MOLECULAR AND CELLULAR BIOLOGY, Feb. 1995, p. 1034–1048 Vol. 15, No. 2
0270-7306/95/$04.00+0

A Dynamic Balance between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and Retinoic Acid Receptor:Retinoid X Receptor Heterodimers Regulates Oct-3/4 Expression in Embryonal Carcinoma Cells

ETTI BEN-SHUSHAN, HAVA SHARIR, ELI PIKARSKY, AND YEHUDIT BERGMAN*

The Hubert H. Humphrey Center for Experimental Medicine and Cancer Research, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Received 21 July 1994/Returned for modification 26 September 1994/Accepted 31 October 1994

The Oct-3/4 transcription factor is a member of the POU family of transcription factors and, as such, probably plays a crucial role in mammalian embryogenesis and differentiation. It is expressed in the earliest stages of embryogenesis and repressed in subsequent stages. Similarly, Oct-3/4 is expressed in embryonal carcinoma (EC) cells and is repressed in retinoic acid (RA)-differentiated EC cells. Previously we have shown that the Oct-3/4 promoter harbors an RA-responsive element, RAREoct, which functions in EC cells as a binding site for positive regulators of transcription and in RA-differentiated EC cells as a binding site for negative regulators. Our present results demonstrate that in P19 and RA-treated P19 cells, the orphan receptors ARP-1/COUP-TFII and EAR-3/COUP-TFI repress Oct-3/4 promoter activity through the RAREoct site in a dose-dependent manner. While the N-terminal region of the ARP-1/COUP-TFII receptor is dispensable for this repression, the C-terminal domain harbors the silencing region. Interestingly, three different RA receptor:retinoid X receptor (RAR:RXR) heterodimers, RARα:RXRα, RARβ:RXRα, and RARβ:RXRβ, specifically bind and activate Oct-3/4 promoter through the RAREoct site in a ligand-dependent manner. We have shown that antagonism between ARP-1/COUP-TFII or EAR-3/COUP-TFI and the RAR:RXR heterodimers and their intracellular balance modulate Oct-3/4 expression. Oct-3/4 transcriptional repression by the orphan receptors can be overcome by increasing amounts of RAR:RXR heterodimers. Conversely, activation of Oct-3/4 promoter by RAR:RXR heterodimers was completely abolished by EAR-3/COUP-TFI and by ARP-1/COUP-TFII. The orphan receptors bind the RAREoct site with a much higher affinity than the RAR:RXR heterodimers. This high binding affinity provides ARP-1/COUP-TFII and EAR-3/COUP-TFI with the ability to compete with and even displace RAR:RXR from the RAREoct site and subsequently to actively silence the Oct-3/4 promoter. We have shown that RA treatment of EC cells results in up-regulation of ARP-1/COUP-TFII and EAR-3/COUP-TFI expression. Most interestingly, in RA-treated EC cells, the kinetics of Oct-3/4 repression inversely correlates with the kinetics of ARP-1/COUP-TFII and EAR-3/COUP-TFI activation. These findings are in accordance with the suggestion that these orphan receptors participate in controlling a network of transcription factors, among which Oct-3/4 is included, which may establish the pattern of normal gene expression during development.

Embryonic development of multicellular organisms is thought to be controlled by sequential activation and repression of a complex cascade of regulatory genes. The POU domain family of transcription factors contains a divergent POU-type homeodomain and thus forms a subfamily of the homeodomain proteins. The POU transcription factors harbor the conserved POU-specific domain amino terminal of the homeodomain. These two domains are connected by a short variable linker region, and together they form the 160-amino-acid POU domain. POU domain proteins are believed to exert developmental functions both in early embryogenesis and in cell-type-specific terminal differentiation events (47, 62).

The Oct-3/4 transcription factor exhibits several features that implicate it as playing a role in mammalian embryogenesis and differentiation. First, it belongs to the POU family of transcription factors (at least some of which are pivotal regulators of development and proliferation of specific cell types). Second, its expression pattern is most interesting. It is expressed in primordial germ cells, in oocytes, and in totipotent/pluripotent stem cells of the pregastrulatory embryo (40, 48, 52). By 8.5 days postfertilization, Oct-3/4 is almost undetectable in somatic cells (48, 50, 51). The Oct-3/4 gene is also expressed in embryonal carcinoma (EC) cells, and its expression is down-regulated in EC cells which are induced to differentiate with retinoic acid (RA) (40, 48, 51). In addition, Oct-3/4 expression is extinguished at the transcriptional level in somatic cell hybrids between EC and fibroblast cells (1, 54). Third, Oct-3/4 expression is regulated by RA through several RA-responsive elements (RAREs) located in the promoter-enhancer region. Expression of the Oct-3/4 gene is controlled by a cell-specific enhancer, RARE1, which contributes to the RA-mediated repression and is located 1.2 kb upstream of the initiation sites (41). Unlike the RARE1 region, which does not contain typical recognition sequences of RA receptors (RAR), the Oct-3/4 promoter harbors a RARE (RAREoct). We have previously shown that the RAREoct shows different DNA-binding and DNase I footprint patterns with nuclear proteins isolated from undifferentiated versus RA-differentiated EC cells. Interestingly, we and others have shown that the RAREoct mediates the RA-induced repression in RA-differentiated EC cells (43, 53).

RA and other retinoids are substances known to influence various aspects of embryogenesis, growth, differentiation, and homeostasis (35, 57). RA affects the transcription of genes in part by acting through two types of nuclear receptors: RARs

* Corresponding author. Phone: 972-2-758362. Fax: 972-2-414583. Electronic mail address: YBERG@HUJIMD.

(isoforms α, β, and γ) (4, 20) and retinoid X receptors (RXRs; isoforms α, β, and γ) (16, 28, 31). Besides the ligand-activated receptors, the receptor superfamily also comprises many orphan receptors for which specific ligands have not yet been defined (11, 14). Two of the better-known orphan receptors are ARP-1/COUP-TFII and EAR-3/COUP-TFI, which play both positive and negative roles in gene regulation upon binding to various regulating elements (6, 7, 17, 21, 30, 36, 58). While the biological role of mammalian ARP-1/COUP-TFII and EAR-3/COUP-TFI is not known, the highly homologous *Drosophila* seven-up gene has been shown to determine photoreceptor cell fates (38). The ARP-1/COUP-TFII and EAR-3/COUP-TFI genes are closely related, with an overall amino acid identity of 87%. The DNA-binding domains and the putative ligand domains have 88 and 97% amino acid identity, respectively (60). Despite their strong sequence homology, they are products of different genes located on different chromosomes (25, 37).

In this report, we provide evidence that EAR-3/COUP-TFI and ARP-1/COUP-TFII down-regulate Oct-3/4 promoter activity. In contrast, RARα:RXRβ, RARβ:RXRα, and RARβ:RXRβ heterodimers stimulate Oct-3/4 activity in P19 cells in the presence of RA. These heterodimers and the orphan receptor proteins bind specifically to the RAREoct site. However, EAR-3/COUP-TFI and ARP-1/COUP-TFII bind this sequence with a higher affinity than the above-mentioned RAR:RXR heterodimers. Furthermore, repression of Oct-3/4 promoter activity by the orphan receptors is overcome by coexpression of RAR:RXR in the presence of RA. The ability of these heterodimers to activate the Oct-3/4 promoter is antagonized by coexpression of ARP-1/COUP-TFII or EAR-3/COUP-TFI. These results indicate that regulation of Oct-3/4 expression is controlled by the balance of the intracellular levels of EAR-3/COUP-TFI, ARP-1/COUP-TFII, RARα,β, and RXRα,β nuclear proteins. Most interestingly, our findings show that upon RA differentiation of EC cells, EAR-3/COUP-TFI and ARP-1/COUP-TFII receptor genes are activated. Thus, all of the above-mentioned receptors are coexpressed within RA-treated P19 (P19/RA) cells. Moreover, the kinetics of the down regulation of Oct-3/4 expression following RA treatment is the inverse of the kinetics of induction of ARP-1/COUP-TFII and EAR-3/COUP-TFI expression in P19/RA cells. These orphan receptors may play a fundamental role in RA-induced differentiation of EC cells and in regulating expression of transcription factors, such as the Oct-3/4 gene, whose function may be critical for normal embryogenesis.

MATERIALS AND METHODS

Cells. Murine EC cells, P19 cells, and Cos-1 cells were maintained in Dulbecco's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Plasmids and oligonucleotides. p0.4oct-CAT was constructed by inserting the *Xba*I-*Msp*I fragment spanning positions −412 to +39 of the Oct-3/4 promoter region upstream to the chloramphenicol acetyltransferase (CAT) reporter gene in pJFCAT1 (12). The p0.4octR*-CAT construct is identical to the above-described plasmid except for four point mutations inserted in the RAREoct site (the mutated sequence is GGCCcGtcGTgAAGGCTAGA, with the lowercase letters denoting mutated nucleotides). Site-directed mutagenesis was performed as previously described (39). The oligonucleotides used for DNA binding assays are as follows: RARE, 5′-AAGGGTTCACCGAAAGTTCACTCGCAT-3′; RAREoct, 5′-GGCCAGAGGTCAAGGCTAGA-3′; OCTA, 5′-CGTACTAAT TTGCATTTCTA-3′; and mutated RAREoct, 5′-GGCCCGTCGTGAAGGCT AGA-3′. Expression constructs harboring complete coding regions of RARα, RARβ (27, 66), RXRα, RXRβ, and RXRγ in pSG5 have been described elsewhere (26). The constructs pMT2-ARP-1 and pMT2-EAR3, which express ARP-1/COUP-TFII and EAR-3/COUP-TFI, have previously been described (24, 25). The vectors which express an amino- or carboxyl-terminus deletion and the DNA-binding-domain derivatives of ARP-1, designated pMT2-ARP-1-ΔA1, pMT2-ARP-1-ΔA6, and pM2-ARP-1-ΔA7, respectively, have also been described previously (25).

DNA transfections. P19 cells were transfected by the calcium phosphate precipitation method (64). Cells (5×10^5) were plated 24 h before transfection and then transfected with 10 μg of CAT reporter plasmids together with 0.1 to 10 μg of vectors expressing full-length cDNAs of the indicated nuclear receptors and 0.5 to 1 μg of β-galactosidase (β-Gal) reference plasmid to correct for differences in transfection efficiency. For transfection into RA-treated cells, 1 μM RA was added at the time of transfection. Medium with or without RA was refreshed 16 to 20 h after transfection. After additional 20 to 24 h, cells were harvested for CAT assays. CAT activity was measured by using [^{14}C]chloramphenicol (53 mCi/mmol; Amersham International, Amersham, United Kingdom) as the substrate in the presence of acetyl coenzyme A at 37°C for 16 h. [^{14}C]chloramphenicol was separated from its acetylated forms by silica thin-layer chromatography and quantitated on a PhosphorImager by using ImageQuant software.

Cos-1 cells were transfected by the DEAE-dextran method (55) with 10 μg of plasmid containing the expression vector of the indicated nuclear receptor.

Nuclear receptor binding analyses. In vitro transcription of RARα, RXRβ, RARβ, EAR-3/COUP-TFI, and ARP-1/COUP-TFII was done as previously described (1). mRNA (1 μg) was added to a rabbit reticulocyte lysate (Promega). Of a 50-μl total reaction mixture, 2.5 to 5 μl was used for analysis in mobility shift DNA binding assays. Whole-cell extracts (WCE) were prepared from transfected Cos-1 cells by lysing the cells in 100 μl of high-salt extraction buffer (0.4 mM KCl, 20 mM Tris-HCl [pH 8.0], 2 mM dithiothreitol, 20% [vol/vol] glycerol) by three cycles of freeze-thaw. The extracts were centrifuged at 10,000 × *g* for 15 min at 4°C to remove cell debris. The following inhibitors were added: phenylmethylsulfonyl fluoride (1 mM), antipain (0.3 μg/ml), leupeptin (1 μg/ml), and trypsin inhibitor (0.5 μg/ml). One to 10 μg of WCE was incubated with 0.3 ng of end-labeled oligonucleotide (30,000 cpm) in the presence of 10 mM Tris (pH 7.8), 14% glycerol, 74 mM KCl, 4 mM dithiothreitol, and 2 μg of poly(dI-dC).

For determination of the dissociation constant (K_d) values for ARP-1/COUP-TFII, EAR-3/COUP-TFI, and RARα:RXRβ heterodimers, gel retardations were performed with a constant amount of Cos-1 protein extracts expressing these receptors and increasing concentrations of radiolabeled oligonucleotide RAREoct probe. After gel electrophoresis, the gels were fixed, dried, and exposed to X-ray film. The radioactive bands corresponding to the bound and free oligonucleotide were quantitated on a PhosphorImager. Data were analyzed by the method of Scatchard (49).

