Differential modulation of transcriptional activity of oestrogen receptors by direct protein–protein interactions with retinoid receptors

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Control of oestradiol-responsive gene regulation by oestrogen receptors (ERs) may involve complex cross-talk with retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Recently, we have shown that ERα directly interacts with RARα and RXRα through their ligand binding domains (LBDs). In the present work, we extend these results by showing that ERβ binds similarly to RARα and RXRα but not to the glucocorticoid receptor, as demonstrated by the yeast two-hybrid tests and glutathione S-transferase pull-down assays. These direct interactions were also demonstrated in gel-shift assays, in which the oestrogen response element (ERE) binding by ERα was enhanced by the RXRα LBD but was abolished by the RARα LBD. In addition, we showed that RARα and RXRα bound the ERE as efficiently as ERα, suggesting that competition for DNA binding may affect the transactivation function of the ER. In transient transfection experiments, co-expression of RARα or RXRα, along with ERα or ERβ, revealed differential modulation of the ERE-dependent transactivation, which was distinct from the results when each receptor alone was co-transfected. Importantly, when the LBD of RARα was co-expressed with ERα, transactivation of ERα on the ERE was repressed as efficiently as when wild-type RARα was co-expressed. Furthermore, liganded RARα or unliganded RXRα enhanced the ERα transactivation, suggesting the formation of transcriptionally active heterodimer complexes between the ER and retinoid receptors. Taken together, these results suggest that direct protein–protein interactions may play major roles in the determination of the biological consequences of cross-talk between ERs and RARα or RXRα.

INTRODUCTION

Oestrogen plays an important role in a large array of biological processes such as development, reproduction and sexual differentiation [1,2]. Perturbations of oestrogen signal transduction have been suggested to contribute to tumour progression and eventual development of an hormone-independent and more aggressive phenotype in human breast cancer. Retinoids have been shown to effectively restrict oestrogen effects on breast cancer cells *in vitro* and *in vivo* [3,4]. Interestingly, the responses to all-*trans*-retinoic acid (RA) appeared to correlate with the expression of the oestrogen receptor (ER) [5–8], suggesting potential cross-talk between ER and retinoic acid receptors (RARs) and/or retinoid X receptors (RXRs).

The ER, RARs and RXRs belong to the nuclear hormone receptor superfamily, a group of transcriptional regulatory proteins linked by conserved structure and function [9,10]. The receptors are direct regulators of transcription that function by binding to specific DNA sequences in promoters of target genes called hormone response elements. The conventional oestrogen response elements (EREs) consist of inverted repeats of a common half-site [5′-(A/G)GGTCA-3′] spaced by three nucleotides, whereas retinoic acid response elements (RAREs) are comprised of direct repeats of the same half-site spaced by two or five nucleotides [10–12]. Whereas, ER apparently binds EREs only as homodimers, RARs bind RAREs with high affinity as heterodimers with RXRs [11–13]. Based on this high affinity binding, the RAR/RXR heterodimers have been considered to be the functionally active forms of these receptors *in vivo* [13–15]. RXRs also heterodimerize with many other nuclear receptors including thyroid hormone receptors, vitamin D receptor and peroxisome proliferator activated receptor [13–16]. In addition, thyroid hormone receptor, peroxisome proliferator activated receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF) and small heterodimer partner have been reported to form heterodimers with other receptors [11,16–20]. Although heterodimerization with RXR usually results in enhanced transactivation, COUP-TF and short heterodimer partner seem to function primarily as transcriptional repressors [20–22]. Heterodimer formation could generate an extremely diverse group of receptors, particularly considering the fact that multiple genes and multiple isoforms exist, and their expression depends on cell type and the stage of growth and development. Thus the protein–protein interaction between nuclear receptors could mediate a wide range of cross-talk, so generating a significant diversity in gene regulation.

We have recently discovered that the ER directly interacts with RARα and RXRα through their ligand-binding domains (LBDs) [23]. In the present study, we extend these results by showing that the recently described ER isotype, ERβ [24], which resulted in the renaming of the conventional ER as ERα, also shows similar binding to RARα and RXRα. These interactions were demonstrated in gel-shift assays as well as co-transfection assays, in which expression of RARα or RXRα, along with ERα or ERβ, revealed differential modulation of the ERE-dependent transactivation, distinct from the results when each receptor alone was co-transfected. Thus we concluded that direct protein–protein interactions play major roles in the cross-talk between ERs and RARα or RXRα.

Abbreviations used: RA, retinoic acid; ER, oestrogen receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; ERE, oestrogen response element; RARE, retinoic acid response element; GR, glucocorticoid receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; LBD, ligand binding domain; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase.

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MATERIALS AND METHODS

Hormones

All-*trans*-RA, 9-*cis*-RA and oestradiol were purchased from Sigma (St. Louis, MO, U.S.A.). Retinoids (10 mM) and oestradiol (1 mM) stock solutions were made in DMSO/ethanol (1:1, v/v) and ethanol respectively, and were maintained at −20 °C. Further dilutions were made in cell culture medium or in appropriate buffer before use.

Plasmids

The receptor expression plasmids, pECE-ERα, pECE-RARα, pECE-RXRα and a C-terminal deletion mutant of RXRα (RXR$\alpha\Delta$AF2) were as described previously [11,25–27]. To ensure optimal detection of oestradiol-induced ERα activity, an ERα-Val expression vector [28] was used in the experiments. pECE-ERβ [24] was constructed by inserting *Eco*RI and *Sal*I fragments of pCMX-ERβ into the corresponding restriction sites of pECE. RARα-LBD was constructed by inserting a *Bam*HI and *Eco*RI fragment from pECE-RARα into the corresponding restriction sites of pECE. The ERE-*tk*-chloramphenicol acetyltransferase (CAT) as well as the RARE reporter constructs (i.e., TREpal-*tk*-CAT, CRBPI-*tk*-CAT, and βRARE-*tk*-CAT) were as described previously [27,29–31].

Yeast two-hybrid assay

EGY48 cells, the lexA–β-gal (β-galactosidase) reporter construct, the LexA- and B42-parental vectors were as reported previously [23]. B42 fusions to the LBDs of the glucocorticoid receptor (GR), RARα, RXRα-LBD and ERβ were as described previously [23]. The mouse ERβ [24] was subcloned into *Eco*RI and *Sal*I sites of the LexA vector by PCR using *Eco*RI and *Xho*I-site-bearing primers to construct LexA/ERβ. The co-transformation and β-gal expression assays in yeast were performed as described previously [23]. For each experiment, at least six separate transformants from each transformation were transferred to indicator plates containing X-gal.

