we replaced RXR α AB by a synthetic peptide (NH₂-NAWVAWRNRCK-COOH) and added identical amounts of 9-*cis*-RA to a peptide solution which gave the same initial fluorescence. Only a marginal linear decrease of fluorescence, indicative of inner filtering, could be observed (Fig. 4A, *inset a*, open symbols). The 9-*cis*-RA-dependent quenching of RXR α AB fluorescence was corrected and analyzed according to Cogan *et al.* (1976). In several experiments, a dissociation constant (K_d) of 14 ± 6 nM ($n = 5$) was determined, which is the same range of K_d values reported by others using different techniques (Heyman *et al.*, 1992; Levin *et al.*, 1992; Allegretto *et al.*, 1993; Allenby *et al.*, 1993). Most importantly, however, from the slope of the Cogan plot we could conclude in all cases that between 90 and 98% of the purified receptor preparations was able to bind 9-*cis*-RA.

9-*cis*-RA Can Stimulate the Binding Efficiency of RXR Produced in Vitro, but No Ligand Effect Is Seen with Functional Receptor Produced in *E. coli*, Sf9, or COS-1 Cells—It has been reported previously that 9-*cis*-RA binding stimulated the binding of RXR homodimers to cognate response elements (Zhang *et al.*, 1992b). We were able to reproduce these observations when using *in vitro* made RXR (Fig. 5A), even though the effect was not as drastic as described by Zhang *et al.* (1992b). However, when we used purified RXR α AB expressed in *E. coli* which was more than 90% functional with respect to ligand binding (see above), 9-*cis*-RA had no or little effect on the efficiency of binding to DR1 (Fig. 5C, compare lanes 1 and 2; we noted some ligand effect in early purifications that yielded lower quality RXR α AB; data not shown). Similarly, His₆-RXR α AB purified to near homogeneity from baculovirus-infected cells (lanes 5 and 6) or transiently expressed in COS-1 cells (lanes 3 and 4) did not show significant changes in DR1 binding efficiency upon exposure to 9-*cis*-RA. Note, however, that in all cases the mobility of the RXR α AB-DR1 complexes increased in the presence of ligand, indicating that the majority of receptor molecules could indeed bind the ligand. The absence of a ligand effect was not only limited to amino-terminally truncated receptors, since the same observation was made with full-length RXR expressed in, and partially purified from, *E. coli* (lanes 7 and 8) or COS-1 cells (lanes 9 and 10). As expected, heterodimers formed between RXR α AB and RAR α AB bound with similar efficiency to DR5 in the presence and absence of 9-*cis*-RA, irrespectively of whether they were expressed in *E. coli*, Sf9, or COS-1 cells (Fig. 4D).

It could be argued that COS-1 cell-derived RXR or His₆-RXR α AB extracts may be contaminated with endogenous RAR and form heterodimers whose DNA binding was reported to be insensitive to ligand exposure (Zhang *et al.*, 1992b). However, in that case RXR α AB-RAR complexes of intermediate mobility should have been formed, which was not the case (compare the mobilities of pure RXR α AB produced in *E. coli* with that expressed in COS-1 cells in Fig. 5C, lanes 1–4, and with that of