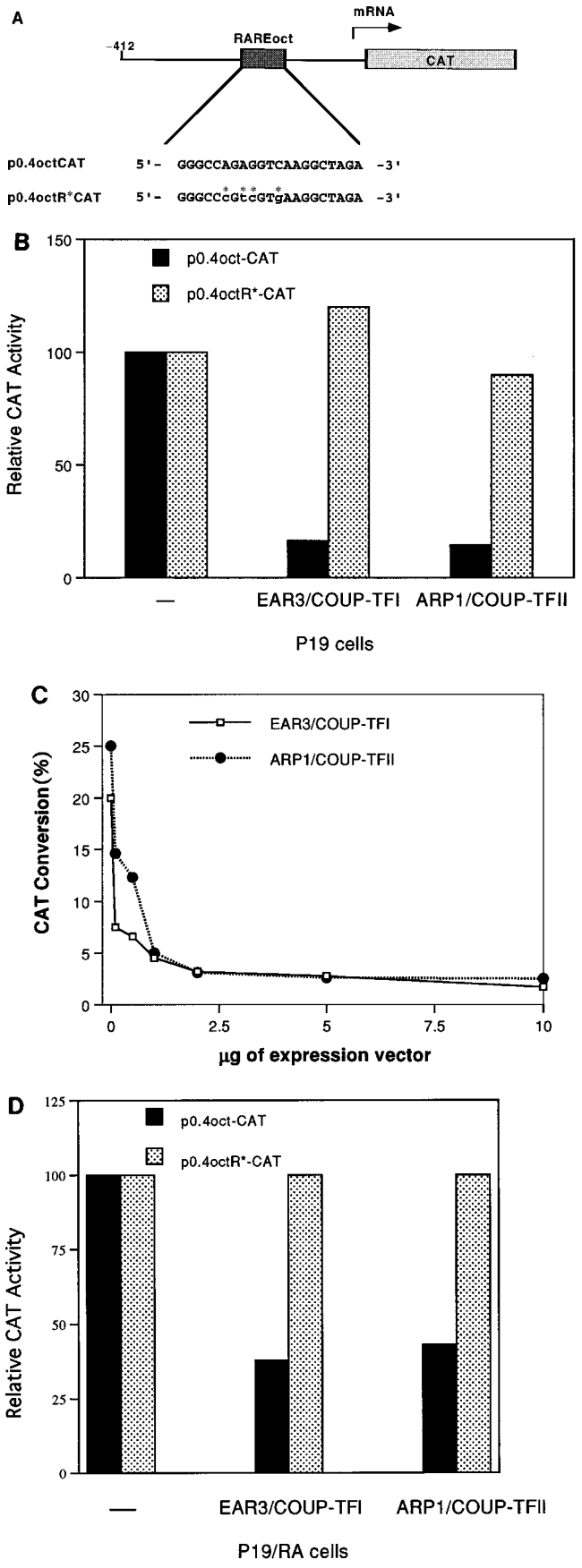
For the competition experiments, 5 μg of protein of Cos-1 WCE containing RARα and RXRβ proteins and nontransfected Cos-1 extract to equalize reaction conditions were mixed on ice in 5 μl of binding buffer and incubated for 20 min. Increasing amounts of ARP-1-ΔA1 or ARP-1-ΔA7 competitor receptor extract were then added, and the mixture was incubated for 30 min on ice. Subsequently ^{32}P-labeled RAREoct oligonucleotide was added, and incubation continued for a further 20 min at room temperature. For the displacement experiment, 5 μg of protein of RARα and RXRβ receptor extract was mixed with labeled oligonucleotide in 5 μl of binding buffer and incubated for 20 min on ice. The samples were transferred to room temperature, and increasing amounts of challenging ARP-1-ΔA1 or ARP-1-ΔA7 receptor extract or wild-type control extract were added. In supershift experiments, the bound samples were transferred to ice and incubated with 1 μl of anti-COUP-TFI antibodies (61) or with an unrelated antibody for 20 min. Protein-DNA complexes were analyzed on prerun 4% polyacrylamide–0.25× Tris-borate-EDTA gels. Gels were dried and exposed to X-ray film at −70°C.

DNase I footprinting assays. For DNase I footprinting assays, an *Avr*II-*Bst*EII fragment (250 bp) isolated from either the p0.4oct-CAT or p0.4octR*-CAT construct was labeled, using the Klenow fragment and [α-^{32}P]dCTP at the *Avr*II site, to a specific activity of greater than 10,000 cpm/ng of DNA. Probes were incubated with 20 μg of protein of WCE in 40 μl of reaction mixture containing 10 mM Tris (pH 7.8), 4% glycerol, 30 mM KCl, 0.5 mM dithiothreitol, and 100 ng of poly(dI-dC). After incubation for 30 min at room temperature, DNase I (0.5 to 1 U; Boehringer Mannheim) diluted in 50 mM $MgCl_2$–10 mM $CaCl_2$ was added for 1 min. The reaction was stopped with the addition of 150 μl of stop solution containing 200 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 33 μg of yeast tRNA per ml. DNA was extracted with phenol-chloroform, ethanol precipitated, and analyzed on a denaturing 6% polyacrylamide gel. Gels were dried and autoradiographed with an intensifying screen at −70°C. Sequencing lanes of the same probes were generated by the Maxam-Gilbert procedure (34).

RNA analyses. Total RNA (15 μg) was electrophoresed on a 1% agarose–formaldehyde gel and blotted onto Nytran filters. Hybridizations were performed under standard conditions (32). Filters were washed at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS and autoradiographed with an intensifying screen at −70°C.

RESULTS

Inhibition of Oct-3/4 expression by ARP-1/COUP-TFII and EAR-3/COUP-TFI through the RAREoct site. We determined the effects of EAR-3/COUP-TFI and ARP-1/COUP-TFII on Oct-3/4 expression. There were several reasons for studying



the effects of these two orphan receptors on the expression of Oct-3/4. First, these orphan receptors generally display higher relative binding affinities for RAREs of the type found in RAREoct, which are direct repeats of the A/GGGTCA motif separated by 1 bp (7, 21, 25). Second, it has been previously shown that ARP-1/COUP-TFII and EAR-3/COUP-TFI have the capacity to negatively regulate the expression of several genes containing RAREs in their regulatory regions (7, 21, 58). The EAR-3/COUP-TFI and ARP-1/COUP-TFII genes are closely related, with an overall amino acid identity of 87%. However, the N-terminal regions of these two proteins have limited similarities, and thus it is possible that these proteins may be capable of mediating different regulatory processes. We cotransfected the p0.4oct-CAT construct, containing the Oct-3/4 promoter fragment (Fig. 1A), with the control plasmid pRSV-βgal and either an ARP-1/COUP-TFII or EAR-3/COUP-TFI expression vector into P19 cells. CAT activity in these cells was determined, normalized by using the corresponding β-Gal activities, and expressed as bar graphs in Fig. 1B. Cotransfection of the EAR-3/COUP-TFI or ARP-1/COUP-TFII expression vector inhibited the CAT activity driven by the Oct-3/4 promoter by six- and sevenfold, respectively (Fig. 1B). The observed repression was not due to nonspecific effects, such as promoter competition between the orphan receptors expression vectors and the Oct-3/4 promoter, since equal amounts of empty expression vector containing no receptor genes were added to the cotransfection reaction to which ARP-1/COUP-TFII or EAR-3/COUP-TFI was not added. In subsequent cotransfection experiments, the amount of DNA was equalized in a similar fashion. To ensure that the repression of Oct-3/4 promoter activity by EAR-3/COUP-TFI or ARP-1/COUP-TFII was through the RAREoct site, we cotransfected a reporter gene driven by an Oct-3/4 promoter in which the RAREoct site was mutated, p0.4octR*-CAT (Fig. 1A), with or without the orphan receptor expression vectors. This mutated version of the RAREoct fails to bind nuclear proteins from P19 and P19/RA cells (43). The results shown in Fig. 1B clearly demonstrate that mutation of the RAREoct site completely abolished repression of Oct-3/4 promoter activity

FIG. 1. EAR-3/COUP-TFI and ARP-1/COUP-TFII repress Oct-3/4 promoter activity. (A) Map of the wild-type p0.4oct-CAT and mutated p0.4octR*-CAT reporter plasmids. The *Xba*I-*Msp*I fragment spanning positions −412 to +39 of the Oct-3/4 promoter region was inserted upstream of the CAT reporter gene. (B) The indicated reporter plasmids (5 μg) were transfected with a β-Gal-containing reference plasmid into P19 cells with either an EAR-3/COUP-TFI or ARP-1/COUP-TFII expression vector or with vector alone (−). The amount of DNA added to each transfection was equalized by adding empty vector (for this as well as for the following experiments). After 48 h, cells were harvested and lysed, and CAT activities were determined. The percent conversion to the acetylated forms of each separate transfection was normalized to the β-Gal activity. The values for percent conversion, presented as means ± standard deviations, corresponding to p0.4oct-CAT in the absence or presence of EAR-3/COUP-TFI or ARP/COUP-TFII are 19.1 ± 2.2, 3.0 ± 0.2, and 2.6 ± 0.14, respectively. CAT activity of each construct alone in the absence of the nuclear receptors was arbitrarily set at 100%. Relative CAT activity represents CAT activity relative to that obtained from p0.4oct-CAT and p0.4octR*-CAT, respectively. The bar graphs are representative of four transfections. (C) Effects of increasing amounts of EAR-3/COUP-TFI and ARP-1/COUP-TFII on Oct-3/4 promoter activity. P19 cells were cotransfected with p0.4oct-CAT and increasing amounts of either EAR-3/COUP-TFI or ARP-1/COUP-TFII, and CAT activity was measured and assayed as described for panel B. (D) EAR-3/COUP-TFI and ARP-1/COUP-TFII repress Oct-3/4 promoter activity in RA-differentiated P19 cells. The indicated plasmids were cotransfected with a β-Gal-containing reference plasmid into P19 cells in the presence of RA. The values for percent conversion, presented as means ± standard deviations, corresponding to p0.4oct-CAT in the absence or presence of EAR3/COUP-TFI or ARP1/COUP-TFII are 3.24 ± 0.6, 1.1 ± 0.15, and 1.3 ± 0.2, respectively. Relative CAT activity was quantitated as described for panel B. The bar graphs are representative of five independent experiments.

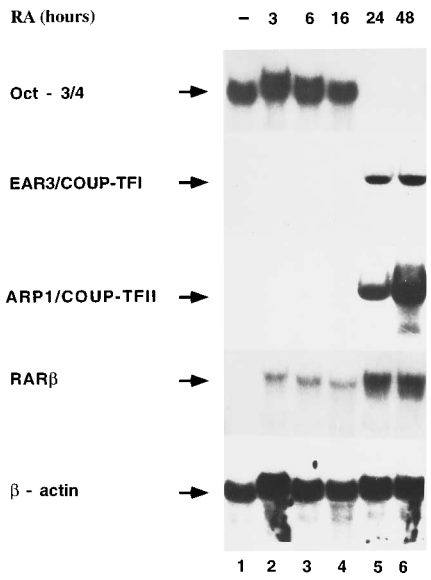


FIG. 2. Coexpression of ARP-1/COUP-TFII and EAR-3/COUP-TFI in RA-differentiated P19 cells. RNA (15 μg) isolated from P19 cells treated for 0, 3, 6, 16, 24, and 48 h with RA was electrophoresed on 1% agarose–formaldehyde gels, transferred to Nytran filters, and hybridized with Oct-3/4, RARβ, and β-actin cDNAs and with *Bam*HI fragments isolated from the 3′ regions of the ARP-1/COUP-TFII and EAR-3/COUP-TFI probes which are unique for each probe (kindly provided by J. Ladias). The blot was initially hybridized with the Oct-3/4 cDNA probe and then stripped and sequentially rehybridized with the indicated probes.

by the ARP-1/COUP-TFII or EAR-3/COUP-TFI expression vector.

To determine whether the inhibition of the Oct-3/4 promoter by ARP-1/COUP-TFII or EAR-3/COUP-TFI was dose dependent, a constant amount (5 μg) of p0.4oct-CAT construct was cotransfected with increasing amounts of the ARP-1/COUP-TFII or EAR-3/COUP-TFI expression vector (Fig. 1C). CAT activity was progressively repressed by increasing amounts of these orphan receptor expression vectors. Thus, inhibition of the Oct-3/4 promoter is dependent on the amount of the transfected ARP-1/COUP-TFII or EAR-3/COUP-TFI expression vector.

This repression of Oct-3/4 promoter activity is similar to that observed when the ARP-1/COUP-TFII or EAR-3/COUP-TFI expression vector was cotransfected with the p0.4oct-CAT construct into P19/RA cells (Fig. 1D). However, the average magnitude of repression (2.5- to 3-fold) was lower, probably because of the presence of endogenous EAR-3/COUP-TFI and ARP-1/COUP-TFII proteins in P19 cells treated for 48 h with RA (see below). In these cells too, inhibition is mediated through the RAREoct site, since mutations of this element completely abolished repression of Oct-3/4 promoter activity by ARP-1/COUP-TFII or EAR-3/COUP-TFI (Fig. 1D). It is therefore concluded that in P19 and P19/RA cells, EAR-3/COUP-TFI and ARP-1/COUP-TFII expression vectors inhibit Oct-3/4 promoter activity through the RAREoct site in a dose-dependent manner.

ARP-1/COUP-TFII and EAR-3/COUP-TFI are coexpressed in P19/RA cells. The physiological significance of the foregoing observations was tested by studying the expression patterns of ARP-1/COUP-TFII and EAR-3/COUP-TFI in undifferentiated and in RA-differentiated P19 cells. We performed Northern (RNA) blot analysis on mRNA purified from P19 and P19/RA cells. As shown in Fig. 2, Oct-3/4 mRNA is detected in P19 cells and in P19 cells which were treated with RA for 3, 6, and 16 h. Following 24 h of RA treatment, there was a sharp decrease in Oct-3/4 mRNA to almost undetectable levels. Transcription of RARβ increased 3 h after the addition of RA to the culture, as previously reported (45). Therefore, RARβ mRNA is induced by RA treatment while Oct-3/4 mRNA is still expressed at high levels. Similar data were observed in run-on experiments (data not shown). Interestingly, EAR-3/COUP-TFI and ARP-1/COUP-TFII mRNAs are almost undetectable in P19 cells and hardly detectable after 16 h of RA treatment. In contrast, following 24 h of RA treatment, there is a sharp increase in the levels of EAR-3/COUP-TFI and ARP-1/COUP-TFII transcripts. Thus, treatment of P19 cells with RA causes repression of Oct-3/4 expression and induction of EAR-3/COUP-TFI and ARP-1/COUP-TFII expression with striking inverse kinetics.