Glutathione S-transferase (GST) pull-down assays

GST fusion proteins were produced in *Escherichia coli* and purified using glutathione-Sepharose affinity chromatography essentially as described previously [23]. GST proteins were bound to glutathione-Sepharose 4B beads (Pharmacia, NJ, U.S.A.) in binding buffer [50 mM KPO_4, pH 6.0/100 mM KCl/10 mM $MgCl_2$/10 % (v/v) glycerol containing 10 mg/ml *E. coli* extract and 0.1 % (v/v) Tween 20]. Beads were washed once with binding buffer and incubated for 60 min at 4 °C in the same buffer with equivalent amounts of various proteins labelled with [^{35}S]methionine by *in vitro* translation. Non-bound proteins were removed by three washes with binding buffer without *E. coli* extract, and specifically bound proteins were eluted with 50 mM GSH in 0.5 M Tris/HCl, pH 8.0. Eluted proteins were resolved by PAGE and visualized by fluorography.

Gel-shift assay

Receptor proteins synthesized *in vitro* were incubated with ^{32}P-labelled oligonucleotides in a reaction mixture (20 μl) containing 10 mM Hepes, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 10 % (v/v) glycerol and 1 μg of poly(dI-dC) at 25 °C for 20 min. The reaction mixtures were then applied to a non-denaturating polyacrylamide (5 %) gel containing 0.5 × TBE (1 × TBE = 0.089 M Tris/borate/2 mM EDTA). The oligonucleotide used as a probe encoding the consensus ERE sequences was: (5′-TCAGGTCACTGTGACCTGA-3′). The oligonucleotides were labelled by Klenow fragments of DNA polymerase and the labelled oligonucleotides were purified by gel electrophoresis [25].

Tissue culture and transient-transfection assays

CV-1 and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium and minimal essential medium respectively, supplemented with 10 % (v/v) fetal-calf serum in a CO_2 incubator. A modified calcium phosphate precipitation procedure was used for transient transfection [25,26]. Briefly, CV-1 cells (50000 cells/well) were seeded in a 24-well culture plate and reporter plasmid (100 ng), β-gal expression vector (150 ng) and various combinations of receptor expression vectors were mixed with carrier DNA (pBluescript) to a total of 1 μg of DNA/well. MCF-7 cells (125000 cells/well) were seeded in a 12-well culture plate and reporter plasmid (250 ng) and β-gal expression vector (300 ng) were mixed with pBluescript to a total of 2.5 μg of DNA/well. After 24 h of hormone treatment, CAT activity was determined [25,26] (c.p.m., normalized for transfection efficiency by the corresponding β-gal activity, were expressed as relative CAT activity). For statistical analysis, one-way analysis of variance with the Tukey-Kramer multiple comparisons test was performed using GraphPad Instat® (GraphPad Software, San Diego, CA, U.S.A.). A value of $P < 0.05$ was considered statistically significant.

RESULTS

ERβ directly interacts with RARα and RXRα

We have shown recently that the ERα interacted with a subset of nuclear receptors including RARα and RXRα [23]. These results led us to examine whether the recently described isotype of ER, ERβ [24,32,33], also interacted with these receptors. As shown in Table 1, a LexA fusion to ERβ alone was transcriptionally inert. However, co-expression of B42 fusions to the LBD of RXRα (B42/RXR-LBD) and RARα (B42/RARα), but not to the LBD of GR (B42/GR-LBD), led to significant activation of *LacZ* reporter gene expression, suggesting that ERβ specifically interacted with RARα and RXRα. Interestingly, the LexA/ERβ-mediated transactivations with co-expression of B42/RXR-LBD or B42/RARα were stimulated in the presence of both oestradiol

Table 1 Interactions between ERβ and retinoid receptors in yeast

The indicated B42 and LexA plasmids were transformed into a yeast strain containing an appropriate *LacZ* reporter gene. At least six separate transformants from each transformation were transferred to indicator plates containing X-gal, and reproducible results were obtained using colonies from a separate transformation. + + +, intense blue colonies after 2 days of incubation indicating strong interaction; + +, light blue colonies after 2 days of incubation indicating intermediate interaction; +, light blue colonies after more than 2 days of incubation indicating weak interaction; −, white colonies indicating no interaction; n.d., not determined.

	LexA/-		LexA/ERβ		
Ligand ...	None	None	Oestradiol	9-*cis*-RA	Oestradiol + 9-*cis*-RA
B42/-	−	−	−	−	−
B42/GR-LBD	−	−	−	n.d.	n.d.
B42/RXRα-LBD	−	+	+	+	+ +
B42/RARα	−	+	+	+	+ +
B42/ERβ	−	+ +	+ + +	n.d.	n.d.

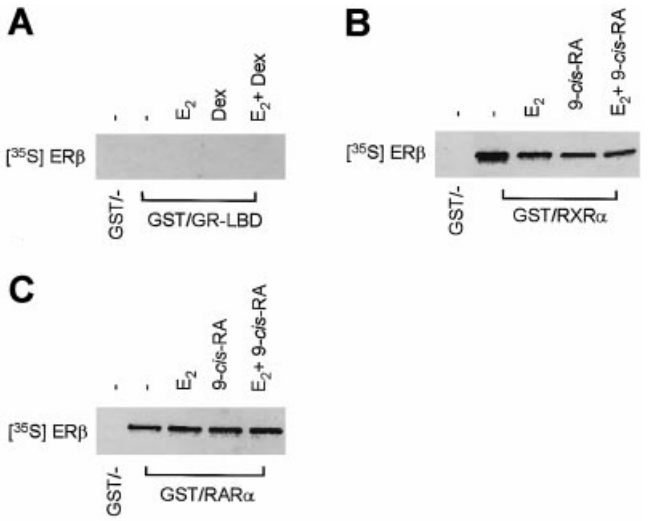

Figure 1 Analysis of the interactions of ERβ and retinoid receptors by GST pull-down assays

ERβ labelled with [35S]methionine by *in vitro* translation were incubated with glutathione-Sepharose beads containing GST alone (GST/—), GST fusions to GR-LBD (**A**), RXRα (**B**) or RARα (**C**). Beads were washed and the specifically bound material was eluted with GSH and resolved by SDS/PAGE. Oestradiol (E_2) and/or dexamethasone (Dex) (10^{-7} M) or 9-*cis*-RA (10^{-7} M) were added as indicated, —, denotes no hormone added.

