Retinoid receptors cause distortion of the retinoic acid response element in the phosphoenolpyruvate carboxykinase gene promoter

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Functional retinoic acid response elements (RAREs) have been described wherein the direct repeats are separated by 1, 2 or 5 bp (termed DR1, DR2 and DR5 respectively). We have previously shown that retinoic acid receptor/retinoid X receptor (RAR/RXR) binds a DR1 RARE within the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter and is the *trans*-acting complex that mediates the retinoic acid (RA) response. However, the mechanism of *trans*-activation is unknown. The consequences of RAR/RXR binding to the PEPCK RARE were examined using a circular permutation analysis as a first step to explore the possible role of DNA conformational changes in the RA response. The RAR/RXR heterodimer produced a distortion angle of 78°. The DNA distortion was shown to be at the centre of the PEPCK RARE; RA did not affect the severity of the

distortion angle or the location of the distortion centre. Monomers and homodimers of RAR also distorted the DNA, but to a lesser extent than did RAR/RXR. The results of a phasing analysis demonstrated that RAR/RXR heterodimers did not induce a static DNA bend, in either the presence or the absence of RA. A cyclization kinetics assay was employed to show that RAR/RXR binding affected DNA ring closure in a phase-sensitive, RA-insensitive, manner. Taken together, these observations support the idea that RAR/RXR heterodimers distort the structure of the PEPCK RARE, at least in part, by altering DNA flexibility. The conformational change in the PEPCK RARE upon RAR/RXR binding has implications for how RAR/RXR heterodimers recognize various RARE structures.

INTRODUCTION

Retinoic acid receptors (RARs) are members of the steroid/thyroid/retinoid superfamily of nuclear hormone receptors. They bind to their cognate response elements in *cis* and alter the transcriptional initiation rates of target genes in a ligand-dependent manner (for reviews, see [1–3]). The RAR, along with the thyroid hormone receptor (T₃R), the 1,25-dihydroxyvitamin D₃ receptor (VDR) and the peroxisome-proliferator-activated receptor (PPAR), are members of a subfamily of nuclear hormone receptors that is distinguished by binding to DNA elements that consist of direct repeats (DRs) of AGGTCA or related sequences. Additionally, these receptors bind DNA primarily as a heterodimeric complex with the retinoid X receptor (RXR), itself a member of the steroid/thyroid/retinoid superfamily [4–9]. The spacing between the half-sites is an important, but not mandatory, requirement for determining hormone receptor specificity for response element binding [10]. In general, hormone response elements with AGGTCA DR half-sites separated by 1 bp bind to RXR homodimers and PPAR/RXR heterodimers, and DR half-sites separated by 3 and 4 bp bind to T₃R/RXR and VDR/RXR respectively [11–13]. RAR/RXR heterodimers have more choices than the other members of this subfamily, since they interact with retinoic acid response elements (RAREs) with spacings of 1, 2 and 5 bp (designated DR1, DR2 and DR5 respectively) [10,14].

We previously demonstrated that 2 µM retinoic acid (RA) induces a 3-fold increase in the transcription rate of the endogenous phosphoenolpyruvate carboxykinase (PEPCK) gene in H4IIE rat hepatoma cells. This response is modest, since RAR is not abundant in H4IIE cells; when a RAR expression vector is co-transfected with PEPCK promoter–chloramphenicol acetyltransferase (CAT) constructs in transient transfections, there is a

robust response to RA [15,16]. Utilizing this co-transfection system, the PEPCK RARE was initially localized between positions –468 and –431 relative to the transcription initiation site. To more finely delineate the PEPCK RARE, a series of block mutations was used to locate the element between positions –451 and –433 bp. This core sequence mediates a RA response when inserted upstream of a heterologous promoter [15]. The minimal functional boundaries of the PEPCK RARE are coincident with those of the accessory factor 1 (AF1) element which is an integral component of the PEPCK glucocorticoid response unit. Mutations that abolish the ability of this element to serve an accessory role in the glucocorticoid response also abolish its ability to moderate a RA response, and vice versa [15,17]. Thus AF1/RARE plays a critical role in two distinct hormone response pathways. The PEPCK RARE contains two imperfect DR half-sites, which we have named sites B and C. The B site, beginning at –451 (5' to 3'), has the sequence TGACCT (this is the anti-sense version of the canonical AGGTCA half-site sequence described above) and the C site, beginning 1 bp downstream, has the sequence TGGCCG [15]. We have recently shown, by methylation interference studies, that monomers and homodimers of RAR bind only to the B site. However, the RAR/RXR heterodimer interacts with both half-sites of the PEPCK RARE, and both half-sites must be occupied to confer a complete RA response. Furthermore, RAR/RXR binds to the PEPCK RARE much more avidly than either protein alone. Based on these observations, we have concluded that the RAR/RXR heterodimer is the functional *trans*-activating complex for the RA response of the PEPCK gene [16].