ARP-1/COUP-TFII and EAR-3/COUP-TFI specifically bind the RAREoct site. To test directly whether the ARP-1/COUP-TFII and EAR-3/COUP-TFI orphan receptor proteins can bind the RAREoct sequence, we transfected Cos-1 cells with the EAR-3/COUP-TFI or ARP-1/COUP-TFII expression vector and analyzed their DNA-binding properties by electrophoretic mobility shift assays (EMSAs). The results in Fig. 3A show that EAR-3/COUP-TFI and ARP-1/COUP-TFII indeed form a very prominent retarded complex with RAREoct oligonucleotide (lanes 4 and 5, respectively). As expected from their predicted sizes, ARP-1/COUP-TFII and EAR-3/COUP-TFI complexes exhibited similar mobilities. These complexes comigrated with the complex generated between RAREoct oligonucleotide and WCE prepared from P19/RA cells (compare lane 2 and lanes 4 and 5, lanes 6 and 10, and lanes 11 and 15). The comigrating complex present in P19/RA WCE contains a COUP transcription factor(s) (either ARP-1, EAR-3, or both), since a polyclonal anti-COUP-TF antibody (lane 17), and not a nonspecific antibody (lane 18), supershifted this complex (compare lane 16 with lane 17). The absence of a complete supershift of the retarded complex by the anti-COUP-TF antibody may indicate either that the antibodies failed to recognize particular forms of COUP-TFs or that this complex contains additional proteins that bind the RAREoct site. The specificity of DNA binding was assessed by competition of the binding to the labeled RAREoct probe with an excess of unlabeled oligonucleotides. Binding of the orphan receptor proteins to RAREoct was competed for by unlabeled RAREoct oligonucleotide (lanes 7 and 12) but not by the unrelated OCTA sequence (lanes 9 and 14) or by the mutated version of the RAREoct site, RARE*oct (lanes 8 and 13). These data confirm that the mutations inserted in the RAREoct site prevent binding of ARP-1/COUP-TFII and EAR-3/COUP-TFI to the RARE*oct sequence.

To better understand the effects of these orphan receptors on the Oct-3/4 promoter, we carried out DNase I footprinting experiments using the wild-type Oct-3/4 promoter (Fig. 3B, WT probe) and the RAREoct-mutated promoter (Fig. 3B, R* probe). These fragments were subjected to footprinting with WCE isolated from Cos-1 cells transfected with ARP-1/COUP-TFII or EAR-3/COUP-TFI. Both WCEs protected an identical region between nucleotides −55 and −25 of the Oct-3/4 wild-type promoter. This region includes the Sp1 site, the RAREoct site harboring the two direct repeats separated by one nucleotide, and an additional 7 bp 3′ to the RAREoct which includes the third direct repeat separated by no nucleotides (lanes 5 and 7). WCE from untransfected Cos-1 cells protected a region between nucleotides −55 and −41 that encompasses the Sp1-binding site (lanes 4 and 6). Using the mutated Oct-3/4 promoter fragment as a probe, WCE from

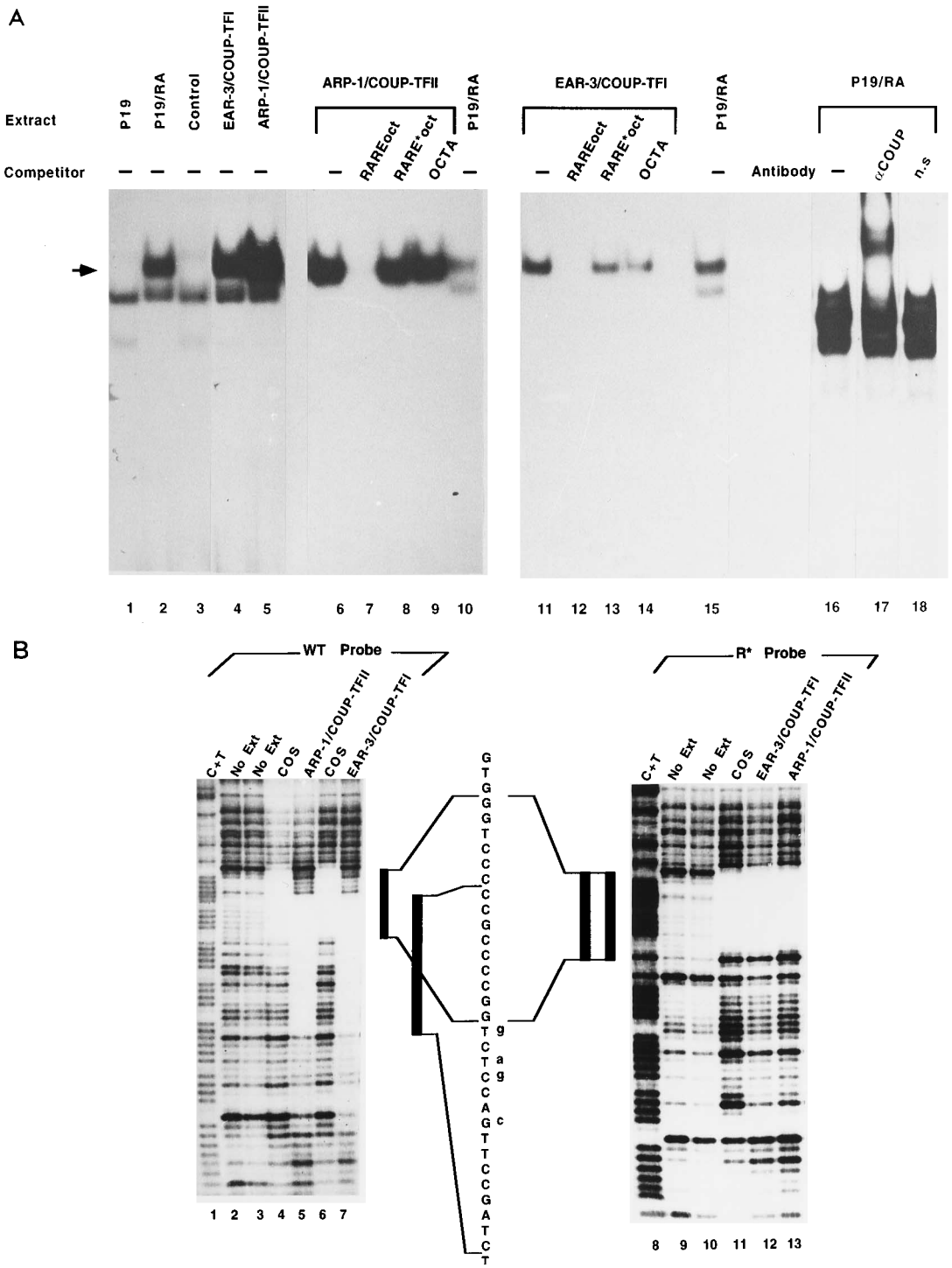


FIG. 3. ARP-1/COUP-TFII and EAR-3/COUP-TFI bind specifically to the RAREoct site. (A) The ^{32}P-labeled RAREoct oligonucleotide was incubated with WCE prepared from P19 cells (lane 1), P19/RA cells (lanes 2, 10, and 16 to 18), Cos-1 cells (lane 3), and Cos-1 cells transfected with either EAR-3/COUP-TFI (lanes 4 and 11 to 14) or ARP-1/COUP-TFII (lanes 5 to 9). Binding reactions were performed in the absence of competitors (lanes 1 to 6, 10, 11, and 15 to 18) or in the presence of a 50-fold molar excess of unlabeled RAREoct (lanes 7 and 12), RARE*oct (lanes 8 and 13), or OCTA (lanes 9 and 14) oligonucleotide or in the presence of 1 μl of anti-COUP-TF (α COUP; lane 17) or nonspecific (n.s.; lane 18) antibodies. The free DNA was run off the bottom of the gel. The arrow indicates the ARP-1/COUP-TFII and EAR-3/COUP-TFI complexes. The faster-migrating band observed with some of the transfected Cos-1 extracts originates from the untransfected Cos-1 cells. The identities of this band and of the bands with similar mobilities found in the P19 and P19/RA extracts are still unclear. The indicated complex originating from P19/RA cells observed in lanes 2, 10, and 15 was separated in lanes 16 to 18 to the two slowly migrating bands as a result of a longer electrophoresis separation. (B) DNase I footprinting of the upper strand of wild-type (WT) and RAREoct (R*) site-directed mutated Oct-3/4 promoter. ^{32}P-labeled probes were incubated in the absence (lanes 2, 3, 9, and 10) or in the presence of WCEs prepared from untransfected (lanes 4, 6, and 11) or ARP-1/COUP-TFII-transfected (lanes 5 and 13) or EAR-3/COUP-TFI-transfected (lanes 7 and 12) Cos-1 cells. Lanes 1 and 8 are Maxam-Gilbert C+T sequencing ladders. The protected regions are boxed, and the corresponding sequences are indicated. The lowercase letters represent the four mutations inserted in the RAREoct site. Ext, extract.

transfected and untransfected Cos-1 cells protected an identical region between nucleotides −55 and −41, which encompasses the Sp1-binding site only (lanes 11 to 13). Thus, with the mutated probe, the marked difference in the protection pattern observed with the wild-type RAREoct probe between WCE prepared from Cos-1 cells transfected with ARP-1/COUP-TFII or EAR-3/COUP-TFI versus Cos-1 cells alone is clearly eliminated. This finding suggests that mutations in the RAREoct site prevent ARP-1/COUP-TF-II and EAR-3/COUP-TFI from binding but still allow Sp1 to bind. These results are analogous to our previously published data regarding footprinting of the wild-type and mutated Oct-3/4 promoter fragment with P19 and P19/RA nuclear proteins (43).

RAR:RXR heterodimers bind the RAREoct site and activate the Oct-3/4 promoter. We have shown previously that the RAREoct site exhibits extensive homology to sequences previously identified in RA-responsive genes such as those encoding CRBPII, CRBPI, apolipoprotein A1 (ApoA1), ApoCIII, PEPCK, RARβ, and RARγ (43). These sites bind various transcriptional regulators which belong to the RAR and RXR families. To determine whether various RAR and RXR proteins have the ability to bind to the RAREoct sequence, we cotransfected Cos-1 cells with expression vectors harboring RARα,β and RXRα,β,γ, separately, and analyzed their DNA-binding properties by EMSA. Labeled RAREoct oligonucleotide hardly formed specific retarded complexes with WCE generated from Cos-1 cells transfected with a single receptor gene (either RAR or RXR; Fig. 4A, lanes 6, 8, and 10, and data not shown). Accordingly, our cotransfection experiments indicate that in P19/RA cells, RARα slightly induced Oct-3/4 promoter expression through the RAREoct site (data not shown). Our results differ from recently published data showing that RARα represses Oct-3/4 promoter activity (56). However, this repression was ligand independent and was observed in RAC 65 cells (which contain a mutation in the endogenous RARα gene). Furthermore, the repression was not through the RAREoct site, which may indicate that this suppression operates through an indirect mechanism which is different from the RARα activation of Oct-3/4 expression observed by us in P19/RA cells.

It has previously been shown that RARs alone are inefficient DNA binders and require auxiliary nuclear proteins for effective response element interaction. RXRs are such auxiliary proteins. Thus, we examined whether heterodimers between the RARs and RXRs can bind the RAREoct site. Heterodimers formed between RARα or RARβ and the different RXRs did bind to the labeled RAREoct oligonucleotide (Fig. 4A, lanes 1 to 3, 5, 7, and 9). The fastest-migrating band can be attributed to the binding of Cos-1 proteins. The bands were specifically competed for by an unlabeled RAREoct oligonucleotide and not by an unrelated oligonucleotide (data not shown). Having established that all the above-studied RAR:RXR heterodimers can bind the RAREoct site, the next question that we addressed was the functional consequences of this binding. We examined the ability of the six different RAR:RXR heterodimers to control transcription from the Oct-3/4 promoter. These constructs were cotransfected into P19 cells together with a CAT reporter gene driven by the Oct-3/4 promoter and a β-Gal plasmid in the presence or absence of RA. In the absence of RA, Oct-3/4 promoter activity was not affected by these heterodimers (data not shown). However, in the presence of 10^{-6} M RA, RARα:RXRβ, RARβ:RXRα, and RARβ:RXRβ heterodimers up-regulated CAT activity driven by the wild-type Oct-3/4 promoter by 4.5-, 2.7-, and 7.2-fold, respectively, above the baseline level, which is defined as the level of expression in the absence of cotransfected receptor (Fig. 4B). This up-regulation by RARα or RARβ is