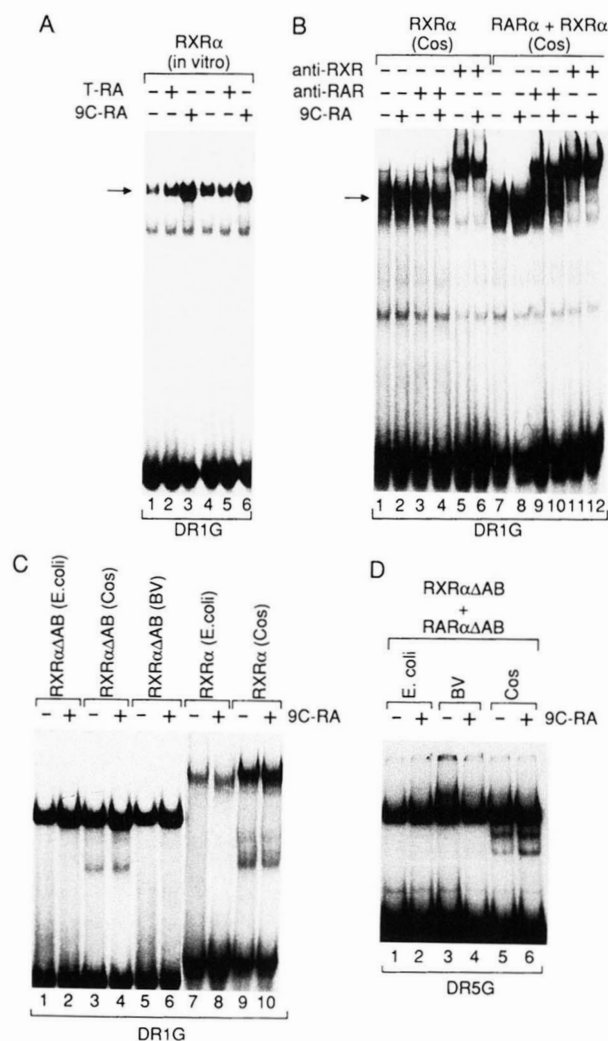


FIG. 5. 9-cis-RA stimulates DR1 binding of *in vitro* made, but not functionally homogeneous or COS-1-cell expressed RXR. Panel A, RXR was produced by *in vitro* transcription and translation and used for gel retardation without (lanes 1 and 4) or with exposure to 1 μ M all-*trans*-RA (T-RA) (lanes 2 and 5) or 1 μ M 9-*cis*-RA (lanes 3 and 6) using DR1 as a probe. The arrow indicates the specific complex. Panel B, gel retardation with crude extracts of COS-1 cells transiently transfected with RXR (lanes 1–6) or RXR plus RAR (lanes 7–12) and the DR1 probe. Extracts were prepared either from cells that had never been incubated with ligand or from cells that were incubated with 1 μ M 9-*cis*-RA (lanes 2, 4, 6, 8, 10, 12). In the latter case all buffers used contained the same concentration of 9-*cis*-RA to exclude ligand dissociation. Antibody supershifts confirmed the absence of significant amounts of RAR in the complexes formed with the RXR-programmed COS-1 cell extract (lanes 3 and 4, compare with lanes 9 and 10) and the identity of the RXR-DR1 complexes (lanes 5 and 6). Note the slightly increased mobility of the complexes upon ligand binding (see also panel C). Panel C, more than 90% pure and functionally homogeneous His₆-RXR α Δ AB (lanes 1, 2, 5 and 6), partially purified RXR (lanes 7 and 8), or crude extracts of His₆-RXR α Δ AB (lanes 3 and 4) or RXR (lanes 9 and 10) expressed in COS-1 cells were used for gel retardation with or without exposing the proteins to 1 μ M 9-*cis*-RA, as indicated at the top. Note that the ligand-bound complexes migrate faster than those formed with the aporeceptor. Panel D, gel retardation assays with the DR5 probe and RXR-RAR heterodimers formed from purified His₆-RXR α Δ AB expressed in *E. coli* (lanes 1 and 2) or Sf9 cells (lanes 3 and 4), or crude extracts of His₆-RXR α Δ AB-expressing COS-1 cells (lanes 5 and 6), to which the same amounts of *E. coli*-expressed and purified His₆-RAR α Δ AB had been added before exposing the samples to 9-*cis*-RA when indicated at the top.

full-length RXR in lanes 7 and 8). Moreover, anti-RXR antibodies supershifted COS-1 cell-derived RXR-DR1 complexes (Fig. 5B, compare lanes 1 and 2 with lanes 5 and 6), but antibodies directed against RAR did not (lanes 3 and 4). As a positive

control, RXR-RAR heterodimeric complexes with DR1 were shifted by antibodies directed against both RXR and RAR (lanes 9–12). We thus conclude that 9-*cis*-RA binding does not alter the efficiency of binding of RXR homodimers to cognate DR1 response elements.