The mechanism of ligand-induced *trans*-activation by steroid receptor family members is poorly understood. An important question is: how do ligand-activated receptors communicate

Abbreviations used: RARE, retinoic acid response element; DR, direct repeat; PEPCK, phosphoenolpyruvate carboxykinase; RA, all-*trans* retinoic acid; RAR, retinoic acid receptor; T₃R, thyroid hormone receptor; VDR, 1,25-dihydroxyvitamin D₃ receptor; PPAR, peroxisome-proliferator-activated receptor; RXR, retinoid X receptor; AF1, accessory factor 1; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; LBD, ligand-binding domain.

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with the basal transcriptional machinery when they may be bound to DNA hundreds or thousands of base pairs upstream (or downstream) from the transcription start site? An emerging paradigm holds that certain proteins are capable of binding to and bending DNA at strategic sites in the upstream regulatory regions of genes. In this manner, DNA-bending proteins may configure the DNA into a loop such that *trans*-acting factors bound to DNA far upstream of the transcription start site are positioned near to the basal machinery (for a review, see [18] and references therein). Furthermore, several transcription factors bend DNA and may themselves contribute to the overall shape of the upstream regulatory regions to which they bind [19–23].

As a first step to explore the possible role of DNA conformational changes in the response of the PEPCK gene to RA, we tested whether RAR/RXR heterodimers altered the conformation of the PEPCK RARE. Two common electrophoretic techniques, circular permutation and phasing analysis, are used to detect DNA distortion and bending [24,25]. Circular permutation analysis detects distortions in the protein–DNA complex such as DNA bending, increased DNA flexibility and extended protein structures [23,26–28]. Phasing analysis distinguishes between DNA bending and other structural distortions, and can be used to determine the magnitude and orientation of directed DNA bends [23,28]. Transcription factors shown to bend DNA by these techniques include Fos, Jun, Myc, Max, ATF and POU proteins [19–23]. Shapiro and colleagues, using circular permutation analysis, demonstrated that the oestrogen DNA-binding domain (DBD), and later the full-length receptor, distort an oestrogen response element [29,30]. Leidig et al. [31] found that T₃R distorts thyroid hormone response elements and showed a correlation between the magnitude of the distortion and the functional capacity of the T₃RE. Moreover, Lu et al. [32] demonstrated that RAR/RXR heterodimers produce a static bend when bound to a DR5-type RARE.

In the present paper we demonstrate that RAR/RXR binding to the PEPCK RARE results in a distortion that is not a static DNA bend. Results obtained from circular permutation analysis, phasing analysis and cyclization kinetics assays suggest that RAR/RXR binding to the PEPCK RARE may cause an increase in the flexibility of the DNA. These observations provide insight into how RAR/RXR heterodimers interact with DNA, and perhaps into how RAR and RXR interact with each other.

MATERIALS AND METHODS

RAR and RXR preparation

Human RAR α was expressed in *Escherichia coli* and purified according to the method of Forman et al. [33]. Mock bacterial extract was prepared by subjecting bacterial lysates from cells without expression vectors to the same purification protocol. The bacterial expression vector pET8CRAR α , and BL21 DE3 pLYSs host cells, were gifts from Dr. Herbert Samuels (New York University Medical Center, New York, NY, U.S.A.). The expression vector pMT2-RXR α [34] was a gift from Dr. Sotirios Karathanasis (Lederle Labs, Pearl River, NY, U.S.A.). An *Eco*RI fragment containing the RXR coding region was excised and subcloned into puc19. RXR was translated *in vitro* using a Promega rabbit reticulocyte lysate kit following *in vitro* synthesis of capped RNA using an Ambion MAXIscrip kit. Since large concentrations of RXR were required for the cyclization assay, DNA encoding the cDNA for human RXR α was amplified by the PCR using the following two primers (5' to 3'): GGCCC-ATATGGACACCAACATTTCTGCC and CCGAGATC-TCTAAGTCATTTGGTGCGGCGCC. The resulting fragment was digested with *Nde*I and *Bgl*II and ligated into the *Nde*I and

*Bam*HI sites of the bacterial expression vector pET-3a [35], such that the 11-amino-acid fusion peptide was removed. The resulting vector, pET3aRXR α , was transformed into BL21 DE3 pLYSs host cells. The protein was then expressed and purified using the method of Forman et al. [33]. The expressed RXR was slightly truncated (49 kDa rather than 51–54 kDa [36]), probably due to an alternative start site or proteolysis, but behaved identically in the circular permutation and phasing assays to RXR expressed in reticulocyte lysates (results not shown). Both RAR and RXR expressed from bacteria comprised greater than 85% of the protein in the mixture, as determined by densitometry of Coomassie Brilliant Blue-stained SDS/PAGE gels.