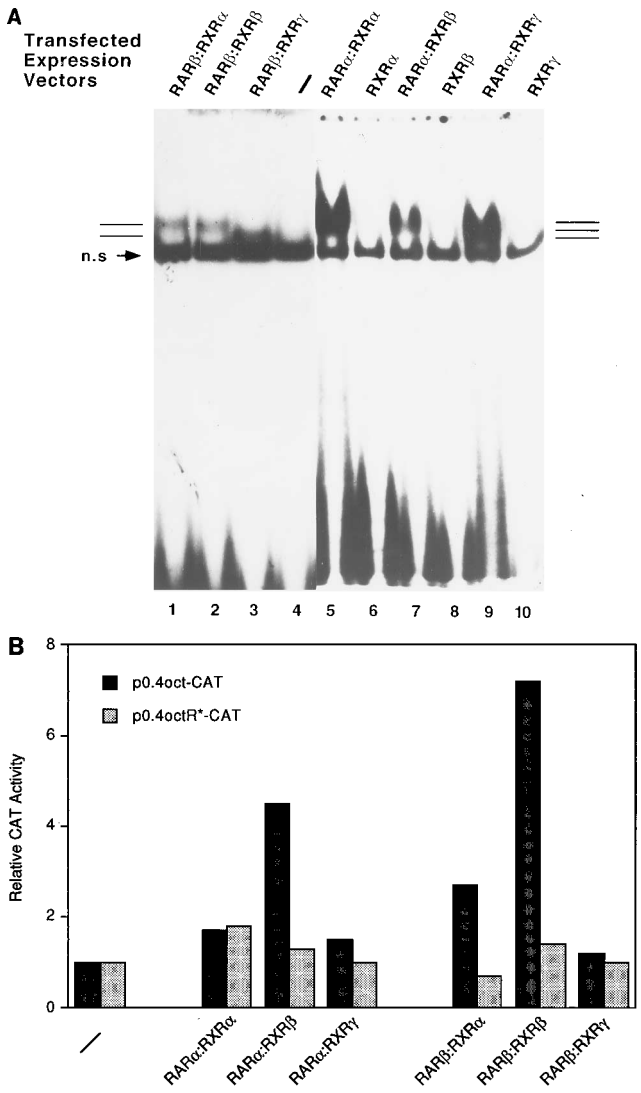


FIG. 4. RAR-RXR heterodimers bind to the RAREoct site and activate the Oct-3/4 promoter in P19/RA cells. (A) ^{32}P-labeled RAREoct was incubated with extracts prepared from either untransfected Cos-1 cells (lane 4) or Cos-1 cells transfected with the indicated expression plasmids. The arrow indicates a complex present in untransfected Cos-1 cells (n.s [nonspecific]). The lines indicate the various transfected heterodimers. (B) P19/RA cells were cotransfected with a β-Gal-containing reference plasmid and the reporter plasmids p0.4oct-CAT and p0.4octR*-CAT in the absence (−) or presence of the indicated nuclear receptor expression plasmids. CAT activity was measured and quantitated as described in the legend to Fig. 1B. The percent conversion values corresponding to p0.4oct-CAT in the absence or presence of the various nuclear receptor expression vectors (from left to right) are 2.9, 4.9, 13.1, 4.3, 7.8, 20.9, and 3.5, respectively. The percent conversion values of the corresponding transfection with p0.4octR*-CAT are 1.9, 3.4, 2.5, 1.9, 1.3, 2.7, and 1.9, respectively. CAT activity of p0.4oct-CAT and p0.4octR*-CAT in the absence of nuclear receptors was arbitrarily set as 1. Relative CAT activity represents CAT activity in the presence of the nuclear receptors relative to that obtained from the reporter plasmids alone. The bar graphs are representative of three independent transfections differing by less than 15%.

dependent on the presence of both RXR and RA. The other RAR:RXR heterodimers either activated the Oct-3/4 promoter to a lower extent or did not activate at all. The up-regulation of the Oct-3/4 promoter is through the RAREoct site, since mutations of this element abolished activation of Oct-3/4 promoter activity by the various transfected RAR:RXR heterodimers (Fig. 4B). It is therefore concluded that in

P19 cells, specific RAR:RXR heterodimers bind to RAREoct and activate the Oct-3/4 promoter in response to treatment of the cells with RA.

RAREoct binds EAR-3/COUP-TFI and ARP-1/COUP-TF-II preferentially. Since ARP-1/COUP-TFII, EAR-3/COUP-TFI, RARα:RXRβ, RARβ:RXRα, and RARβ:RXRβ bind the RAREoct oligonucleotide and, even more importantly, regulate the activity of the Oct-3/4 promoter, the relative binding of each complex for the RAREoct site was determined. For this purpose, we incubated the labeled RAREoct oligonucleotide with WCE proteins derived from Cos-1 cells transfected with the indicated expression vectors and analyzed the complexes by EMSA (Fig. 5A). The results show that the orphan receptors bind to RAREoct site with a higher affinity (at least 10 times greater) than that of the RAR:RXR heterodimers. To ensure that the difference in the binding of these receptors, as seen by comparing lanes 2 and 3 with lanes 4 to 6, is not due to a difference in the amount of proteins expressed in the transfected Cos-1 cells, we performed an EMSA with the RARE as a radioactive probe (Fig. 5B). This analysis indeed shows that ARP-1/COUP-TFII, EAR-3/COUP-TFI, and the various RAR:RXR heterodimers bind to RARE with similar affinities (compare lanes 2, 6, and 7 with lanes 3 to 5 and 8 to 10). To address the possibility that the formation of these complexes could be influenced by factors (either positive or negative) present in the Cos-1 WCE, RARα:RXRβ, RARβ:RXRβ, and EAR-3/COUP-TFI were in vitro transcribed. Reticulocyte lysates programmed with these mRNAs form specific retardation complexes with the RAREoct sequence. The results show that similarly to the data described above, the in vitro-transcribed and -translated EAR-3/COUP-TFI binds to the RAREoct site with a higher affinity than that of the RAR:RXR heterodimers (Fig. 5C). Moreover, the intensity of these complexes was only slightly affected upon addition of untransfected Cos-1 WCE (data not shown).

To assess the DNA-binding affinities of ARP-1/COUP-TFII, EAR-3/COUP-TFI, and RARα:RXRβ heterodimers to the RAREoct site, the binding of Cos-1 cell extracts containing these various receptors separately was determined as a function of RAREoct oligonucleotide concentration. The amounts of RAREoct in these complexes (bound) were determined and plotted against the amounts of nonbound (free) RAREoct oligonucleotide in each lane (Fig. 5D and E). Subsequent Scatchard analysis of these data revealed that under these conditions, the K_ds for binding of ARP-1/COUP-TFII and EAR-3/COUP-TFI to the RAREoct site are 5.2 and 2.5 nM, respectively (Fig. 5D). The data show that ARP-1/COUP-TFII and EAR-3/COUP-TFI bind to the RAREoct site with similar affinities. As indicated in Fig. 5E, the K_d for RARα:RXRβ is 167 nM. We were unable to perform an accurate Scatchard analysis to determine the K_d value for the RARβ:RXRβ heterodimers because of their poor binding. These assays reveal that the orphan receptors bind to the RAREoct site with an affinity approximately 30 times greater (at least) than that of RARα:RXRβ heterodimers.

Antagonism between the effects of ARP-1/COUP-TFII, EAR-3/COUP-TFI, and RAR:RXR heterodimers on Oct-3/4 promoter activity. We have shown above that the Oct-3/4 promoter was inhibited by ARP-1/COUP-TFII and EAR-3/COUP-TFI and activated by three specific RAR:RXR heterodimers. All of these nuclear receptors bind to the RAREoct site and, moreover, are present in RA-differentiated P19 cells (Fig. 2 and data not shown) and thus might interfere with the regulatory effect of each other. A corollary of this prediction is that transcriptional inhibition by the orphan receptors can be overcome by increasing amounts of cotransfected RAR:RXR heterodimers. Therefore, we cotransfected P19 cells with p0.4oct-CAT reporter construct, the control vector pRSV-βgal, and various combinations of ARP-1/COUP-TFII, EAR-3/COUP-TFI, RARα:RXRβ, and RARβ:RXRβ in the presence of RA (since the RAR:RXR heterodimers regulate Oct-3/4 activity only in the presence of RA). The total amount of DNA in each transfection reaction was kept constant by compensating with an empty expression vector. In P19/RA cells, 0.1 μg of the EAR-3/COUP-TFI or ARP-1/COUP-TFII expression vector represses Oct-3/4 promoter activity by 4- or 2.5-fold, respectively (Fig. 6A). Increasing amounts (1 to 10 μg) of the RARβ:RXRβ expression vector restored CAT activity levels to those observed in the absence of the orphan receptors expression vectors. Thus, the transcriptional repression observed with ARP-1/COUP-TFII and EAR-3/COUP-TFI can be abolished by increasing the level of RARβ:RXRβ in the cell.

We have shown above (Fig. 4B) that three specific RAR:RXR heterodimers activate the Oct-3/4 promoter in the presence of RA. We wanted to determine whether EAR-3/COUP-TFI and ARP-1/COUP-TFII can interfere with the activation of the Oct-3/4 promoter by RAR:RXR heterodimers. For these experiments, P19/RA cells were cotransfected with the p0.4oct-CAT construct, a constant amount of RARβ:RXRβ (5 μg), the control vector pRSV-βgal, and increasing amounts of either ARP-1/COUP-TFII or EAR-3/COUP-TFI. The results show that activation of the Oct-3/4 promoter by RARβ:RXRβ expression vectors was completely overcome with either ARP-1/COUP-TFII or EAR-3/COUP-TFI (Fig. 6B).

We then asked whether a different heterodimer, RARα:RXRβ, behaves similarly to the RARβ:RXRβ and is also able to interfere with repression of Oct-3/4 promoter by ARP-1/COUP-TFII and EAR-3/COUP-TFI. At this stage, we performed experiments of the kind described above, using the RARα:RXRβ heterodimers. The results showed that whereas the addition of RARβ:RXRβ restored CAT activity levels to those observed in the absence of the orphan receptor expression vectors, increasing the amount of the cotransfected RARα:RXRβ heterodimers to 5 and 10 μg activated the Oct-3/4 promoter above the basal level (Fig. 7A). Thus, the transcriptional repression observed with the orphan receptors can be abolished by increasing the level of RARα:RXRβ in the cell. Conversely, transactivation by RARα:RXRβ was completely repressed by increasing amounts of ARP-1/COUP-TFII or EAR-3/COUP-TFI in a dose-dependent manner (Fig. 7B). The results from Fig. 6 and 7 demonstrate that antagonism between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and RARα:RXRβ and RARβ:RXRβ heterodimers modulates expression of the Oct-3/4 promoter in P19/RA cells.

ARP-1/COUP-TFII displaces RARα:RXRβ heterodimers from the RAREoct site. There are several possible mechanisms through which ARP-1/COUP-TFII and EAR-3/COUP-TFI can repress Oct-3/4 promoter activity. It is possible that these orphan receptors antagonize Oct-3/4 transcriptional activation by RAR:RXR heterodimers through an indirect mechanism by heterodimerization with either RAR or RXR (2, 22, 63). Thus, we performed EMSAs with extracts prepared from Cos-1 cells transfected with the different combinations of nuclear receptors and with in vitro-transcribed and -translated receptors. We could not detect DNA-binding heterodimers between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and either RARα,β or RXRβ (data not shown). Transfection of P19 cells with ARP-1/COUP-TFII and RXRβ expression vectors repressed Oct-3/4 promoter activity to the same extent as transfection of ARP-1/COUP-TFII alone. The same results were obtained