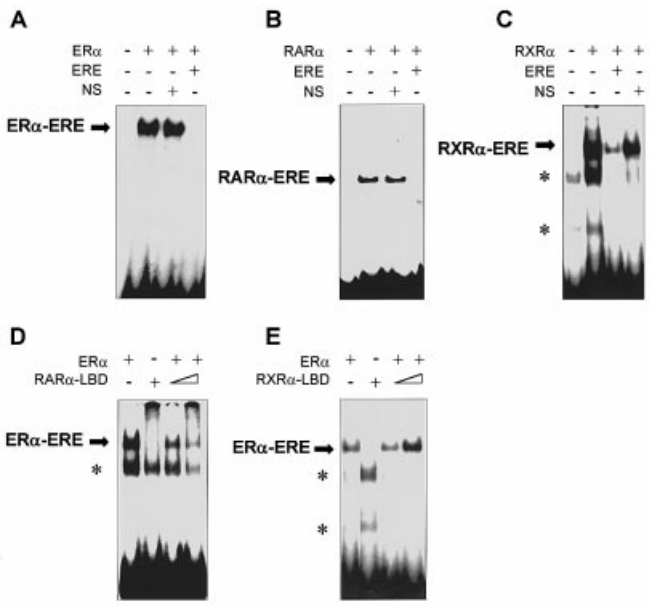

Figure 2 Gel-shift assays to study interactions of ERα with RARα and RXRα

ERα, translated *in vitro*, was incubated alone or together with purified GST-fusion proteins to the LBD of RARα or RXRα, as indicated. Reticulocyte lysate (2 μl) containing ERα, RARα or RXRα, 0.2 μl of the purified RARα-LBD or RXRα-LBD alone, or 2 μl of ERα together with 0.2 μl or 1 μl of RARα-LBD or RXRα-LBD were used for the assays, as indicated. The reaction mixtures were incubated with 32P-labelled ERE and analysed by gel-shift assay. ERE and NS indicate excess of unlabelled ERE and non-specific oligonucleotide respectively. Asterisks indicate non-specific binding.

and 9-*cis*-RA. This stimulation, however, was abolished when a B42 fusion to a mutant RXR deleted for the AF2 domain [27] was used (results not shown), indicating that it reflected ligand-dependent transcriptional stimulation of the AF2 domain and not ligand-dependent enhancement of the protein–protein interactions. These ligand-independent interactions were further confirmed *in vitro* using GST pull-down assays (Figure 1). GST fusions to the LBD of the GR as well as full-length RARα and RXRα were expressed, purified and tested for interaction with a full-length rat ERβ translated *in vitro* or luciferase labelled with [35S]methionine. As expected, luciferase was unable to interact with any of the GST proteins (results not shown). However, ERβ readily interacted with GST–RARα and GST–RXRα in a ligand-independent manner (Figure 1). ERβ did not interact with GST alone or with GST–GR-LBD, as expected. Taken together, we concluded that ERβ constitutively binds to RARα and RXRα through direct protein–protein interactions.

Interactions of ERα with RARα and RXRα using gel-shift assays

Direct protein–protein interactions between ERα and retinoid receptors were further examined in gel-shift assays by using a 32P-labelled oligonucleotide encoding consensus ERE sequences and *in vitro* translated ERα, RARα and RXRα as well as bacterially expressed and purified GST-fusion proteins to various RARα and RXRα constructs. RARα or RXRα alone bound ERE as efficiently as ERα (Figure 2). The specificity of each binding complex was also confirmed with displacement by excess unlabelled ERE and super-shifting by ERα- or GST-specific antibodies (Figure 2; and results not shown). As shown in Figures 2(D) and 2(E), the ERα–ERE binding was inhibited by the LBD of RARα (RARα-LBD) in a dose-dependent manner, whereas it was specifically enhanced by the LBD of RXRα (RXRα-LBD). Similar results were also obtained with LBDs of RARα or RXRα translated *in vitro*, which were not fused to GST (results not shown). These results demonstrate that the direct interactions of ERα with RARα or RXRα through the LBDs are capable of affecting the ER–ERE interactions at least *in vitro*. Despite extensive efforts, however, we were not able to find any intermediate heterodimer bindings when ERα was mixed with either a full-length RARα or RXRα together (results not shown).

Co-expression of RARα modulates ERα-mediated transactivation

For transient transfection experiments, we employed an ERE-*tk*-CAT reporter construct that contained the consensus ERE sequences from the vitallogenin A2 gene [28]. The reporter itself was not activated by either 9-*cis*-RA or oestradiol in CV-1 cells without co-transfection of receptors. As expected, co-transfection of CV-1 cells with ERα alone led to an approx. 8-fold activation of the reporter gene expression by oestradiol (Figure 3A). Co-transfection of CV-1 cells with RARα alone also led to approx. 4-fold activation of the reporter gene expression by 9-*cis*-RA, consistent with the ERE binding of RARα in gel-shift assays. Higher doses of ERα or RARα alone did not induce further induction of the reporter gene activity (results not shown). The -fold activation of RARα on ERE induced by 9-*cis*-RA was approx. 50% of that of ERα induced by oestradiol. When increasing amounts of RARα were co-expressed, the oestradiol-dependent transactivation of ERα was significantly inhibited in a dose-dependent manner (Figure 3A). However, the oestradiol-dependent transactivation by ERα was significantly stimulated when 9-*cis*-RA was present. With 50 ng of liganded-RARα co-transfected, the oestradiol-dependent transcription level was stimulated approx. 2.5-fold relative to the level obtained with ERα alone. It is notable that this level was equal to approx. 600% of the 9-*cis*-RA-induced transactivation of RARα on