Mobility gel shift assay

High- and low-stringency mobility gel shift systems were employed as previously described [16]. The double-stranded oligonucleotide representing the PEPCK RARE (AF1) was described previously [16]. A typical high-stringency binding reaction contained 2.5×10^4 c.p.m. of the labelled probe, 10 mM Hepes (pH 7.8), 50 mM NaCl, 1 mM spermidine, 10 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P40 and 2 μ g of poly(dI-dC)·(dI-dC). The reaction conditions were identical for circular permutation assays and phasing analysis, except that an additional 5 μ g of poly(dI-dC)·(dI-dC) was added to each reaction. A typical low-stringency reaction contained 1.0×10^4 c.p.m. of the labelled probe, 25 mM Tris/HCl (pH 7.8), 0.5 mM EDTA, 88 mM KCl, 10 mM 2-mercaptoethanol, 0.1 μ g of aprotinin, 0.05% Triton X-100, 10% glycerol and 0.5 μ g of poly(dI-dC)·(dI-dC).

Circular permutation analysis

The parent plasmid pPL32 [37] was used to construct the plasmids used in the circular permutation assay. As illustrated in Figure 1, an *Nde*I fragment containing the PEPCK RARE was excised from pPL32 and ligated to form a tandem repeat, which was subsequently inserted into the *Nde*I site of puc19 to form the plasmid pucCP. Digestion of pucCP with each of the five restriction enzymes shown in Figure 1 generated five 169 bp fragments, each containing the PEPCK RARE located at a different site relative to the ends of the probe. The restricted fragments were dephosphorylated with calf intestinal phosphatase, end-labelled with [γ -³²P]ATP using polynucleotide kinase (NEB) and purified by gel electrophoresis. Intrinsic bend standards [38] were obtained from Tom Kerppola and Tom Curran (Roche Institute of Molecular Biology, Nutley, NJ, U.S.A.). Flexure angles were calculated according to the method of Kerppola and Curran [28], using the Macintosh Kalideograph curve-fitting program to generate best-fit cosine functions.

Phasing analysis

The phasing analysis vectors were constructed as follows. The *Eco*RI and *Hind*III sites of the plasmid puc19 were destroyed by restriction with these enzymes and were made blunt-ended with mung bean nuclease. The *Nde*I fragment mentioned above (containing unique *Eco*RI and *Hind*III sites) was inserted into the modified puc19 plasmid. This plasmid was then digested with *Hind*III and *Eco*RI, and oligonucleotides with *Hind*III and *Eco*RI ends were inserted to generate what we have designated the 'pucphase' series of plasmids. The oligonucleotides contained three phased A·T tracts, which together generate an intrinsic DNA bend of approx. 54° [38]. The plasmids were named by the number of base pairs from the centre of the PEPCK RARE to the centre of the directed intrinsic bend. Thus the plasmids

Figure 1 Construction of circular permutation probes

A 169 bp *NdeI*–*NdeI* fragment of the reporter vector pPL32 was ligated into the *NdeI* site of puc19 as a tandem repeat. The *NdeI*–*NdeI* fragment contains five unique restriction sites spaced nearly equally along the sequence. The tandem repeat was digested with the corresponding restriction enzymes, generating five 169 bp probes that were circularly permuted with respect to the PEPCK RARE. The restriction sites are *NdeI* (N), *HindIII* (H), *EcoRI* (E), *PvuII* (P) and *A1wNI* (A).

pucphase-35, pucphase-37, pucphase-40, pucphase-42 and pucphase-44 contained the sense-strand oligonucleotides AGCTTGCAAAAACGGGCAAAAACGGGCAAAAACGG, AGCTTGCAAAAACGGGCAAAAACGGGCAAAAACGTCG, AGCTTGCAAAAACGGGCAAAAACGGGCAAAAACGTCGACG, AGCTTGCAAAAACGGGCAAAAACGGGCAAAAACGTCGACACG and AGCTTGCAAAAACGGGCAAAAACGGGCAAAAACGTCGACTGACG, and the anti-sense oligonucleotides (5' to 3') AATTCGTTTTTGCCCGTTTTTGCCCGTTTTTGCA, AATTCGACGTTTTTGCCCGTTTTTGCCCGTTTTTGCA, AATTCGTCGACGTTTTTGCCCGTTTTTGCCCGTTTTTGCA, AATTCGTGTCGACGTTTTTGCCCGTTTTTGCCCGTTTTTGCCCGTTTTTGCA and AATTCGTCAGTCGACGTTTTTGCCCGTTTTTGCCCGTTTTTGCA respectively.