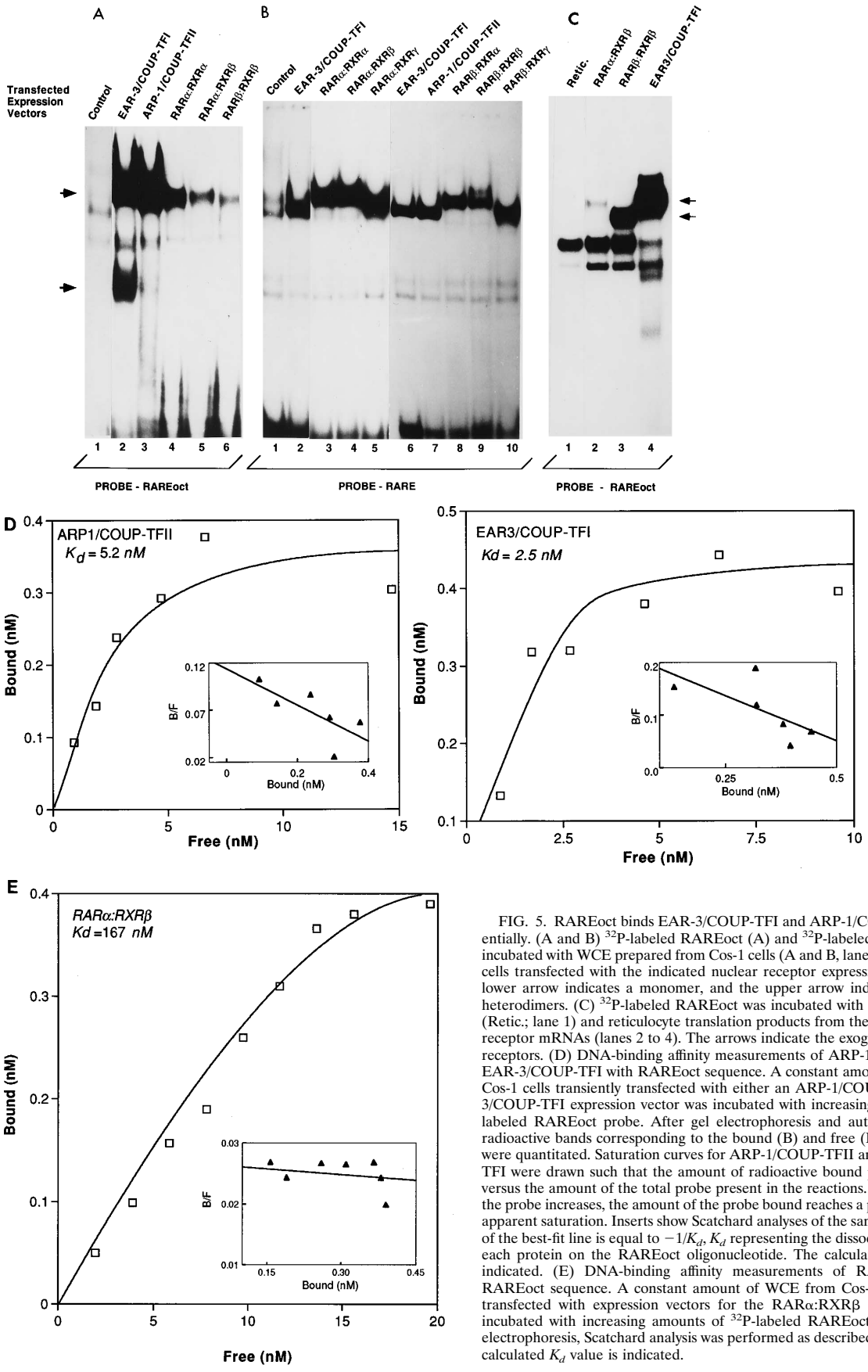


FIG. 5. RAREoct binds EAR-3/COUP-TFI and ARP-1/COUP-TFII preferentially. (A and B) ^{32}P-labeled RAREoct (A) and ^{32}P-labeled RARE (B) were incubated with WCE prepared from Cos-1 cells (A and B, lanes 1) or from Cos-1 cells transfected with the indicated nuclear receptor expression plasmids. The lower arrow indicates a monomer, and the upper arrow indicates homo- and heterodimers. (C) ^{32}P-labeled RAREoct was incubated with reticulocyte lysate (Retic.; lane 1) and reticulocyte translation products from the indicated nuclear receptor mRNAs (lanes 2 to 4). The arrows indicate the exogenously translated receptors. (D) DNA-binding affinity measurements of ARP-1/COUP-TFII and EAR-3/COUP-TFI with RAREoct sequence. A constant amount of WCE from Cos-1 cells transiently transfected with either an ARP-1/COUP-TFII or EAR-3/COUP-TFI expression vector was incubated with increasing amounts of ^{32}P-labeled RAREoct probe. After gel electrophoresis and autoradiography, the radioactive bands corresponding to the bound (B) and free (F) oligonucleotide were quantitated. Saturation curves for ARP-1/COUP-TFII and EAR-3/COUP-TFI were drawn such that the amount of radioactive bound probe was plotted versus the amount of the total probe present in the reactions. As the amount of the probe increases, the amount of the probe bound reaches a plateau, indicating apparent saturation. Inserts show Scatchard analyses of the same data. The slope of the best-fit line is equal to $-1/K_d$, K_d representing the dissociation constant of each protein on the RAREoct oligonucleotide. The calculated K_d values are indicated. (E) DNA-binding affinity measurements of RARα:RXRβ with RAREoct sequence. A constant amount of WCE from Cos-1 cells transiently transfected with expression vectors for the RARα:RXRβ heterodimers was incubated with increasing amounts of ^{32}P-labeled RAREoct probe. After gel electrophoresis, Scatchard analysis was performed as described for panel D. The calculated K_d value is indicated.

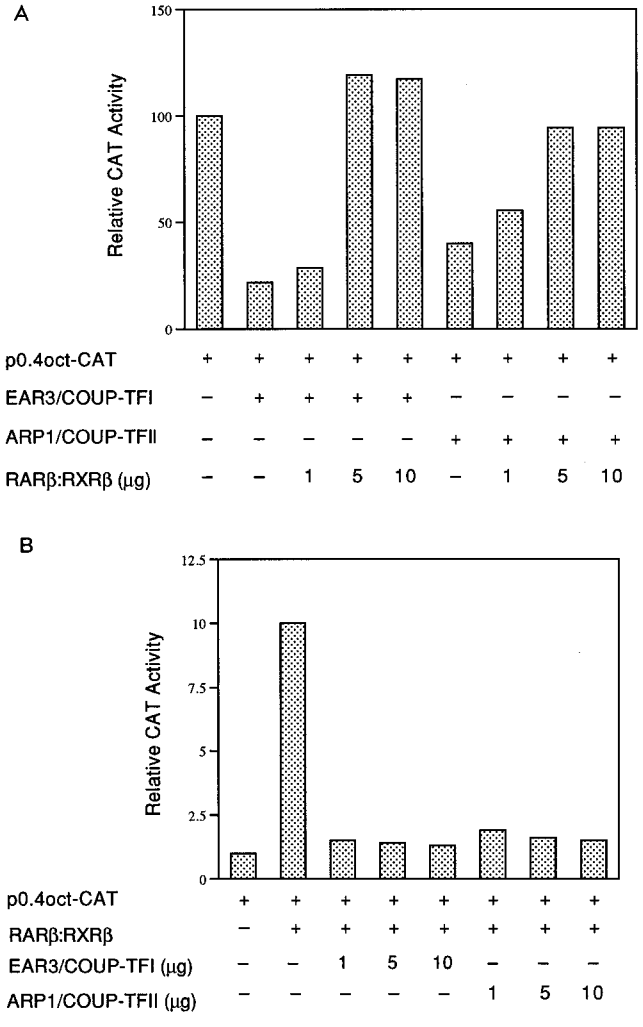


FIG. 6. Antagonism between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and RARβ:RXRβ heterodimers modulates Oct-3/4 promoter activity. (A) RARβ:RXRβ heterodimers oppose ARP-1/COUP-TFII and EAR-3/COUP-TFI repression of the Oct-3/4 promoter. P19/RA cells were transiently transfected with the reporter plasmid p0.4oct-CAT (5 μg), a β-Gal-containing reference plasmid (0.5 μg), a constant amount of ARP-1/COUP-TFII or EAR-3/COUP-TFI (0.1 μg), and the indicated increasing amounts of RARβ:RXRβ expression plasmids. Transfection efficiency and CAT activity were monitored and assayed as described in the legend to Fig. 1B. The percent conversion values corresponding to p0.4oct-CAT in the absence or presence of the various nuclear receptor expression vectors (from left to right) are 3.5, 0.8, 1.0, 4.2, 4.1, 1.4, 1.9, 3.3, and 3.3, respectively. The data shown represent the averages of two independent experiments differing by less than 10%. (B) ARP-1/COUP-TFII and EAR-3/COUP-TFI oppose RARβ:RXRβ transactivation of the Oct-3/4 promoter. P19/RA cells were transiently transfected with p0.4oct-CAT (5 μg), a β-Gal-containing reference plasmid (0.5 μg), a constant amount of RARβ:RXRβ (5 μg:5 μg), and increasing amounts of either an EAR-3/COUP-TFI or ARP-1/COUP-TFII expression plasmid. Transfection efficiency and CAT activity were monitored and assayed as described in the legend to Fig. 1B. The percent conversion values corresponding to p0.4oct-CAT in the absence or presence of the various nuclear receptor expression vectors (from left to right) are 2.4, 27.9, 3.6, 3.9, 3.2, 4.5, 3.1, and 3.6, respectively. CAT activity of p0.4oct-CAT in the absence of nuclear receptors was arbitrarily set as 1. Relative CAT activity represents CAT activity of p0.4oct-CAT in the presence of nuclear receptors relative to that obtained from p0.4oct-CAT alone. The data shown represent the averages of two independent experiments differing by less than 12%.

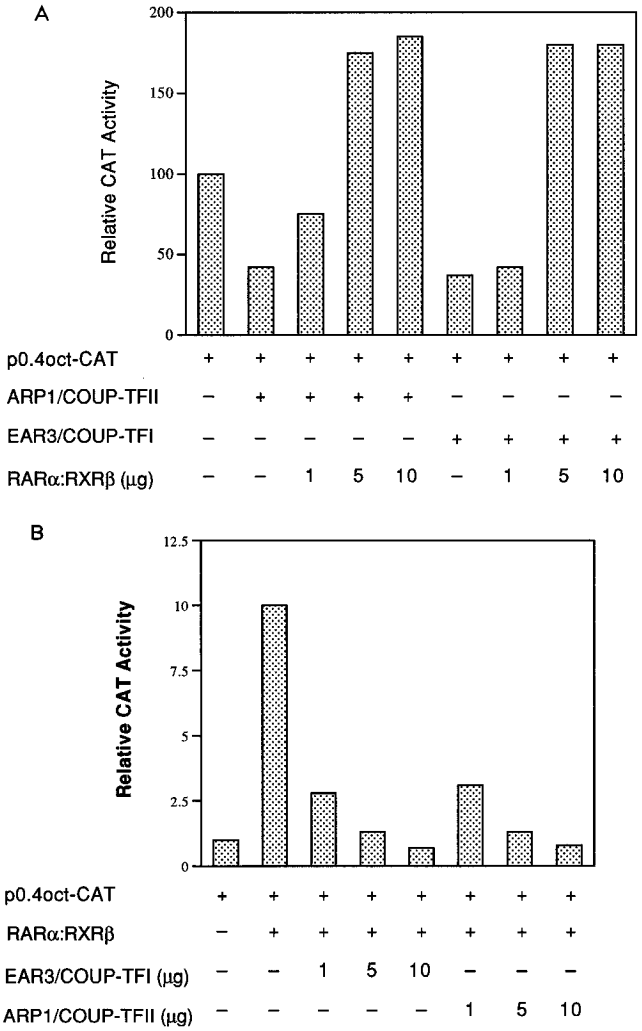


FIG. 7. Antagonism between ARP-1/COUP-TFII, EAR-3/COUP-TFI, and RARα:RXRβ heterodimers modulate Oct-3/4 promoter activity. (A) RARα:RXRβ heterodimers oppose ARP-1/COUP-TFII and EAR-3/COUP-TFI repression of the Oct-3/4 promoter. P19/RA cells were transiently transfected with the reporter plasmid p0.4oct-CAT (5 μg), a β-Gal-containing reference plasmid (0.5 μg), a constant amount of ARP-1/COUP-TFII or EAR-3/COUP-TFI (1 μg), and increasing amounts of RARα:RXRβ expression plasmids. Transfection efficiency and CAT activity were monitored and assayed as described in the legend to Fig. 1B. The percent conversion values corresponding to p0.4oct-CAT in the absence or presence of the various nuclear receptor expression vectors (from left to right) are 3, 1.3, 2.2, 5.2, 5.5, 1.1, 1.3, 5.4, and 5.4, respectively. The data shown represent the averages of two independent experiments differing by less than 15%. (B) ARP-1/COUP-TFII and EAR-3/COUP-TFI oppose RARα:RXRβ transactivation of the Oct-3/4 promoter. P19/RA cells were transiently transfected with p0.4oct-CAT (5 μg), a β-Gal-containing reference plasmid (0.5 μg), a constant amount of RARα:RXRβ (5 μg:5 μg), and increasing amounts of either an EAR-3/COUP-TFI or ARP-1/COUP-TFII expression plasmid. Transfection efficiency and CAT activity were monitored and assayed as described in the legend to Fig. 1B. The percent conversion values corresponding to p0.4oct-CAT in the absence or presence of the various nuclear receptor expression vectors (from left to right) are 3.2, 32, 9.0, 4.2, 2.2, 9.9, 4.2, and 2.6, respectively. Relative CAT activity was calculated as described in the legend to Fig. 6B. The data shown represent the averages of two independent experiments differing by less than 10%.

with the EAR-3/COUP-TFI expression vectors (data not shown).

Having ruled out the formation of heterodimers between the orphan receptors and either RARα,β or RXRβ as the mode of inhibition of Oct-3/4 transcriptional activation, we turned our attention to another possible mechanism involving displacement of RAR:RXR heterodimers from the RAREoct site. To test this proposition directly, we examined whether ARP-1/COUP-TFII could compete with or even displace RARα:

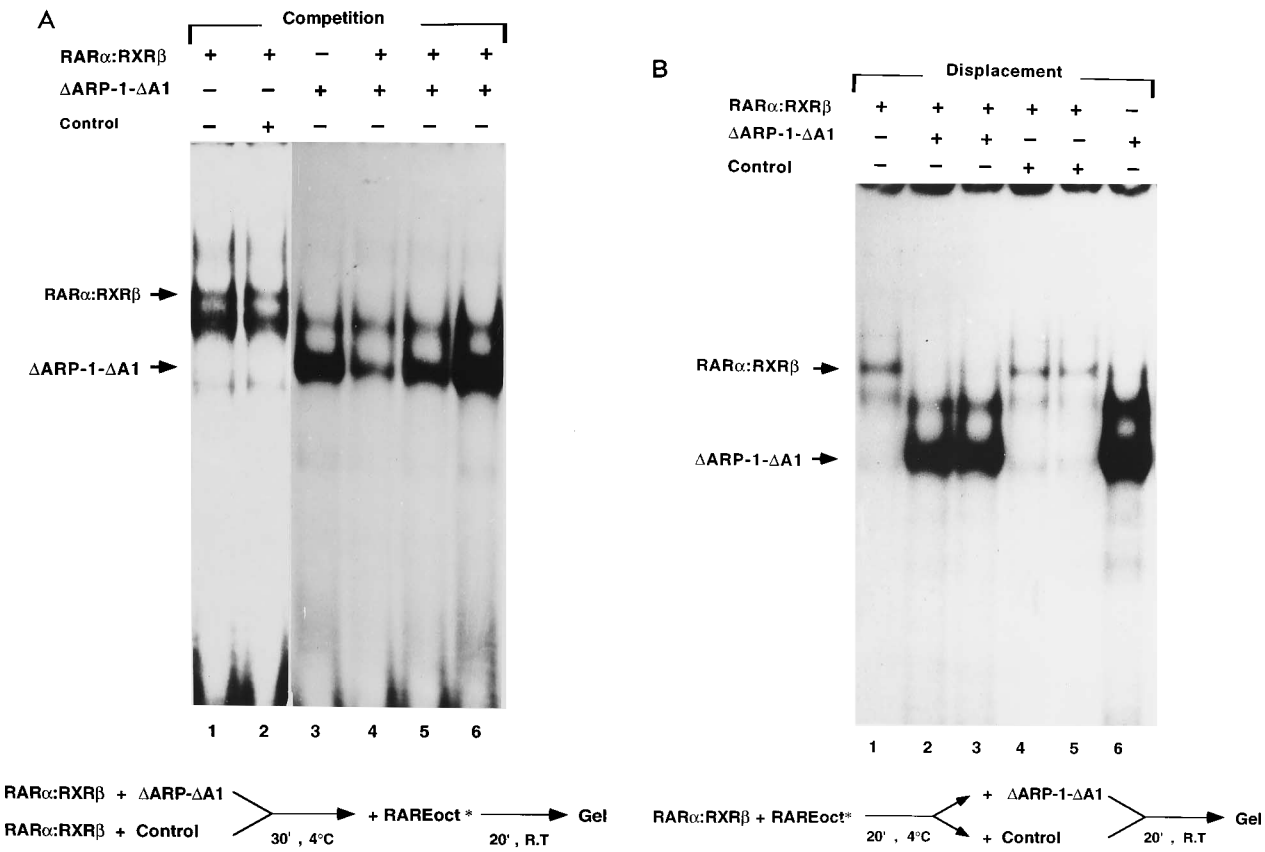


FIG. 8. ARP-1-ΔA1 competes with and displaces RARα:RXRβ heterodimers from the RAREoct site. (A) ARP-1-ΔA1 competes with RARα:RXRβ for the RAREoct site. RARα:RXRβ extracts were incubated with increasing amounts of extracts (0.5, 1, and 2 μg) containing competing ARP-1-ΔA1 (lanes 4 to 6) and ^{32}P-labeled RAREoct oligonucleotide. DNA-protein complexes were separated by gel electrophoresis. Lane 1, RARα:RXRβ-transfected Cos-1 extract; lane 2, RARα:RXRβ-transfected Cos-1 extract in the presence of a control extract; lane 3, ARP-1-ΔA1-transfected Cos-1 extract. The DNA-protein complex that migrates between the two marked complexes originates from the untransfected Cos-1 WCE and varies in strength. (B) Dissociation of RARα:RXRβ-RAREoct ternary complex by ARP-1-ΔA1. RARα and RXRβ (5 μg:5 μg)-containing extracts and ^{32}P-labeled RAREoct oligonucleotide were mixed, and ternary complexes were allowed to form at 4°C for 20 min. The reaction mixtures were transferred to room temperature (R.T), and increasing amounts of ARP-1-ΔA1-containing extracts (0.5 and 1 μg; lanes 2 and 3, respectively) or control Cos-1 extracts (0.5 and 1 μg; lanes 4 and 5, respectively) were added for 20 min prior to gel electrophoresis. Lane 1, RARα:RXRβ extract alone; lane 6, ARP-1-ΔA1 extract alone. The upper and lower arrows indicated the positions of the hetero- and homodimers formed, respectively.

RXRβ heterodimers from the RAREoct site. Since the RARα:RXRβ and ARP-1/COUP-TFII proteins exhibit similar mobilities in EMSA, we used an N-terminal deletion mutant of ARP-1 (designated ARP-1-ΔA1 [25]). The different electrophoretic mobilities of RARα:RXRβ- and ARP-1-ΔA1-DNA complexes facilitated their discrimination and identification. Competition for binding to the RAREoct site was analyzed by mixing a constant amount of extract containing RARα:RXRβ proteins (5 μg:5 μg) with an increasing amount of ARP-1-ΔA1 (Fig. 8A, lanes 4 to 6, 0.5, 1, and 2 μg, respectively) and analyzing their binding to a limited amount of labeled RAREoct oligonucleotide. RARα:RXRβ generated a complex which was completely abolished upon the addition of ARP-1-ΔA1 (Fig. 8A; compare lanes 1 and 2 with lanes 4 to 6). In contrast, increasing amounts of RARα:RXRβ heterodimers to 10 μg did not interfere with the formation of ARP-1-ΔA1-RAREoct complex (data not shown). These results are not due to differences in the amount of proteins expressed in the transfected Cos-1 cells, since WCE from the ARP-1-ΔA1- and RARα:RXRβ-transfected cells show similar complex formation on the RARE oligonucleotide isolated from the RARβ promoter (data not shown). These results are consistent with the above-described data showing the preferential binding of ARP-1/COUP-TFII to the RAREoct site. To extend on these findings, we analyzed whether ARP-1-ΔA1 could challenge an already formed complex between RARα:RXRβ heterodimers and the RAREoct DNA. A ternary RARα:RXRβ-RAREoct complex was allowed to form prior to the addition of increasing amounts of ARP-1-ΔA1 or control extract. Subsequently, the mixtures were incubated for an additional 20 min and the complexes were revealed by EMSA. The RARα:RXRβ-RAREoct ternary complex was stable for >1 h in the presence of control extracts. However, addition of an extract containing ARP-1-ΔA1 (0.5 to 1 μg, 10-fold less than the amount of RARα:RXRβ) virtually dissociated the RARα:RXRβ-RAREoct complex (Fig. 8B; compare lanes 1, 4, and 5 with lanes 2 and 3). This result indicates that the RARα:RXRβ-RAREoct complex is not a very strong complex but may have a fast off rate. Thus, even a low level of ARP-1-ΔA1 protein can displace the activator RARα:RXRβ from the RAREoct site. In conclusion, the combined competition and displacement data strongly suggest that the RAR:RXR heterodimers and the orphan receptors bind to the RAREoct site in a mutually exclusive manner.

ARP-1/COUP-TFII actively represses Oct-3/4 expression. To determine whether the inhibition of Oct-3/4 promoter ac-

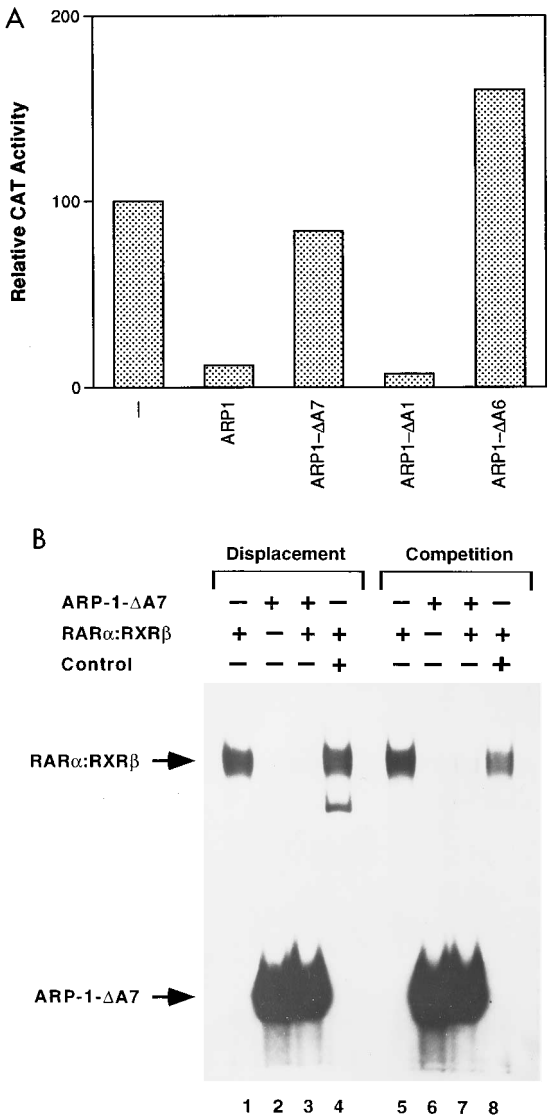


FIG. 9. ARP-1/COUP-TFII actively represses expression of the Oct-3/4 promoter. (A) P19 cells were cotransfected with p0.4oct-CAT reporter construct (10 μg) and a β-Gal-containing reference plasmid (0.5 μg) in the absence or presence of 5 μg of wild-type ARP-1, N-terminal-deleted ARP-1 (ARP-1-ΔA1), ARP-1 DNA-binding domain (ARP-1-ΔA7), and C-terminal-deleted ARP-1 (ARP-1-ΔA6). CAT activity was measured and quantitated as described in the legend to Fig. 1B. The percent conversion values corresponding to p0.4oct-CAT in the absence or presence of ARP-1 expression vectors in P19 cells (from left to right) are 7.5, 0.9, 6.3, 0.5, and 9.7, respectively. (B) The ARP-1-ΔA7 DNA-binding domain can compete with and displace RARα:RXRβ heterodimers from the RAREoct site. For the displacement experiments, RARα:RXRβ (5 μg:5 μg)-containing extracts and ^{32}P-labeled RAREoct oligonucleotide were mixed at 4°C for 20 min. The reaction mixtures were transferred to room temperature, and ARP-1-ΔA7 (ARP-1 DNA-binding domain only)-containing extract (1 μg; lane 3) or control Cos-1 extract (1 μg; lane 4) was added for 20 min prior to gel electrophoresis. For the competition experiments, RARα:RXRβ (5 μg:5 μg)-containing extracts were mixed with ARP-1-ΔA7-containing extract (1 μg; lane 7) or control Cos-1 extract (1 μg; lane 8). Lanes 1 and 5, RARα:RXRβ extract alone; lanes 2 and 6, ARP-1-ΔA7 extract alone. The positions of the various DNA-protein complexes are indicated. The free probe was run off the bottom of the gel to maximize separation of different DNA-binding complexes.

tivity was exerted by a passive mechanism, i.e., competition or displacement of a bound activator from the RAREoct site, or by an active repression mechanism caused by the bound ARP-1/COUP-TFII protein, we cotransfected P19 cells with the p0.4oct-CAT construct and various ARP-1 expression vectors (Fig. 9A). The expression vectors contained either the entire wild-type gene, the DNA-binding domain only (ΔA7), the N-terminal deletion mutant (ΔA1), or the C-terminal deletion mutant (ΔA6). As shown above, the N-terminal-truncated ARP-1 mutant was able to displace the RARα:RXRβ heterodimers from the RAREoct site (Fig. 8B) and, even more importantly, was able to repress p0.4oct-CAT activity in P19 cells (Fig. 9A). Thus, the ARP-1 N-terminal region is dispensable for Oct-3/4 repression. Interestingly, the C-terminal-truncated ARP-1 mutant which contains the DNA-binding domain was unable to repress Oct-3/4 expression. Our results thus show that the C-terminal region of ARP-1/COUP-TFII contains the silencing domain. Similarly, COUP-TFI, the thyroid receptor α isoform (TRα), TRβ, and RARα also possess active transcriptional silencing domains within their C-terminal ligand-binding domains (5). The ARP-1-ΔA7 expression vector, containing the DNA-binding domain alone, was unable to repress Oct-3/4 promoter activity (Fig. 9A). Since ARP-1-ΔA7 efficiently displaced and competed for RARα:RXRβ heterodimers from the RAREoct site (Fig. 9B; compare lanes 1 and 4 with lane 3 and lanes 5 and 8 with lane 7), we conclude that merely the displacement of an activator from the RAREoct site is insufficient to repress Oct-3/4 promoter activity. Thus, the orphan receptor is likely to interact with components of the Oct-3/4 transcriptional machinery, such as basal transcription factors or other factors that bind sites located in Oct-3/4 regulatory regions.

DISCUSSION

The Oct-3/4 gene is expressed at the earliest stages of embryogenesis and is repressed in subsequent stages (48, 51). Thus, it is a good candidate for involvement in regulation of initial decisions in differentiation. The discovery of the mechanisms which regulate Oct-3/4 transcription will probably add to our understanding of mammalian differentiation during the early stages of embryonic development.

Transcription of the Oct-3/4 gene is controlled by a complex interaction of positive and negative regulatory elements. We and others have recently characterized a regulatory element within the Oct-3/4 promoter, RAREoct, which reduces transcriptional activity of the Oct-3/4 promoter (43, 53). We have previously shown that in undifferentiated P19 cells, RAREoct functions either as a binding site for a positive regulator of transcription or as a modulator of a nearby transcriptional activator, probably Sp1. In contrast, in RA-differentiated P19 cells, the RAREoct site functions as a binding site for a negative regulator(s). While this report was being written, two groups of investigators reported that the ARP-1/COUP-TFII and EAR-3/COUP-TFI orphan receptors repress Oct-3/4 promoter activity in P19 cells (53, 56). We have shown that in both P19 and P19/RA cells, increasing the levels of ARP-1/COUP-TFII and EAR-3/COUP-TFI represses the Oct-3/4 promoter activity. Moreover, this repression is dose dependent and is mediated through the RAREoct site. Our mobility shift and DNase I footprinting assays show that EAR-3/COUP-TFI and ARP-1/COUP-TFII form very prominent specific complexes with RAREoct oligonucleotides and protect a specific region of the Oct-3/4 promoter. The data show that the RAREoct is a preferential binding site for EAR-3/COUP-TFI and ARP-1/COUP-TFII. As previously reported, ARP-1/COUP-TFII and EAR-3/COUP-TFI can heterodimerize (7). However, cotransfection of these two receptors with a reporter gene, regulated by the Oct-3/4 promoter, did not reveal better repression than by transfection of each receptor alone (data not shown). This

finding suggests that even if they do form heterodimers on the RAREoct DNA, these heterodimers are not more efficient at repression than the homodimers.