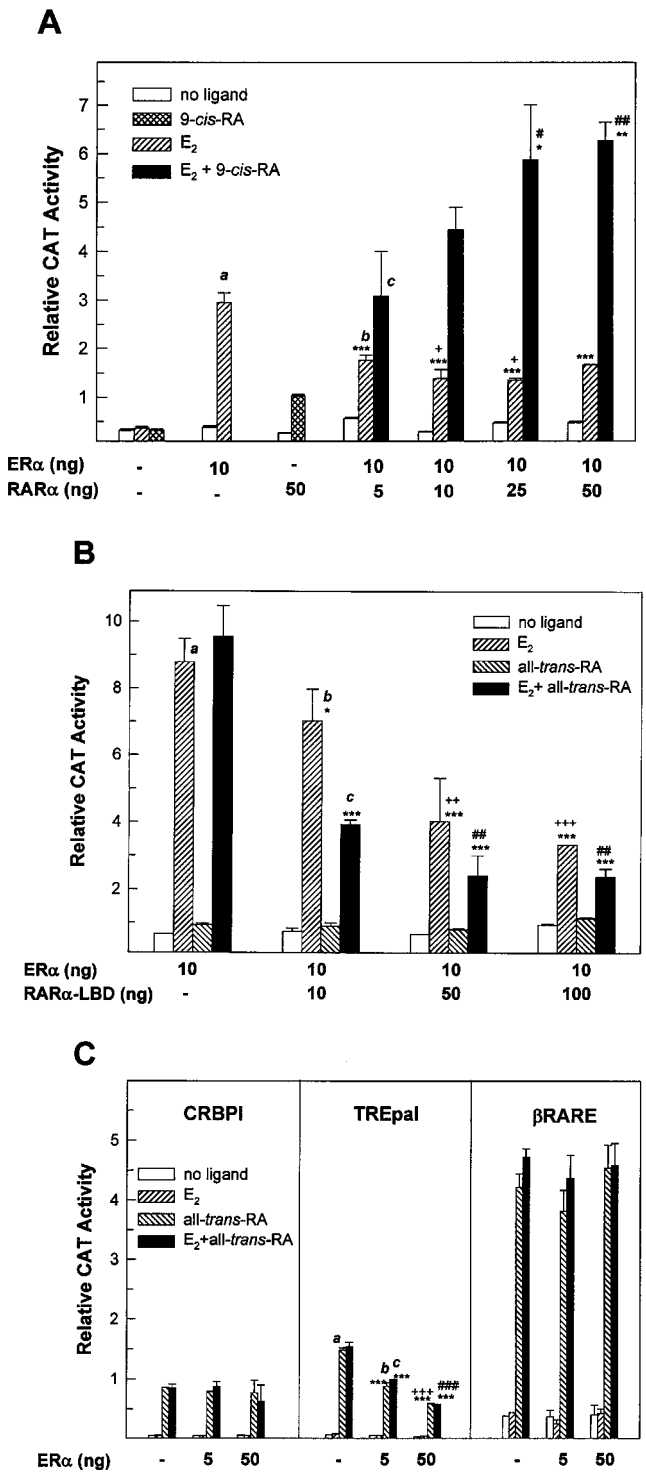

Figure 3 RARα and all-*trans*-RA modulated transcriptional activity of ERα in CV-1 cells

ERE-*tk*-CAT (0.1 μg), TRE-*tk*-CAT (0.1 μg), CRBPI-*tk*-CAT (0.1 μg) and βRARE-*tk*-CAT (0.1 μg), were co-transfected with the indicated combinations of receptor expression vectors into CV-1 cells as described in the Materials and methods section. (**A**) ERE-*tk*-CAT with pECE-ERα and/or pECE-RARα. Transfected cells were treated with oestradiol (10^{-8} M) (E_2), 9-*cis*-RA (10^{-7} M) or both, as indicated, and assayed 24 h later for CAT activity. Co-transfection of RARα inhibited ERα activity on the ERE. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ compared with *a*. +, $P < 0.05$ compared with *b*. ##, $P < 0.01$; #, $P < 0.05$ compared with *c*. (**B**) ERE-*tk*-CAT with pECE-ERα and/or pECE-RARα-LBD. Transfected cells were treated with oestradiol (10^{-8} M) (E_2), all-*trans*-RA (10^{-7} M) or both, as indicated, and assayed 24 h later for CAT activity. Co-transfection of RARα-LBD inhibited ERα activity on the ERE. ***, $P <$ 0.001 and *, $P < 0.05$ compared with *a*. +++, $P < 0.001$ and ++, $P < 0.01$ compared with *b*. ##, $P < 0.01$ compared with *c*. (**C**) CRBPI, TREpal, βRARE with ERα. Transfected cells were treated with oestradiol (10^{-8} M) (E_2), all-*trans*-RA (10^{-7} M) or both, as indicated, and assayed 24 h later for CAT activity. ERα differentially modulated transcriptional activity of RARα on RAREs. ***, $P < 0.001$, +++, $P < 0.001$, and ###, $P < 0.001$ compared with *a*, *b* and *c* respectively. The results, in each case, are the means ± S.E.M. ($n = 4$).

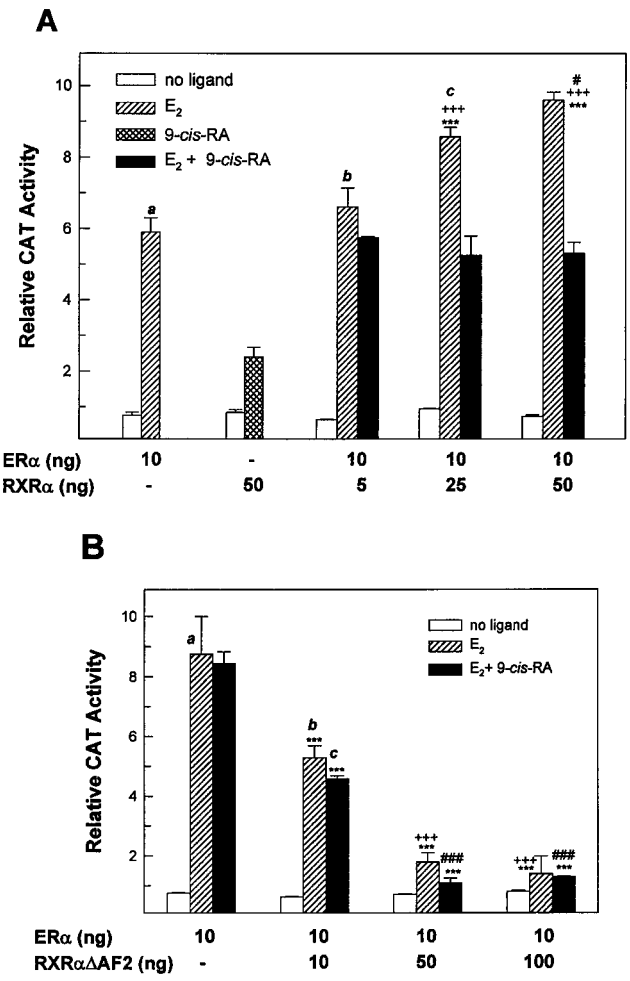

Figure 4 RXRα and 9-*cis*-RA modulated transcriptional activity of ERα in CV-1 cells