Cyclization assay

The vectors utilized for the cyclization kinetics assay were constructed from the pucphase vectors as follows. The sense

oligonucleotide AGCTTAAAAACGGGCAAAAACGGGCAAAAACAGATCTGA and the antisense oligonucleotide (5' to 3') AGCTTCAGATCTGTTTTGCCCCGTTTTTGCCCGTTTTT were annealed to provide *HindIII* compatible ends, a unique *BglII* site and phased A·T tracts which created an intrinsic bend of approx. 54°. Pucphase-35 and -40 plasmids were restricted with *HindIII* and ligated to the annealed oligonucleotides to create pcircle-35 and pcircle-40. Thus two sets of sequences with intrinsic bends were positioned in phase to produce a 108° bend [38]. The plasmids pcircle-35 and pcircle-40 were used as templates in PCR reactions to create RARE mutants as follows. An upstream primer, GGGAAATCCATATGGTGCACCTCTCAGTAC, and a mutant RARE primer (5' to 3'), GTGTCACCTCCCACTTCCAAAGGGAATGAGAAGGGAATTC, were used in a PCR reaction to generate a 'megaprimer' [39]. The megaprimer was gel-purified and used with a downstream primer (5' to 3'), GGGAAATCCATATGCTGCTGGCTGCAC, in a second PCR reaction which, after restriction with *NdeI*, yielded fragments identical to the *NdeI* fragments of pcircle-35 and pcircle-40, except that the RAR/RXR binding sites were mutated. These fragments were ligated into pGEM 5 and the resulting plasmids were designated pcircle-m35 and pcircle-m40. The probes for the cyclization assay were generated by PCR, where the downstream primer (5' to 3') CGGCCGATCGATCCACAGCTGTGAGGTGTAC and the upstream primer CATGGAATCGATGCTCTGATGCCGCATAGTTAAGC were used with pcircle-40 or pcircle-m40 to generate 165 bp probes (see below). A second upstream primer, CATGGAATCGATAATCTGCTCTGATGCCGCATAGT, was used with pcircle-35 or pcircle-m35 and the downstream primer to generate two additional 165 bp probes. Since all of the probes were the same length, they all had the same torsional alignment.

The cyclization probes were labelled using the PCR. The reaction mix contained 10 mM KCl, 20 mM Tris/HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 % Triton X-100, 125 μM each of dGTP, dCTP and dTTP, 62.5 μM dATP, 30 μCi [α -³²P]dATP (3000 Ci/mmol), 0.2 μM oligonucleotide primers, 2 ng of DNA template and 2 units of Vent polymerase (NEB). The reaction proceeded for 30 cycles, each consisting of 20 s at 94 °C, 20 s at 64 °C and 20 s at 72 °C. The PCR products were extracted with phenol, precipitated overnight at –20 °C and digested for 2 h with 30 units of *ClaI*. The DNA fragments were purified on 5% (w/v) polyacrylamide gels, run in 1 × TBE [50 mM Tris/HCl (pH 8.0), 50 mM boric acid, 1 mM EDTA] and electroeluted. The cyclization kinetics assay was performed as described by Kahn and Crothers [40], except that the total reaction volume was 10 μl, the final concentration of T4 ligase was 5 units/μl and the reaction took place at 16 °C for up to 1 h. Gel-purified ligation products were shown to be circular because they exhibited resistance to exonuclease *Bal31*, and digestion with *BglII* generated a single linear 165 bp fragment (results not shown). The products of the ligation reactions were quantified using a Bio-Rad GS-250 Molecular Imager in conjunction with the Bio-Rad Molecular Analyst software.

RESULTS

Retinoid receptors cause a distortion of the RARE

The consequences of the binding of RAR or RAR/RXR complexes to the PEPCK RARE were initially explored using circular permutation analysis [24]. Five 169 bp DNA fragments containing the natural PEPCK RARE were constructed such that the element was located at various distances from the ends of the fragment (Figure 1). Thus the fragments were circularly

Figure 2 Circular permutation analysis of the RAR/RXR-RARE complex

(a) Mobility gel shift of the RAR/RXR-RARE complex using circularly permuted probes. Approx. 100 fmol of partially purified RAR and 2 μ l of reticulocyte lysate programmed with RXR mRNA were incubated with 32 P-end-labelled circularly permuted probes. The reaction mixtures contained either 2 μ M RA or the vehicle, DMSO (Control). The distortion angles were calculated to be $78.3 \pm 1.3^\circ$ ($n = 4$, mean $\pm$ S.E.M.) for control and $77.5 \pm 2.0^\circ$ ($n = 4$) for RA-treated receptor complexes using the formula $Acp = 1 - \cos(k\alpha/2)$ [28], where Acp is the circular permutation amplitude, k is an electrophoretic condition coefficient and α is the estimated distortion angle. (b) Distortion centre analysis. The migration, as a percentage of the maximum, of the RAR/RXR-shifted circularly permuted probes from (a) (control lanes) was plotted as a function of the nucleotide position (in bp) of the *NdeI*-*NdeI* fragment, taken as an arbitrary standard. The curve was generated using a polynomial function from DeltaGraph; error bars represent the S.E.M.s from four independent experiments. The dashed lines are extrapolations from the linear portions of the curve; the open box represents the position of the PEPCK RARE. The restriction sites are *NdeI* (N), *HindIII* (H), *EcoRI* (E), *PvuII* (P) and *AflwNI* (A).