DISCUSSION

Pure and Functionally Homogeneous RXR—This study was aimed at preparing large amounts of receptor for structural studies. Although multiple bacterial, yeast, invertebrate, and vertebrate expression systems have been scrutinized, only the bacterial T7 and the BV system of Studier and Moffatt (1986) and Summers (O'Reilly *et al.*, 1992), respectively, yielded sufficient amounts of recombinant receptor. Proteolytic “hot spots” in the A/B region of all RARs and RXRs tested (in both expression systems) prompted us to purify A/B-truncated RXR. To optimize and speed up the purification procedure, histidine tags were attached to the amino terminus of the protein for Ni²⁺ chelating chromatography (for details see “Materials and Methods”). Three purification procedures (Fig. 1) yielded roughly equivalent amounts of protein, devoid of degradation products. Clearly, purification of His₆-RXR Δ AB by chelating chromatography from *E. coli* was the simplest and most rapid procedure yielding mg amounts of receptor protein.

One of the most important requirements for crystallization is that the protein be not only free of impurities and degradation products, but that all molecules in the purified preparation adopt the same structure. For a nuclear receptor, the simplest measurement for such a structural homogeneity is to determine the functional homogeneity of a purified preparation with respect to ligand and DNA binding. It is well known that ligand binding is one of the most sensitive functions of a nuclear receptor which can be easily lost during purification. To determine functional homogeneity, we had to develop a novel ligand binding assay, based on fluorescence quenching, since we found that all methods involving a separation of free and bound ligand affected ligand binding. Quenching of protein fluorescence by a bound retinoid has been used previously to determine binding of retinoids to various proteins including binding of RA to recombinant cellular retinoic acid-binding proteins (Noy and Xu, 1990; Noy and Blaner, 1991; Fiorella and Napoli, 1991; Chen *et al.*, 1993). Indeed, using this method, we found that nearly 98% of the purified His₆-RXR Δ AB bound 9-*cis*-RA. Moreover, it also heterodimerized quantitatively with RAR (data not shown).

Although the bacterially expressed RXR bound with an identical binding site repertoire to direct and inverted repeats, its affinity to the cognate DR1 was reduced as compared with receptor expressed in COS-1 cells. Interestingly, receptor produced in Sf9 cells also bound less efficiently to DR1 as compared with RXR from COS-1 cells. Note that we have excluded that RXR-RAR heterodimers (formed with endogenous COS-1 cell RAR) could contribute to DR1 binding. Thus, either vertebrate cell-specific factors stabilize the binding of RXR to DR1, or vertebrate cell-specific post-translational modification can increase the binding efficiency of RXR to DR1 elements.

The DNA Binding of RXR Is Not Altered by 9-*cis*-RA—9-*cis*-RA has been reported previously to be mandatory for efficient DNA binding of RXR homodimers to DR1 elements (Zhang *et al.*, 1992b). From these studies the existence of a signaling pathway was inferred which would depend on the 9-*cis*-RA-induced formation of RXR homodimers and thus be distinct from the one constituted by RXR-RAR heterodimers. Although we observed some ligand effect on the efficiency of DR1 binding of *in vitro* made RXR, no such effect was seen with purified RXR from *E. coli* or Sf9 cells nor with RXR expressed in COS-1 cells. Importantly, we have unequivocally demon-

strated that the RXR purified from *E. coli* is fully functional for ligand binding. In keeping with our results, the absence of a ligand effect on DNA binding of RXR has been concluded from fluorescence anisotropy measurements of RXR-DR1 complexes formed in the presence and absence of 9-*cis*-RA.² It is possible that a fraction of the *in vitro* made receptor is partially unfolded and that ligand binding induces the proper structure for efficient DNA binding.

Clearly, the DNA binding characteristics of RXR in the absence and presence of 9-*cis*-RA refute the concept that 9-*cis*-RA is requisite for RXR homodimer formation and DNA binding *in vitro*. However, this does not rule out the possibility that RXR homodimers may constitute a 9-*cis*-RA-dependent signaling pathway. Indeed, evidence supporting this hypothesis comes from our recent observation that RXR can efficiently activate transcription from DR1-based reporter genes in yeast cells that lack endogenous RARs (Heery *et al.*, 1994). This activation was similar to that seen with the RXR-RAR heterodimer with the same reporter gene but less than the activation seen with the heterodimer and DR5-based promoters (Heery *et al.*, 1993, 1994). Thus, it is possible that *in vivo* RXR homodimers can act as transcription factors with properties distinct from those of RXR-RAR heterodimers, since transactivation will be induced solely by 9-*cis*-RA, but not all-*trans*-RA.

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