(**A**) ERE-*tk*-CAT (0.1 μg) was co-transfected with pECE-ERα (10 ng) in the presence of the indicated amounts of RXRα expression vector (pECE-RXRα) into CV-1 cells as described in the Materials and methods section. Transfected cells were treated with oestradiol (10^{-8} M) (E_2), 9-*cis*-RA (10^{-7} M) or both and assayed 24 h later for CAT activity. Co-transfection of RXRα enhanced ERα activity on ERE. ***, $P < 0.001$, +++, $P < 0.001$, and #, $P < 0.05$ compared with *a*, *b* and *c* respectively. (**B**) ERE-*tk*-CAT (0.1 μg) was co-transfected with pECE-ERα (10 ng) in the presence of the indicated amounts of pCMX-RXRαΔAF2 into CV-1 cells as described in the Materials and methods section. Transfected cells were treated with oestradiol (10^{-8} M) (E_2), 9-*cis*-RA (10^{-7} M) or both and assayed 24 h later for CAT activity. Co-transfection of RXRαΔAF2 inhibited ERα activity on ERE. ***, $P < 0.001$, +++, $P <$ 0.001, and ###, $P < 0.001$ compared with *a*, *b* and *c* respectively. The results are the means ± S.E.M. ($n = 4$).

ERE, without ERα co-transfected. Interestingly, co-expression of the RARα-LBD that contains the protein–protein-interaction domain with other nuclear receptors did not show this synergy with ERα but repressed the reporter activity as efficiently as the wild-type RARα (Figure 3B). These results indicate that RARα-

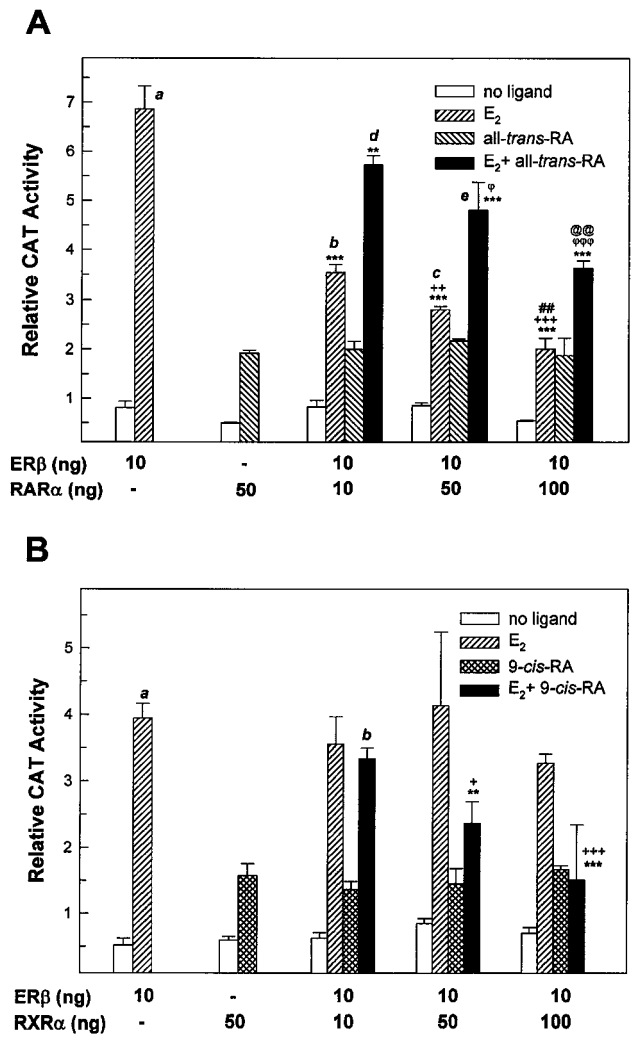

Figure 5 Modulation of transcriptional activity of ERβ induced by RARα (A) or RXRα (B)

ERE-*tk*-CAT (0.1 μg) was co-transfected with the ERβ expression vector (pECE-ERβ, 10 ng), in the presence of the indicated amounts of (**A**) pECE-RARα or (**B**) pECE-RXRα, into CV-1 cells as described in the Materials and methods section. Transfected cells were treated with (**A**) oestradiol (10^{-8} M) (E_2), all-*trans*-RA (10^{-7} M) or both or (**B**) oestradiol (10^{-8} M) (E_2), 9-*cis*-RA or both, and assayed 24 h later for CAT activity. In (**A**), ***, $P < 0.001$ and **, $P < 0.01$ compared with *a*. + + +, $P < 0.001$ and + +, $P < 0.01$ compared with *b*. ##, $P < 0.01$ compared with *c*. φφφ, $P < 0.001$ and φ, $P < 0.05$ compared with *d*. @@, $P < 0.01$ compared with *e*. In (**B**), ***, $P < 0.001$ and **, $P < 0.01$ compared with *a*. + + +, $P < 0.001$ and +, $P < 0.05$ compared with *b*. The results are the means ± S.E.M. ($n = 4$).