permuted with respect to the location of the PEPCK RARE. Figure 2(a) shows a circular permutation analysis of the constructs depicted in Figure 1 bound to RAR/RXR. The binding of RAR/RXR caused the circularly permuted fragments to migrate relatively more slowly when the heterodimers were bound to the RARE in the centre of the fragment, whereas the RAR/RXR-RARE complex migrated more rapidly when located near the ends (compare lanes E and N, Figure 2a). The resulting 'frown' pattern is indicative of DNA distortion, which may be caused by DNA bending [24], increased DNA flexibility

Figure 3 Circular permutation analysis comparing RAR alone with RAR/RXR heterodimers

Approx. 100 fmol of RAR was incubated with 32 P-end-labelled circularly permuted probes, either alone or with the addition of 2 μ l of RXR-programmed reticulocyte lysates and 6 μ g of poly(dI-dC)·(dI-dC). The reactants were run on a low-stringency gel system as described in the Materials and methods section (and in [16]), prior to autoradiography. The k factor [28] for this gel system was calculated to be 0.77. The calculated distortion angles for the RAR monomer and dimer bands were $75.6 \pm 7.2^\circ$ ($n = 3$, mean $\pm$ S.E.M.) and $67.1 \pm 6.9^\circ$ ($n = 3$) respectively. The RAR/RXR distortion angle in this gel system was $97.4 \pm 0.4^\circ$ ($n = 3$). The restriction sites are *NdeI* (N), *HindIII* (H), *EcoRI* (E), *PvuII* (P) and *AflwNI* (A).

[23,28] or extended protein structures (rigid rods extending perpendicular to the long axis of the DNA) [26,27]. The centre of the DNA distortion can be determined by plotting the migration of the circularly permuted probes versus the position of the binding site relative to an arbitrarily chosen reference point. Figure 2(b) shows that the centre of the RAR/RXR-mediated DNA distortion is near the midpoint of the PEPCK RARE.

Although circular permutation analysis cannot distinguish between DNA bending, increased flexibility [23,28] or an extended protein structure [26,27], it can be used to quantify the distortion caused by the binding of RAR/RXR to the PEPCK RARE. This allowed us to determine whether RA caused an alteration in the extent of DNA distortion. The distortion angle induced by RAR/RXR binding was quantified using a variation of the Thompson-Landy equation [38] derived by Kerppola and Curran [28] (see the legend to Figure 2). The k factor was derived from a calibration curve generated with the use of intrinsic bend standards, which consist of DNA fragments containing phased A·T tracts that produce a known magnitude of DNA bending [38]. The k factor for the experiments represented in Figure 2(a) was calculated to be 0.87. The distortion angle for RAR/RXR in the absence of RA was $78.3 \pm 1.3^\circ$ ($n = 4$), and in the presence of RA it was $77.5 \pm 2.0^\circ$ ($n = 4$). Thus the presence of RA made no difference to the RAR/RXR-mediated distortion angle, nor did it alter the centre of DNA distortion (results not shown).

RAR/RXR induces a greater distortion angle than does RAR alone

We have previously shown that RAR alone binds to the PEPCK RARE as a monomer or a homodimer, but only under low-stringency conditions {low poly(dI-dC)·(dI-dC) concentrations and low-ionic-strength running buffer [16]; see also the Materials and methods section}. By contrast, RAR/RXR heterodimers bind to the PEPCK RARE under high-stringency conditions ([16]; Materials and methods section) and appear not to enter the

Figure 4 Phasing analysis of the RAR/RXR-RARE complex

(a) Diagram of phasing probes. The phasing probes contained phased A·T tracts at variable distances from the PEPCK RARE. The diagram illustrates the prediction that a protein-induced directed bend would complement or counteract the intrinsic bend. The result would be a family of protein-DNA complexes, each with a slightly different shape which, in turn, would run with different mobilities through a native acrylamide gel. (b) Mobility gel shift assay of the RAR/RXR-RARE complex using the phasing probes. Approx. 100 fmol of RAR and 2 μ l of RXR-programmed reticulocyte lysate were incubated with 32 P-end-labelled phasing probes in the presence of either 2 μ M RA or vehicle control (DMSO). The reaction mix was loaded on to a 5% native polyacrylamide gel and subjected to autoradiography. The sizes of the probes in bp are indicated below the gel.

gel when run in the low-stringency system. In order to compare RAR/RXR- and RAR-induced DNA distortions, conditions were sought wherein both sets of retinoid receptor complexes could be visualized on the same gel. A single band representing the RAR/RXR heterodimer was apparent when an additional 6 μ g of poly(dI-dC)·(dI-dC) was added to the low-stringency binding reaction mixture containing both RAR and RXR (Figure 3). Both sets of retinoid receptor complexes retarded migration when bound to the centre of the fragment (compare lanes N and E in Figure 3), and the calculated distortion angles for the RAR monomer and dimer bands were $75.6 \pm 7.2^\circ$ ($n = 3$) and $67.1 \pm 6.9^\circ$ ($n = 3$) respectively. In contrast, the RAR/RXR distortion angle in this gel system was $97.4 \pm 0.4^\circ$ ($n = 3$). Thus RAR/RXR, which binds much more avidly to the PEPCK RARE than does RAR alone [16], also generates greater DNA distortion. The different magnitudes of distortion exhibited by RAR/RXR and RAR alone could also be correlated with the RA response, since the former complex (and not RAR alone) is the *trans*-activating complex for the PEPCK RA response [16].