For the first time, we show that while ARP-1/COUP-TFII and EAR-3/COUP-TFI repress Oct-3/4 promoter activity, three different RAR:RXR heterodimers, RARα:RXRβ, RARβ:RXRα, and RARβ:RXRβ, bind and activate the Oct-3/4 promoter through the RAREoct site in a ligand-dependent manner in P19 cells. Interestingly, repression of Oct-3/4 promoter activity by each of the orphan receptors is abolished by increasing the levels of RARβ:RXRβ and RARα:RXRβ heterodimers. Moreover, activation of the Oct-3/4 promoter by the RAR:RXR proteins is eliminated by cotransfecting each of the orphan receptors. Our binding affinity studies indicate that RAREoct binds the orphan receptors with a higher affinity than the RARα:RXRβ heterodimers. This finding is in agreement with our data showing that the inhibition of the RARα:RXRβ-activated Oct-3/4 promoter is more effective with EAR-3/COUP-TFI and ARP-1/COUP-TFII than the activation of the orphan receptors repressed Oct-3/4 promoter by the RAR:RXR heterodimers.

Our study clearly demonstrates that following RA treatment of EC cells, the expression of ARP-1/COUP-TFII and EAR-3/COUP-TFI is strongly activated. Our results are in accordance with the findings that the C-terminal region of COUP-TFI was isolated from neurally differentiated P19 cells by using a subtractive hybridization approach (19). Thus, the copresence of RARα, RARβ, RXRβ, ARP-1/COUP-TFII, and EAR-3/COUP-TFI in P19/RA cells raises the interesting possibility that in differentiated EC cells, containing all of the positive and negative regulators, the net effect on transcription of Oct-3/4 depends on the concentration and affinity of these factors for the RAREoct site as well as on protein-protein interactions with other factors binding to proximal or distal regulatory elements. Thus, the Oct-3/4 gene having in its promoter the RAREoct site, which can interact with both the positive (RAR:RXR) and the negative (ARP-1/COUP-TFII and EAR-3/COUP-TFI) regulators, will be susceptible to the antagonistic action of these proteins. In EC cells, in which the orphan receptors are not expressed, the existing RARα:RXRβ heterodimers can assist in up-regulation of Oct-3/4 promoter activity. This suggestion is compatible with our previous published data showing that the RAREoct site contributes to the transcriptional activation of Oct-3/4 promoter in P19 cells (43). In contrast, in RA-differentiated EC cells, in which ARP-1/COUP-TFII and EAR-3/COUP-TFI, as well as various RAR:RXR heterodimers, are expressed, the orphan receptor binds to the RAREoct site with a stronger affinity, which allows these factors to displace the heterodimers from this site and to exert their transcriptional repression. Our results provide a strong basis for the concept that the RAREoct site is at least one of the points of integration of many diverse signalling pathways influencing Oct-3/4 gene expression.

We have previously shown that in RA-differentiated EC cells and in EC × fibroblast somatic cell hybrids, the shutdown of Oct-3/4 transcription is accompanied by de novo methylation of the 1.3-kb upstream regulatory region and a change in its chromatin structure (1). However, our data show that these changes follow suppression of Oct-3/4 mRNA accumulation and up-regulation of EAR-3/COUP-TFI and ARP-1/COUP-TFII expression. Following 24 h of RA treatment, Oct-3/4 expression is almost completely repressed and expression of the orphan receptors is strongly induced. De novo methylation and a change in the chromatin structure occur only after 4 to 7 days of RA treatment (reference 1 and data not shown). This allows for a particular window in time in which the various receptors can bind the RAREoct site to control Oct-3/4 promoter activity before the gene is structured in an inaccessible form.

Tissue-specific and developmentally regulated gene expression is accomplished by a complex interplay of transcriptional activators and negative regulating proteins. The mechanisms responsible for repression of eukaryotic gene expression are much less well understood than those involved in activation of transcription. In principle, repression of transcription can be achieved by interference with any step in transcriptional activation. Although the COUP element was initially characterized as a positive element, both EAR-3/COUP-TFI and ARP-1/COUP-TFII down-regulate various hormone-regulated genes through their hormone-responsive elements (5, 21, 29, 42, 58, 63). The ability of ARP-1/COUP-TFII and EAR-3/COUP-TFI to bind to diverse spatial arrangements of the A/GGGTCA repeats permits them to repress transcriptional activation of a number of genes regulated by the steroid-thyroid-RA receptor family (5, 7). It has previously been shown that these orphan receptors inhibit gene expression through a number of mechanisms. One mechanism employs direct competition with various transactivators such as HNF-4 and the estrogen receptor for binding to DNA (24, 29, 36). An alternative mechanism of inhibition utilizes a common feature in the molecular biology of TR, vitamin D_3 receptor, and RAR (in addition to binding to A/GGGTCA direct repeats), which is their requirement for a positive coregulator (RXR) in order to accomplish high-affinity DNA binding and maximal transcriptional activity (3, 15, 22, 26, 32, 65, 67). EAR-3/COUP-TFI and ARP-1/COUP-TFII can antagonize their transactivation by a mechanism involving squelching of the auxiliary RXR protein (21). Thus, the negative regulatory function of ARP-1/COUP-TFII and EAR-3/COUP-TFI is intrinsic to this subfamily and appears general for various response elements to which they bind.

Our current study shows that these orphan receptors have the ability to repress a developmentally important gene. There are a number of possible mechanisms whereby ARP-1/COUP-TFII and EAR-3/COUP-TFI could inhibit the activity of the Oct-3/4 promoter. Cotransfection of either ARP-1/COUP-TFII or EAR-3/COUP-TFI together with RXR and the p0.4oct-CAT reporter construct did not inhibit Oct-3/4 promoter activity any better than transfection of each orphan receptor alone (data not shown). Taken together, these results and those of binding experiments which did not show any detectable level of ARP-1/COUP-TFII:RXR heterodimers (data not shown) indicate that antagonism by the orphan receptors of the RAR:RXR transactivation of Oct-3/4 promoter activity is not through titration of the coregulator RXR. Our data clearly show that, in contrast to site C3P in the ApoCIII gene, which binds the activator (HNF-4) and the repressor (ARP-1) with similar affinities (36), the RAREoct site preferentially binds the orphan receptors. The strong correlation observed between effective binding of the orphan receptors to the RAREoct site and their repressive effect provides a very convincing argument that they repress transactivation of Oct-3/4 by RAR:RXR by a competitive binding mechanism. This view is further corroborated by our gel retardation assays, in which we observed that increasing ARP-1/COUP-TFII protein concentrations not only inhibited the binding of RARα:RXRβ heterodimers but also displaced a preformed ternary RAREoct-RARα:RXRβ complex, thus allowing the orphan receptors to bind.

Our studies demonstrating that ARP-1/COUP-TFII and EAR-3/COUP-TFI interfere with the binding and transactivation ability of RAR:RXR heterodimers raise the question of

how these orphan receptors inhibit Oct-3/4 expression following binding the RAREoct site. One possibility is that their ability to displace the activators from the RAREoct site is sufficient to inhibit transcription, i.e., a passive mechanism of inhibition. Our results demonstrating that the ARP-1 DNA-binding domain can displace RAR:RXR heterodimers from RAREoct but cannot repress the Oct-3/4 promoter most probably exclude this possibility. Alternatively, once bound to the RAREoct sequence, the orphan receptors may interfere with signals coming from upstream transcription factor-binding sites such as the Sp1-binding site and affect the basal transcription complex. A prerequisite for this type of repression is the simultaneous binding of a negative regulator and an activator protein to independent sites of the DNA template. Indeed, our DNase I footprint analysis shows that both the Sp1 site and the RAREoct site are occupied. Thus, ARP-1/COUP-TFII or EAR-3/COUP-TFI most probably does not antagonize the interaction of Sp1 with its binding site. Following binding to DNA, the orphan receptors can counteract the activator either by directly contacting the activator protein or by interacting with an adaptor factor which transmits the signal from the activator to the basal machinery complex. In addition, repression may result from direct interaction with one or more of the components of the basal transcription complex. It has previously been shown that COUP-TF exerts its transcriptional effects via the basal transcription factor TFIIB (18, 59). Our study indicates that for these types of interactions, the N-terminal domain of ARP-1/COUP-TFII is dispensable for Oct-3/4 repression. We have shown that, like COUP-TFI, TRα, TRβ, and RARα (5), the C-terminal region of ARP-1/COUP-TFII possesses an active silencing domain which is required for Oct-3/4 repression. Interestingly, two separate repression domains located at the amino- and carboxyl-terminal halves of ARP-1 were required in order to repress the ApoA1 gene expression (13).

While it seems likely that the interplay between RAR:RXR heterodimers, ARP-1/COUP-TFII, and EAR-3/COUP-TFI at the RAREoct site plays a major role in Oct-3/4 gene expression, other factors, for example, unidentified factors which bind to the RAREoct sequence or factors which bind to the adjacent Sp1 site or to the further removed enhancer element, will undoubtedly also be found to influence the regulation of this gene.

Data from several studies show that generally ARP-1/COUP-TFII and EAR-3/COUP-TFI exert negative effects on transcription of various genes, including Oct-3/4. Our data show that in RA-differentiated cells, these orphan receptors are highly expressed. However, previously published data show that there are several genes that are activated in P19/RA cells through their RAREs (8, 9, 45). This finding raises the question of how these genes are activated in the presence of these negatively regulating orphan receptors which can compete with the RAR:RXR heterodimers and eliminate activation. It is likely that the net effect on transcription of the different genes depends on the concentration and affinity of the different receptors for their recognition sites and must take into account the full context of the promoter-enhancer elements. In the case of the RAREoct sequence located in the Oct-3/4 promoter, the binding affinity for the orphan receptors is higher than the affinity of the RAR:RXR heterodimers. Another possibility is that these orphan receptors sensitize certain promoters for RA, as has been proposed for the ApoA1 promoter (63). Alternatively, ARP-1/COUP-TFII and EAR-3/COUP-TFI may also function as positive regulators on certain response elements in the presence of an as yet unidentified ligand or when activated through other pathways (23, 38, 44).

Our study clearly demonstrates that following RA treatment of EC cells (both F9 [data not shown] and P19 cells), ARP-1/COUP-TFII and EAR-3/COUP-TFI expression is up-regulated. It is tempting to suggest that these orphan receptors may be regulated by RA-inducible RARs such as RARβ. It is instructive to note that only after 24 h of RA treatment do RARβ transcripts reach high levels of expression, and it is possible that only at these levels are they capable of up-regulating ARP-1/COUP-TFII and EAR-3/COUP-TFI expression.

Most interestingly, the kinetics of Oct-3/4 repression inversely correlates with the kinetics of activation of the orphan receptors. More explicitly, we have shown that following 24 h of RA treatment, there are no Oct-3/4 transcripts and there is an impressive induction in expression of the orphan receptors. As noted above, Oct-3/4 is expressed during early embryogenesis. By 8.5 days postfertilization, Oct-3/4 is almost undetectable in somatic cells but is found in both male and female primordial germ cells (48, 51). Interestingly, in situ hybridization experiments showed that the expression of mouse COUP-TFI and II began around 8.5 days postcoitum (46). Thus, in both in vitro and in vivo studies, Oct-3/4 expression is down-regulated when ARP-1/COUP-TFII and EAR-3/COUP-TFI expression is up-regulated.

The morphogen RA has dramatic effects on development, such as establishing the anterior-posterior axis in the developing chick limb bud (10, 35, 57). The ability of ARP-1/COUP-TFII and EAR-3/COUP-TFI to take part in RA control mechanisms implicates these orphan receptors as playing a role in development and differentiation. Our study showing that these orphan receptors regulate the expression of the Oct-3/4 gene substantiates this assumption, since Oct-3/4 most probably plays an important role in early embryogenesis. This finding raises the possibility that these orphan receptors participate in building up and regulating a hierarchy of a network of transcription factors which establish normal development. EAR-3/COUP-TFI and ARP-1/COUP-TFII are remarkably homologous to the *Drosophila* seven-up gene. The seven-up gene is involved in the control of cell fate during the generation of neuronal diversity in the eye (38). It is required in photoreceptor cell precursors during eye development, and in its absence, these cells are transformed toward a different cell fate. The conservation between the seven-up and COUP genes enforces the suggestion that the orphan receptors play a role in development. It is tempting to speculate that the functions of Oct-3/4 and these orphan receptors are contradictory. While a possible role for the Oct-3/4 gene is to maintain the pluripotency of EC cells, the function of the orphan receptors may be to specify the fate of RA-treated EC cells.

ACKNOWLEDGMENTS

We thank Pierre Chambon for the RARα, RARβ, RXRα, RXRβ, and RXRγ expression plasmids; John Ladias for the unique ARP-1/COUP-TFII and EAR-3/COUP-TFI probes and the pMARP-1, pMEAR3, ΔA1, ΔA7, and ΔA6 plasmids, and Ming-Jer Tsai for the polyclonal anti-COUP-TFI antibodies. We thank Oded Meyuhas and Joseph Shlomai for a constructive critical reading of the manuscript. We acknowledge the assistance of Gillian Hirst in preparing the manuscript.

This research was supported by the Ehrlich Center for the Study of White Cells, by a career development award from the Israel Cancer Research Fund to Y.B., and by a grant provided by the Swiss Friends of the Hebrew University-Hadassah Medical School, Jerusalem, Israel.