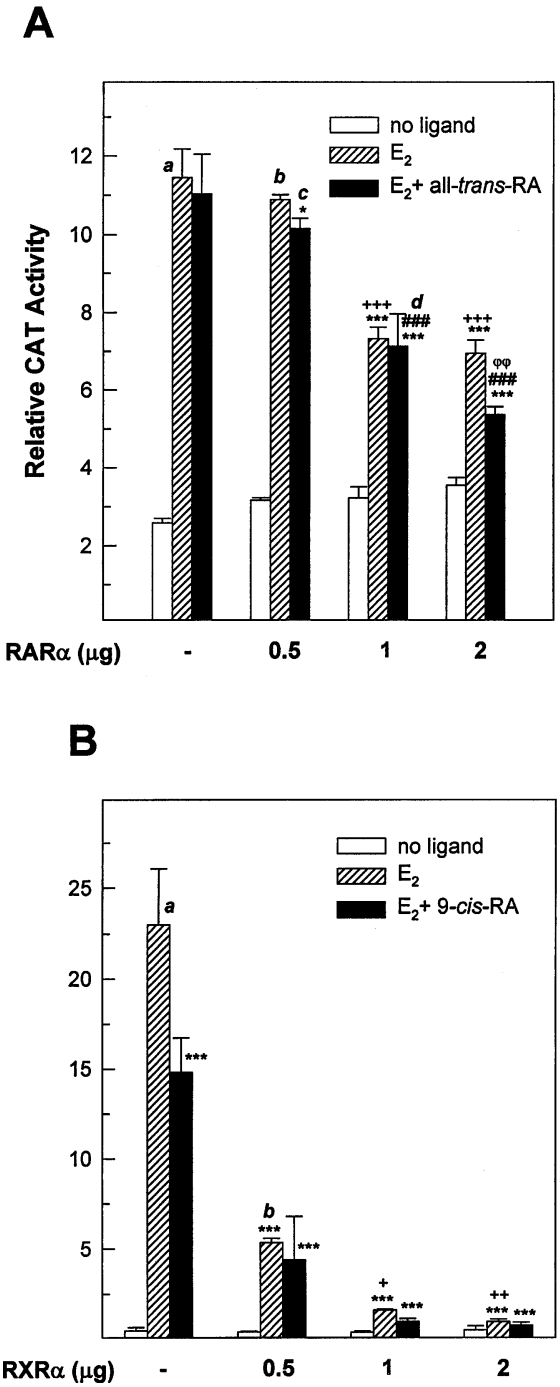

Figure 6 RARα (A) and RXRα (B) repressed transcriptional activity of endogenous ERα present in MCF-7 cells

ERE-*tk*-CAT (0.25 μg) was co-transfected with pECE-RARα or (**B**) pECE-RXRα into MCF-7 cells as described in the Materials and methods section. Transfected cells were treated with oestradiol (10^{-8} M) (E_2) alone or with (**A**) all-*trans*-RA (10^{-7} M) or (**B**) 9-*cis*-RA and assayed 24 h later for CAT activity. In (**A**), ***, $P < 0.001$ and *, $P < 0.05$ compared with *a*. + + +, $P < 0.001$; ###, $P < 0.001$; φφ, $P < 0.01$ compared with *b*, *c* and *d* respectively. In (**B**), ***, $P < 0.001$ compared with *a*. + +, $P < 0.01$ and +, $P < 0.05$ compared with *b*. The results are the means ± S.E.M. ($n = 4$).

LBD may be sufficient to exert the negative effects on the ER activity, whereas the synergy obtained with liganded RARα may require non-LBD domains of RARα. Overall, these results suggest that the direct protein–protein interactions described above (Table 1, Figures 1 and 2) appear to play major roles in the modulation of ERα activity by RARα.

If the inhibition of ERα activity by unliganded-RARα was through direct physical interactions, inhibition of the RAR-mediated transactivation by ERα could also be expected. As shown in Figure 3(C), ERα itself did not affect the RARE reporter gene activities in the absence or presence of oestradiol. Co-transfection of increasing amounts of ERα, however, significantly inhibited the all-*trans*-RA-dependent activation of the TREpal-*tk*-CAT reporter constructs. In contrast, the CRBPI-*tk*-CAT and βRARE-*tk*-CAT gene expressions were not significantly affected. The inhibition may not be due to competitive

DNA binding, since ERα did not bind or transactivate the response element ([11], and results not shown). These results suggest that the direct interactions between ERα and RARα are also capable of modulating the RARα activities, with a subset of RAREs at least.

Co-expression of RXRα modulates ERα-mediated transactivation

Co-transfection of CV-1 cells with RXRα alone led to approx. 3-fold activation of the reporter gene expression by 9-*cis*-RA, consistent with the ERE binding of RXRα in gel-shift assays. This 9-*cis*-RA-induced level of transcription with RXRα on ERE was equal to approx. 40 % of the oestradiol-induced transcription of ERα on ERE. When increasing amounts of unliganded RXRα was co-expressed, the oestradiol-dependent transactivation of ERα was enhanced in a dose-dependent manner (Figure 4A), consistent with the direct protein–protein interactions described above (Table 1, Figures 1 and 2). To test whether the AF2 transactivation function of RXRα was required for this synergy between liganded ERα and unliganded RXRα, we used a mutant RXR specifically impaired for the AF2 domain, RXRΔAF2, described previously [32]. RXRΔAF2 significantly reduced the transcriptional activity of ERα in either the presence or absence of 9-*cis*-RA (Figure 4B), indicating that the AF2 domain of RXR is required for the synergistic activation of liganded ERα and unliganded RXRα.

Modulations of the ERβ-mediated transactivation by RARα and RXRα

ERβ has similarities to the ERα in its structure and function [24,32,33]. However, tissue distribution of the receptors is not identical, although in some tissues it appears to overlap [33]. We tested whether the transactivation function of ERβ was similarly regulated by co-transfected RARα or RXRα. As shown in Figures 5(A) and 5(B), co-expression of RARα or RXRα repressed the transcriptional activity of ERβ in a ligand-independent manner. The transcriptional synergies observed between liganded ERα and liganded RARα or unliganded RXRα were not observed with ERβ, suggesting that transcriptional properties of the potential heterocomplexes of ERβ with RARα or RXRα are different from those of the ERα heterocomplexes.

Co-transfection of RARα and RXRα inhibited transactivations of endogenous ER in MCF-7 cells

We examined whether the retinoid receptors modulate transactivation of endogenous ERα in a hormone-dependent and ER-positive human breast carcinoma cell line, MCF-7. Both RARα and RXRα inhibited the endogenous ER-activity ligand independently (Figures 6A and 6B), which was primarily in agreement with other observations [5,34]. However these results were in marked contrast with the results that were obtained in CV-1 cells (see Figures 3 and 4), suggesting that the interaction of the retinoid receptors and ERα may involve other cellular factor(s), such as co-activators and co-repressors distributed among different cell types. Such factors are at present unknown but may be important cellular determinants that mediate cross-talk between retinoids and oestrogen in a cell-type-specific manner. These findings are in agreement with the anti-proliferative effects of retinoids on ER-positive, hormone-dependent breast cancer cells.