RAR/RXR does not cause a directed DNA bend of the PEPCK RARE

Phasing analysis was used to determine if RAR/RXR caused a static DNA bend. Probes were constructed so that different spacing existed between an intrinsic DNA bend, created with phased A·T tracts, and the PEPCK RARE (Figure 4a; and see

the Materials and methods section). The spacing varied between 35 and 44 bp, a difference that represents a single turn of the DNA helix. If RAR/RXR caused a directed DNA bend, the intrinsic bend of a set of the variably spaced probes would be complemented by the RAR/RXR-induced bend, enlarging the overall bend angle. Conversely, a directed bend of a second set of probes, with spacing either 5 bp shorter or longer than the first set, would counteract the intrinsic bend and decrease the overall bend angle, since the RAR/RXR complex would bind to the opposite face of the DNA helix. These differentially phased RAR/RXR-RARE complexes would then migrate through polyacrylamide gel matrices at different rates, since their shapes would be different (Figure 4a). As shown in Figure 4(b), the RAR/RXR complexes all migrated at approximately the same rate regardless of the phasing, and were unaffected by the presence of RA. Therefore RAR/RXR does not appear to cause static DNA bending of the PEPCK RARE.

RAR/RXR alters the flexibility of the PEPCK RARE

We performed a cyclization kinetics assay to determine whether DNA flexibility, the formation of an extended protein structure or other structural anomalies accounted for the observations noted above. This assay is based on the kinetics of ligation of small DNA fragments (165 bp; see the Materials and methods section) into a circle using T4 DNA ligase [40]. If RAR/RXR binds to the PEPCK RARE with an extended protein conformation, but does not alter the structure of the DNA, the ligation kinetics of the cyclization probes would not be affected. However, if RAR/RXR increases the flexibility of the DNA, the probability of forming a covalently closed circle would change.

To simplify the analysis of the cyclization kinetics assay, we focused on two probes, circle-35 and circle-40, of identical length (165 bp). The probes were constructed so that the spacing between the intrinsic bend and the PEPCK RARE in circle-40 was 5 bp (one half-helical turn) longer than in circle-35. Conditions were found wherein RAR/RXR completely bound the wild-type DNA cyclization probes in a gel shift assay yet permitted efficient ligation (results not shown). When the circle-35 and circle-40 probes were incubated with mock bacterial extract or no protein, the circle-40 probe ligated at a more rapid rate than the circle-35 probe (compare Figures 5b and 5e; and results not shown). The difference in ligation kinetics between the two probes was not due to differences in torsional alignment since the two probes had the same length. The difference was probably due to intrinsic DNA curvature as this assay is very sensitive to minor DNA structural variations [40]. When RAR/RXR bound the circle-40 probe, the rate of cyclization was approximately the same as when the probe was incubated with mock bacterial extract (Figures 5a and 5b). However, as shown in Figures 5(d) and 5(e), when RAR/RXR bound the circle-35 probe the rate of cyclization was reduced when compared with the assay performed with mock bacterial extract. This indicated that the binding of RAR/RXR altered the structure of the DNA, and that the RAR/RXR-mediated alterations in DNA structure were phase-specific. We found no difference in ligation efficiency when RA was added to the binding/ligation reaction (results not shown).

To demonstrate that any differences in the ligation kinetics of the probes in the presence of RAR/RXR were due to binding of the RARE, we constructed cyclization probes (circle-m35 and circle-m40) containing mutations of the RAR/RXR binding sites. Under conditions where 100% of wild-type probes were shifted, only a small percentage of the circle-m35 or circle-m40 probes were shifted by RAR/RXR in gel-shift assays (results not

Figure 5 Cyclization kinetics assay

(a) Approx. 50 fmol of bacterially expressed RAR and RXR, or an equivalent volume of mock bacterial extract, was incubated with 5 fmol of 32 P-labelled circle-40 or circle-m40 cyclization probes. T4 DNA ligase was added at a final concentration of 5 units/ μ l and the reaction was allowed to continue for up to 60 min at 16 °C. The reaction was stopped by addition of EDTA at the indicated times, protein was removed by proteinase K digestion, and the linear DNA and circular ligation products were separated on 5% native polyacrylamide gels. (b) and (c) Quantification of circle-40 and circle-m40 probes. The amount of cyclization probe, either circular or remaining linear, was quantified using a Bio-Rad GS-250 Molecular Imager in conjunction with the Bio-Rad Molecular Analyst software. Cyclization kinetics are represented as a percentage of probe cyclized as a function of time. Curves were fitted with a simple second-order polynomial function using the Cricket Graph program. The results are the means of from four to six independent experiments; error bars represent S.E.M.s. (d) Polyacrylamide gel electrophoretic separation of linear and circular forms of the circle-35 and circle-m35 cyclization probes. The cyclization kinetics assay was performed as described for (a). (e) and (f) The amounts of linear and circular circle-35 and circle-m35 probes were quantified and are represented as described for (b) and (c) respectively.

shown). Neither of the mutant probes exhibited altered ligation kinetics in the presence of RAR/RXR (Figures 5c and 5f). Taken with the previous results, the simplest explanation for the altered cyclization kinetics is that RAR/RXR increases the flexibility of the PEPCK RARE. Since the increased flexibility appears to be phase-sensitive, the flexibility must have a directional preference.