REFERENCES

1. **Ben-Shushan, E., E. Pikarsky, A. Klar, and Y. Bergman.** 1993. Extinction of Oct-3/4 gene expression in embryonal carcinoma × fibroblast somatic cell hybrids is accompanied by changes in the methylation status, chromatin

structure, and transcriptional activity of the Oct-3/4 upstream region. Mol. Cell. Biol. **13:**891–901.
2. **Berrodin, T. J., M. S. Marks, K. Ozato, E. Linney, and M. A. Lazar.** 1992. Heterodimerization among thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, chicken ovalbumin upstream promoter transcription factor and endogenous liver protein. Mol. Endocrinol. **6:**1468–1478.
3. **Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg.** 1992. RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. **11:**1409–1418.
4. **Chambon, P., A. Zelent, M. Petkovich, C. Mendelsohn, P. Leroy, A. Krust, P. Kastner, and N. Brand.** 1991. The family of retinoic acid nuclear receptors, p. 38–45. *In* J.-H. Saurat (ed.), Retinoids: 10 years on. S. Karger, Basel.
5. **Cooney, A. J., X. Leng, S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai.** 1993. Multiple mechanisms of chicken ovalbumin upstream promoter transcription factor-dependent repression of transactivation by the vitamin D, thyroid hormone, and retinoic acid receptors. J. Biol. Chem. **268:**4152–4160.
6. **Cooney, A. J., S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai.** 1991. Chicken ovalbumin upstream promoter transcription factor binds to a negative regulatory region in the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **65:**2853–2860.
7. **Cooney, A. J., S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai.** 1992. Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D_3, thyroid hormone, and retinoic acid receptors. Mol. Cell. Biol. **12:**4153–4163.
8. **de-Groot, R. P., J. Schoorlemmer, S. T. van Genesen, and W. Kruijer.** 1990. Differential expression of jun and fos genes during differentiation of mouse P19 embryonal carcinoma cells. Nucleic Acids Res. **18:**3195–3202.
9. **Edwards, S. A., T. Darland, R. Sosnowski, M. Samuels, and E. D. Adamson.** 1991. The transcription factor, Egr-1, is rapidly modulated in response to retinoic acid in P19 embryonal carcinoma cells. Dev. Biol. **148:**165–173.
10. **Eichele, G.** 1989. Retinoids and vertebrate limb pattern formation. Trends Genet. **5:**246–251.
11. **Evans, R. M.** 1988. The steroid and thyroid hormone receptor family. Science **240:**889–895.
12. **Fridovich-Keil, J. L., J. M. Gudas, I. B. Bryan, and A. B. Pardee.** 1991. Improved expression vectors for eukaryotic promoter/enhancer studies. BioTechniques **11:**572–579.
13. **Ge, R., M. Rhee, S. Malik, and S. K. Karathanasis.** 1994. Transcriptional repression of apolipoprotein AI gene expression by orphan receptor ARP-1. J. Biol. Chem. **269:**13185–13192.
14. **Green, S., and P. Chambon.** 1988. Nuclear receptors enhance our understanding of transcription regulation. Trends Genet. **4:**309–314.
15. **Hermann, T., B. Hoffmann, X. K. Zhang, P. Tran, and M. Pfahl.** 1992. Heterodimeric receptor complexes determine 3,5,3′-triodothyronine and retinoid signaling specificities. Mol. Endocrinol. **6:**1153–1162.
16. **Heyman, R. A., D. J. Mangelsdorf, J. A. Dyck, R. B. Stein, G. Eichele, R. M. Evans, and C. Thaller.** 1992. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell **68:**397–406.
17. **Hwung, Y.-P., D. T. Crowe, L.-H. Wang, S. Y. Tsai, and M.-J. Tsai.** 1988. The COUP transcription factor bind to an upstream promoter element of the rat insulin II gene. Mol. Cell. Biol. **8:**2070–2077.
18. **Ing, N. H., J. M. Beekman, S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley.** 1992. Members of the steroid hormone superfamily interact with TFIIB (S300-II). J. Biol. Chem. **267:**17617–17623.
19. **Jonk, L. J. C., M. E. J. de Jonge, J. M. A. Vervaart, S. Wissink, and W. Kruijer.** 1994. Isolation and developmental expression of retinoic-acid-induced genes. Dev. Biol. **161:**604–614.
20. **Kastner, P., N. Brand, A. Krust, P. Leroy, C. Mendelsohn, M. Petkovitch, A. Zelent, and P. Chambon.** 1992. Retinoic acid nuclear receptors, p. 75–88. *In* J. R. Hinchcliffe et al. (ed.), Developmental patterning of the vertebrate limb. Plenum Press, New York.
21. **Kliewer, S. A., K. Umesono, R. A. Heyman, D. J. Mangelsdorf, J. A. Dyck, and R. M. Evans.** 1992. Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc. Natl. Acad. Sci. USA **89:**1448–1452.
22. **Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans.** 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. Nature (London) **355:**446–449.
23. **Koenig, R. J., M. A. Lazar, R. A. Hodin, G. A. Brent, P. R. Larsen, W. W. Chin, and D. D. Moore.** 1989. Inhibition of thyroid hormone action by non-hormone binding c-erbA protein generated by alternative mRNA splicing. Nature (London) **337:**659–661.
24. **Ladias, J. A. A., M. Hadzopoulou-Cladaras, D. Kardassis, P. Cardot, J. Cheng, V. Zannis, and C. Cladaras.** 1992. Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3. J. Biol. Chem. **267:**15849–15860.
25. **Ladias, J. A. A., and S. K. Karathanasis.** 1991. Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. Science **251:**561–565.
26. **Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J.-Y. Chen, A. Staub, J.-M. Garnier, S. Mader, and P. Chambon.** 1992. Purification, cloning and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68:**377–395.
27. **Leroy, P., A. Krust, A. Zelent, C. Mendelsohn, J.-M. Garnier, P. Kastner, A. Dierich, and P. Chambon.** 1991. Multiple isoforms of the mouse retinoic acid receptor α are generated by alternative splicing and differential induction by retinoic acid. EMBO J. **10:**59–69.
28. **Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, and J. F. Grippo.** 1992. 9-Cis retinoic acid steroisomer binds and activates the nuclear receptor RXRα. Nature (London) **355:**359–361.
29. **Liu, Y., N. Yang, and C. T. Teng.** 1993. COUP-TF acts as a competitive repressor for estrogen receptor-mediated activation of the mouse lactoferrin gene. Mol. Cell. Biol. **13:**1836–1846.
30. **Liu, Y. H., and C. T. Teng.** 1992. Estrogen response module of the mouse lactoferrin gene contains overlapping chicken ovalbumin upstream promoter transcription factor and estrogen receptor binding elements. Mol. Endocrinol. **6:**355–364.
31. **Mangelsdorf, D. J., U. Borgmeyer, R. A. Heyman, J. Y. Zhov, E. S. Ong, A. E. Oro, A. Kakizuka, and R. M. Evans.** 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. **6:**329–344.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato.** 1992. H-2RIIBP (RXRβ) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. EMBO J. **11:**1419–1435.
34. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
35. **Mendelsohn, C., E. Ruberte, and P. Chambon.** 1992. Retinoids in vertebrate limb development. Dev. Biol. **152:**50–61.
36. **Mietus-Snyder, M., F. M. Sladek, G. S. Ginsburg, C. F. Kuo, J. A. A. Ladias, J. E. Darnell, Jr., and S. K. Karathanasis.** 1992. Antagonism between apolipoprotein AI regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol. Cell. Biol. **12:**1708–1718.
37. **Miyajima, N., Y. Kadowaki, S.-I. Fukushige, S.-I. Shimizu, K. Semba, Y. Yamanashi, K.-I. Matsubara, K. Yoyoshima, and T. Yamamoto.** 1988. Identification of two novel members of erbA superfamily by molecular cloning: the gene products of the two are highly related to each other. Nucleic Acids Res. **16:**11057–11074.
38. **Mlodzik, M., Y. Hiromi, U. Weber, C. S. Goodman, and G. M. Rubin.** 1990. The Drosophila seven-up gene, a member of the steroid receptor gene superfamily, controls photoreceptor cell fates. Cell **60:**211–224.
39. **Murinaga, Y., T. Franceschini, S. Inouye, and M. Inouye.** 1984. Improvement of oligonucleotide-directed site-specific mutagenesis using double-stranded DNA. Bio/Technology **2:**636–639.
40. **Okamoto, K., H. Okazawa, A. Okuda, M. Sakai, M. Muramatsu, and H. Hamada.** 1990. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. Cell **60:**461–472.
41. **Okazawa, H., K. Okamoto, F. Ishino, T. Ishino-Kaneko, S. Takeda, Y. Toyoda, M. Muramatsu, and H. Hamada.** 1991. The oct-3 gene, a gene for an embryonic transcription factor, is controlled by a retinoic acid repressible enhancer. EMBO J. **10:**2997–3005.
42. **Paulweber, B., F. Sandhofer, and B. Levy-Wilson.** 1993. The mechanism by which the human apolipoprotein B gene reducer operates involves blocking of transcriptional activation by hepatocyte nuclear factor 3. Mol. Cell. Biol. **13:**1534–1546.
43. **Pikarsky, E., H. Sharir, E. Ben-Shushan, and Y. Bergman.** 1994. Retinoic acid represses Oct-3/4 gene expression through several retinoic acid-responsive elements located in the promoter-enhancer region. Mol. Cell. Biol. **14:**1026–1038.
44. **Power, R. F., J. P. Lydon, O. M. Conneely, and B. W. O'Malley.** 1991. Dopamine activation of an orphan of the steroid receptor superfamily. Science **252:**1546–1548.
45. **Pratt, M. A. C., J. Kralova, and M. W. McBurney.** 1990. A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. Mol. Cell. Biol. **10:**6445–6453.
46. **Qiu, Y., A. J. Cooney, S. C. Kuratani, S. Y. Tsai, and M.-J. Tsai.** 1994. Differential expression of COUP-TFI and II in developing mouse central nervous system. J. Cell. Biochem. **18**(Suppl. B)**:**335.
47. **Rosenfeld, M. G.** 1991. POU-domain transcription factors: Pou-er-ful developmental regulators. Genes Dev. **5:**897–907.
48. **Rosner, M. H., M. A. Vigano, K. Ozato, P. M. Timmons, F. Poirier, P. W. J. Rigby, and L. M. Staudt.** 1990. A POU-domain transcription factor in early stem cell and germ cells of the mammalian embryo. Nature (London) **345:**686–692.
49. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51:**660–672.
50. **Schöler, H. R., R. Balling, A. K. Hatzopoulos, N. Suzuki, and P. Gruss.** 1989.

Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J. **8:**2551–2557.
51. **Schöler, H. R., G. R. Dressler, R. Balling, H. Rohdewohld, and P. Gruss.** 1990. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. EMBO J. **9:**2185–2195.
52. **Schöler, H. R., S. Ruppert, N. Suzuki, K. Chowdhury, and P. Gruss.** 1990. New type of POU domain in germ line-specific protein Oct-4. Nature (London) **344:**435–439.
53. **Schoorlemmer, J., A. van-Puijenbroek, M. van den Eijnden, L. Jonk, C. Pals, and W. Kruijer.** 1994. Characterization of a negative retinoic acid response element in the murine Oct-4 promoter. Mol. Cell. Biol. **14:**1122–1136.
54. **Shimazaki, T., H. Okazawa, H. Fujii, M. Ikeda, K. Tamai, R. D. G. McKay, M. Muramatsu, and H. Hamada.** 1993. Hybrid cell extinction and re-expression of Oct-3 function correlates with differentiation potential. EMBO J. **12:**4489–4498.
55. **Sompayrac, L. M., and K. J. Danna.** 1981. Efficient injection of monkey cells with DNA of simian virus 40. Proc. Natl. Acad. Sci. USA **78:**7575–7578.
56. **Sylvester, I., and H. R. Schöler.** 1994. Regulation of the Oct-4 gene by nuclear receptors. Nucleic Acids Res. **22:**901–911.
57. **Tabin, C. J.** 1991. Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. Cell **66:**199–217.
58. **Tran, P., X.-K. Zhang, G. Salbert, T. Hermann, J. M. Lehmann, and M. Pfahl.** 1992. COUP orphan receptors are negative regulators of retinoic acid response pathways. Mol. Cell. Biol. **12:**4666–4676.
59. **Tsai, S. Y., I. Sagami, H. Wang, M.-J. Tsai, and B. W. O'Malley.** 1987. Interactions between a DNA-binding transcription factor (COUP) and a non-DNA binding factor (S300II). Cell **50:**701–709.
60. **Wang, L.-H., N. H. Ing, S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai.** 1991. The COUP-TF compose a family of functionally related transcription factors. Gene Expression **1:**207–216.
61. **Wang, L.-H., S. Y. Tsai, R. G. Cook, W. G. Beattie, M.-J. Tsai, and B. W. O'Malley.** 1989. COUP transcription factor is a member of the steroid receptor superfamily. Nature (London) **340:**163–166.
62. **Wegner, M., D. W. Drolet, and M. G. Rosenfeld.** 1993. POU-domain proteins: structure and function of developmental regulators. Curr. Opin. Cell Biol. **5:**488–498.
63. **Widom, R. L., M. Rhee, and S. K. Karathanasis.** 1992. Repression by ARP-1 sensitizes apolipoprotein AI gene responsiveness to RXRα and retinoic acid. Mol. Cell. Biol. **12:**3380–3389.
64. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76:**1373–1376.
65. **Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Näär, S. Yun Kim, J.-M. Boutin, C. K. Glass, and M. Rosenfeld.** 1991. RXRβ: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67:**1251–1266.
66. **Zelent, A., A. Krust, M. Petkovich, P. Kastner, and P. Chambon.** 1989. Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin. Nature (London) **339:**714–717.
67. **Zhang, X-K., B. Hoffmann, P. B.-V. Tran, G. Graupner, and M. Pfahl.** 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (London) **355:**441–446.