DISCUSSION

Recently, it has become apparent that a complex network of interaction exists among nuclear receptor superfamily members. In particular, complexities of the regulation of oestrogen-responsive gene expression implicate not only ER and ERE but also other transcriptional factors and DNA binding motifs [35–37]. The potential cross-talk between ER and RAR–RXR had been suspected, since retinoids effectively restricted oestrogen effects on human breast cancer cells *in vitro* and *in vivo* [3,4] and the responses to all-*trans*-RA appeared to correlate with expression of ER [5–8].

Consistently with these results, we clearly demonstrated that ERβ directly interacted with retinoid receptors using the yeast two-hybrid and GST pull-down assays (Table 1 and Figure 2). These results extend those of other reports in which ERα was shown to act as a common interaction partner for a subset of nuclear receptors including oestrogen receptor-related receptor 1, COUP-TF, RARα and RXRα [23,38–40]. However, we were not able to find any intermediate heterodimer binding to ERE between ERα and retinoid receptors in gel-shift assays. Similarly, heterodimers of COUP-TF–ER [41], oestrogen receptor-related receptor 1–ER [42] and short heterodimer partner-ER [42a], readily detected in solution, were not observed to bind EREs in gel-shift assays. In parallel, two recent reports showed interaction of COUP-TF with orphan receptors nur77 [17] and HNF4 [18] only in solution but not when bound to DNA. These results are consistent with the possibility that the ER–retinoid receptors may bind EREs as a heterodimer or a higher-order complex, which are not stable enough to sustain in gel-shift assays. In addition, we showed that RARα and RXRα bound ERE as efficiently as ERα in the conditions employed, suggesting that competitive DNA binding of retinoid receptors to ERE may participate in the modulation of transactivation of ERα.

Our transient transfection data clearly demonstrate that the direct protein–protein interactions of ER with retinoid receptors play major roles in the cross-talk between ER and retinoid receptors. As shown in Figures 3 and 4, co-transfection of RARα or RXRα modulated the transactivation potential of ERα in a negative or positive manner. The negative effects of unliganded RARα on the transcriptional activation of ERα may reflect either competitive binding of the retinoid receptors to ERE (Figure 2) or the intrinsically lower transactivation potential of the putative heterocomplex of ERα–RARα. However, the latter possibility is strongly suggested by the results in which co-transfection of only the LBD of RARα was as efficient as wild-type RARα in repressing ERα activity (Figure 1C), and an excess of RARα-LBD partially abolished the binding of ERα in gel-shift assays (Figure 3). In addition, 9-*cis*-RA has been reported to induce squelching of RXR from transcriptionally active heterodimers, such as RAR–RXR and thyroid hormone receptor–RXR, into RXR homodimers, thus repressing the transcriptional activity of such heterodimers [43]. Therefore, the repression of transcriptional activity of the putative ERα–RXRα heterocomplex by 9-*cis*-RA may have resulted from this 9-*cis*-RA-induced homodimerization of RXRα. Importantly, either liganded RARα or unliganded RXRα enhanced the ERα transactivation by oestradiol (Figures 3 and 4). These synergistic effects are an excellent demonstration of the formation of transcriptionally active heterodimer complexes between ER and retinoid receptors on ERE.

Recent studies suggested that transcriptional regulation by nuclear receptors is controlled by selective recruitment of co-activators in response to ligand [44–47], whereas the co-repressors, such as the silencing mediator for retinoid and thyroid receptors (SMRT) and the nuclear receptor co-repressor (N-CoR), selectively interact with unliganded receptors [48,49]. Selective recruitment of either co-activators or co-repressors, depending on the status of ligand binding, may determine the

transactivation potential of each of the putative ER–RARα or ER–RXRα heterocomplexes. For instance, while unliganded RXRα enhanced ERα activity on ERE reporter gene activity, RXR$\alpha\Delta$AF2, a mutant lacking the AF2 function, significantly repressed the ERα activity (Figure 2C), suggesting that transcriptional co-activators that act through the AF2 domain could be involved in the transcriptional activation of the ERα–RXRα heterocomplex. Furthermore, many of the known transcription cofactors are expressed with tissue- and cell-type specificity *in vivo*. Thus such factors may act as a major determinant in the differential control of cross-talk between ER and retinoid receptors in different cell types. Consistently with this, amplification and overexpression of AIB1, a member of the SRC-1 family, has been observed recently in ER-positive breast and ovarian cancer cells [50]. In addition, unliganded RXRα enhanced ERα activity in CV-1 cells, whereas it was inhibitory in MCF-7 cells (Figures 3, 4 and 6), suggesting the presence of putative intracellular factors which are expressed differentially in these two cell types.

In conclusion, we have shown that direct protein–protein interactions between ER and RARα–RXRα may play major roles in the cross-talk between these receptors. Our results have provided a potential mechanism by which retinoids contribute to the normal physiological modulation of oestrogen function, and by which the alteration of molecular interactions leads to changes in biological responses to oestrogen and retinoids. The physiological and pharmacological implications of the cross-talk between ER and RARα–RXRα described in this work may provide a potential target for development of therapeutics and/or preventive agents against breast cancer.