DISCUSSION

Data obtained from the cyclization kinetics assay demonstrate that RAR/RXR altered the rate of circular ligation of PEPCK RARE-containing DNA fragments (Figure 5). This, along with the circular permutation results showing a relatively large distortion angle (Figure 2) and phasing analysis demonstrating no DNA bending (Figure 4), suggested that RAR/RXR increased the flexibility of the DNA. Figure 6 shows a schematic summary of our results. The RAR/RXR-mediated flexibility has the character of a flexible bend or hinge, where there is a magnitude of flexibility (i.e. a component of the distortion angle) that is restricted within a specific range of motion (which is manifested by phase-sensitive ligation kinetics). While static DNA bending clearly does not contribute to the distortion angle, we suggest that the RAR/RXR-induced DNA distortion of the PEPCK RARE is a combination of DNA flexibility and extended protein structure. However, we cannot rule out the possibility that other structural anomalies, such as DNA twisting, also contribute to the distortion.

Circular permutation analysis was used to demonstrate that RAR/RXR distorts DNA when it binds to the PEPCK RARE (Figures 2 and 3). A phasing analysis (Figure 4) indicated that the distortion was not a directed DNA bend. A similar set of observations has been described for the yeast regulatory factor

GCN4. This protein causes the anomalous electrophoretic migration of circularly permuted fragments, but does not affect the migration of fragments containing intrinsic bends spaced at different intervals from the GCN4 binding site in a phasing analysis. The authors suggested that these observations could be explained either by increased DNA flexibility or by the formation of an extended protein structure [27]. Subsequently, the co-crystal structure of GCN4 bound to its DNA element was solved and while the DNA was found to be in an unaltered B-form, the protein was found to have an α -helix extending as a rigid rod perpendicular from the long axis of the DNA [26]. Thus the extended structure of GCN4 probably produced the anomalous migration of the transcription factor/DNA complex in the circular permutation analysis. Homo- and hetero-dimers of the AP1 and CREB families of transcription factors have large distortion angles ($> 65^\circ$) and very small ($\leq 4^\circ$) bend angles [20]. These observations led Kerppola and Curran to postulate the independent bends model [28]. This model suggests that each protein bends DNA independently and that the overall magnitude and orientation of a directed DNA bend is the vector sum of the bends induced by each of the two dimer partners. One possible scenario outlined in the model is that the combinations of heterodimers that do not bend DNA may have partners that bend DNA in opposite directions, cancelling out each other's effect. These non-bending heterodimers have large distortion angles, presumably because they increase the flexibility of DNA [23,28]. These observations, in several different systems, make it clear that anomalous migration in a circular permutation assay is not necessarily an indication of static DNA bending.

Transcriptional activation by ligand-bound RAR/RXR probably involves protein interactions between the *trans*-activating complex bound at an upstream RARE and the basal transcription

Figure 6 Summary of the effects of RAR/RXR binding on the structure of the PEPCK RARE

(a) The migration of the probes in the circular permutation assay is consistent with at least three possible structural distortions as shown: DNA bending, DNA flexibility or an extended protein structure. (b) Phasing analysis distinguished between directed DNA bending and the two alternative possibilities shown: either increased DNA flexibility or an extended protein structure. (c) The results of the cyclization kinetics assay are consistent with the idea that RAR/RXR increases the flexibility of the DNA. See the text and Figures 2–5 for further details.

machinery, an arrangement that could require looping out of the intervening sequence. DNA looping can be facilitated by properly placed protein-induced DNA bends. For example, the *c-fos* promoter contains three binding sites for the inducer/repressor transcription factor YY1. This protein bends DNA and apparently influences promoter function primarily by affecting the topology of the promoter structure rather than by protein–protein interactions. Thus, by altering the location of the YY1 binding site, the transcription factor can act either as a repressor or as an inducer of *trans*-activation by positioning upstream regulatory factors either further from or nearer to the basal transcriptional machinery respectively [41]. Similar functions have been ascribed to the DNA-bending proteins HMG I(Y) for the interferon- β gene promoter and LEF-1 for the T-cell receptor α enhancer [18,42]. Flexible bends or hinges may be more functionally adaptable than static DNA bending if DNA flexibility also plays a role in creating nucleoprotein structures required for proper transcriptional regulation. A given static bend would put more constraint on the shape the rest of the promoter structure must maintain in order to form a functional nucleoprotein architecture than would increased flexibility.