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REFERENCES

1 Korach, K. S. (1994) Science **266**, 1524–1527
2 Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S. and Smithies, O. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 11162–11166
3 Costa, A. (1993) Eur. J. Cancer **29A**, 589–592
4 Moon, R. C. and Metha, R. G. (1990) Basic Life Sci. **52**, 213–224
5 Demirpence, E., Balaguer, P., Trousse, F., Niclas, J., Pons, M. and Gagne, D. (1994) Cancer Res. **54**, 1458–1464
6 Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C. and Zhang, X.-K. (1995) Mol. Cell. Biol. **16**, 1138–1149
7 Lacroix, A. and Lippman, M. E. (1980) J. Clin. Invest. **65**, 586–591
8 van der Burg, B., van der Leede, B. M., Kwakkenbos-Isbrucker, L., Salverda, S., de Laat, S. W. and van der Saag, P. T. (1993) Mol. Cell. Endocrinol. **91**, 149–157
9 Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995) Cell **83**, 835–839
10 Pfahl, M., Apfel, R., Bendik, I., Fanjul, A., Graupner, G., Lee, M.-O., La-vista, N., Lu, X.-P., Piedrafita, J., Ortiz, M. A. et al. (1994) Vit. Horm. **49**, 327–381
11 Zhang, X.-K., Hoffmann, B., Tran, P., Graupner, G. and Pfahl, M. (1992) Nature (London) **355**, 441–446
12 Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. and Chambon, P. (1992) Cell **68**, 377–395
13 Kliewer, S. A., Umesono, K, Mangelsdorf, D. J. and Evans, R. M. (1992) Nature (London) **355**, 446–449
14 Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K. and Rosenfeld, M. G. (1991) Cell **65**, 1251–1266
15 Bugge, T. H., Pohl, J., Lonnoy, O. and Stunnenberg, H. G. (1992) EMBO J. **11**, 1409–1418
16 Schrader, M., Muller, K. M., Nayeri, S., Kahlen, J. P. and Carlberg, C. (1994) Nature (London) **370**, 382–386
17 Wu, Q., Li, Y., Liu, R., Agadir, A., Lee, M. O., Liu, Y. and Zhang, X. (1997) EMBO J. **16**, 1656–1669
18 Ktistaki, E. and Talianidis, I. (1997) Mol. Cell. Biol. **17**, 2790–2797
19 Bogazzi, F., Hudson, L. D. and Nikodem, V. M. (1994) J. Biol. Chem. **269**, 11683–11686
20 Seol, W., Choi, H.-S. and Moore, D. D. (1996) Science **272**, 1336–1339
21 Cooney, A. J., Tsai, S. Y., O'Malley, B. W. and Tsai, M.-J. (1992) Mol. Cell. Biol. **12**, 4153–4163
22 Tran, P. B. V., Zhang, X-K., Salbert, G., Hermann, T., Lehmann, J. M. and Pfahl, M. (1992) Mol. Cell. Biol. **12**, 4666–4676
23 Lee, S.-K., Choi, H.-S., Song, M.-R., Lee, M.-O. and Lee, J. W. (1998) Mol. Endocrinol. **12**, 1184–1192
24 Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F. and Giguère, V. (1997) Mol. Endocrinol. **11**, 353–365
25 Lee, M.-O., Liu, Y. and Zhang, X.-K. (1995) Mol. Cell Biol. **15**, 4194–4207
26 Pfahl, M., Tzukerman, M., Zhang, X.-K., Helmann, J. M., Hermann, T., Wills, K. N. and Graupner, G. (1990) Methods Enzymol. **189**, 256–270
27 Zavacki, A. M., Lehmann, J. M., Seol, W., Willson, T. M., Kliewer, S. A. and Moore, D. D. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 7909–7914
28 Tzukerman, M., Zhang, X.-K., Hermann, T., Wills, K. N., Graupner, G. and Pfahl, M. (1990) New Biol. **2**, 613–620
29 Hoffman, B., Lehmann, J. M., Zhang, X.-K., Hermann, T., Graupner, G. and Pfahl, M. (1990) Mol. Endocrinol. **4**, 1734–1743
30 Rottman, J. N., Windom, R. L., Nadal-Ginard, B., Mahdavi, V. and Karathanasis, S. K. (1991) Mol. Cell. Biol. **11**, 3814–3820
31 Husmann, M., Hoffman, B., Stump, D. G., Chytil, F. and Pfahl, M. (1992) Biochem. Biophys. Res. Commun. **187**, 1558–1564
32 Pace, P., Taylor, J., Suntharalingam, S., Coombes, R. C. and Ali, S. (1997) J. Biol. Chem. **272**, 25832–25838
33 Kuiper, G., Carlsson, B., Grandien, K., Enmark, E., Hagglad, J., Nilsson, S. and Gustafsson, J. A. (1997) Endocrinology **138**, 863–870
34 Segars, J. H., Marks, M. S., Hirschfeld, S., Driggers, P. H., Martinez, E., Grippo, J. F., Wahli, W. and Ozato, K. (1993) Mol. Cell. Biol. **13**, 2258–2268
35 Gronemeyer, H. (1991) Annu. Rev. Genet. **25**, 89–123
36 Savouret, J.-F., Rauch, M., Redeuilh, G., Sokhavuth, S., Chauchereau, A., Woodruff, K., Parker, M. G. and Milgrom, E. (1994) J. Biol. Chem. **269**, 28955–28962
37 Scott, R. E. M., Wu-Peng, X. S., Yen, P. M., Chin, W. W. and Pfaff, D. W. (1997) Mol. Endocrinol. **11**, 1581–1592
38 Glass, C. K., Holloway, J. M., Devary, O. V. and Rosenfeld, M. G. (1988) Cell **54**, 313–323
39 Joyeux, A., Balaguer, P., Gagne, D. and Nicolas, J. C. (1996) J. Steroid Biochem. Mol. Biol. **58**, 507–515
40 Savouret, J.-F., Rauch, M., Redeuil, H. G., Sokhavuth, S., Chauchereau, A., Woodruff, K., Parker, M. G. and Milgrom, E. (1994) J. Biol. Chem. **269**, 28955–28962
41 Klinge, C. M., Silver, B. F., Driscoll, M. D., Sathya, G., Bambara, R. A. and Hilf, R. (1997) J. Biol. Chem. **272**, 31465–31474
42 Johnston, S. D., Liu, X., Zuo, F., Eisenbraun, T. L., Wiley, S. R., Kraus, R. J. and Mertz, J. E. (1997) Mol. Endocrinol. **11**, 342–352
42a Seol, W., Hanstein, B., Brown, M. and Moore, D. D. (1998) Mol. Endocrinol. **12**, 1551–1557
43 Lehmann, J. M., Zhang, X.-K., Graupner, G., Lee, M.-O., Hermann, T., Hoffmann, B. and Pfahl, M. (1993) Mol. Cell. Biol. **13**, 7698–7707
44 Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S. and Tung, L. (1996) Mol. Endocrinol. **10**, 1167–1177
45 Perlmann, T. and Evans, R. M. (1997) Cell **90**, 391–397
46 Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M.-J. and O'Malley, B. W. (1997) Nature (London) **389**, 194–198
47 Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M., Nakatani, Y. and Evans, R. M. (1997) Cell **90**, 569–580
48 Heinzel, T., Lavinsky, R. M., Mullen, T.-M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W.-M., Brard, G., Ngo, S. D., Davie, J. R. et al. (1997) Nature (London) **387**, 43–48
49 Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L. and Evans, R. M. (1997) Cell **89**, 373–380
50 Anzick, S. L., Konone, J., Walker, R. L., Azorsa, D. O., Tanner, J. J., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M. and Meltzer, P. S. (1997) Science **277**, 965–968

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