We observed that RAR/RXR heterodimers induced a larger distortion angle than did RAR monomers or homodimers, which is consistent with the idea that DNA flexibility may be func-

tionally important (Figure 3). We have previously demonstrated that both RAR monomers and homodimers bind only to the B site, the 5' half-site of the PEPCK RARE. However, RAR/RXR heterodimers bind to both half-sites, and both half-sites must be occupied to effect a RA response. Thus RAR/RXR heterodimers, rather than RAR alone, form the *trans*-activating complex for the PEPCK RARE [16]. RAR monomers and dimers, which bind weakly to only a single half-site and are functionally inert, have distortion angles that are approx. 20° less than those of RAR/RXR heterodimers, so there is a correlation between the magnitude of distortion and the ability of the bound protein complex to promote *trans*-activation. Other correlations between DNA distortion or bending and transcriptional activation have been reported. T₃R binds to homologous sites in the chorionic somatomammotropin and human growth hormone gene promoters. Circular permutation analysis revealed that T₃R induced a larger distortion on the chorionic somatomammotropin element than on the growth hormone element. Thyroid hormone stimulates transcription of the chorionic somatomammotropin gene, but not the growth hormone gene, which suggests that there is a relationship between the magnitude of distortion and transcriptional activity [31]. Kerppola and Curran have also described a correlation between DNA bending of truncated Jun and Fos proteins and the ability of these proteins to induce transcription. They found that the same domains are required for *trans*-activation and DNA bending [43].

Our data suggest that RAR/RXR does not cause a directed DNA bend when bound to the PEPCK RARE, which is a DR1. We believe that these data may provide insight into how nuclear hormone receptors interact with, and potentially bend or increase the flexibility of, DNA. Members of the steroid/thyroid/retinoid superfamily of hormone receptors have modular domain structures which include a DBD connected to a ligand-binding domain (LBD) by a hinge region. The DBDs consist of two zinc fingers, wherein the P-box of the first finger confers sequence specificity and the D-box of the second finger is crucial for dimerization [44]. The DBDs of RAR and T₃R also contain an additional dimerization interface in the second zinc finger (termed the DR-box) which can interact with the P-box of the first zinc finger of RXR [45]. This allows the DBDs of RAR and RXR to interact when bound to direct repeat elements even though they still are oriented with the same polarity on the adjacent DNA half-sites. This arrangement also dictates that RXR is bound to the 5' AGGTCA half-site [45,46]. A second protein–protein interface exists in the LBD of RAR and RXR that allows interaction between the two proteins on either direct repeats or palindromes, because the hinge region allows swivelling of the LBD so that one may be turned 180° with respect to the other [46].

Lu et al. [32] investigated the DNA conformational change induced by RAR/RXR binding to the β RAR RARE, a DR5. In this system, RAR/RXR binding produced a directed bend in the DNA which presumably was required for proper interaction of the RAR and RXR dimerization interfaces [32]. In the case of a DR5, the DBD and LBD dimerization interfaces interact optimally, since the spacing of the half-sites is such that both proteins bind to the same side of the DNA helix. In contrast, when considering a DR1, the LBDs are oriented away from each other, since the DBDs would be situated essentially on opposite faces of the DNA helix. The DBD interaction would also be different in a DR1 compared with a DR5, since a RAR DBD bound to a half-site in a DR1 would be rotationally shifted by 144° compared with the same domain bound to the corresponding half-site in a DR5. The existing data suggest a two-step mech-

anism whereby the co-operative binding of the DBDs of RAR/RXR somehow increases the flexibility of the DNA (perhaps by disrupting base stacking interactions in both DR1s and DR5s). The second step would involve the stabilization of this flexibility into a directed DNA bend by the interactions of the LBDs of the receptor partners (in DR5s only). Thus a RAR/RXR heterodimer binding to a DR5 would have optimal LBD dimerization contacts, which would lead to the formation of a static bend. When RAR/RXR binds to a DR1, the proteins would have suboptimal LBD interactions which would not be able to stabilize a directed bend.

To summarize, we found that RAR/RXR bound to the PEPCK RARE does not induce a directed DNA bend but apparently does increase the flexibility of the DNA. PEPCK is among a growing family of genes whose regulation is controlled by a number of hormones and whose promoters contain complex multihormonal regulatory domains [47]. We, and others, have defined more than 15 binding sites for various transcription factors along the PEPCK gene promoter from -468 to -1 bp relative to the transcription start site, all of which appear to have some functional role in hormonal regulation [17,47-50]. It seems likely that many of these DNA-binding proteins will be found to either bend or increase the flexibility of DNA. Thus, with 15 or 20 proteins binding, bending and flexing the DNA within a 465 bp promoter region, a complex nucleoprotein structure may be formed, stabilized by specific protein-DNA and protein-protein interactions, that is crucial for proper hormonal regulation of the PEPCK gene.

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