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Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization

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The diverse function of retinoic acid (RA) is mediated by its nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). However, the RA response is often lost in cancer cells that express the receptors. Previously, it was demonstrated that the RA response is regulated by the COUP-TF orphan receptors. Here, we present evidence that nur77, another orphan receptor whose expression is highly induced by phorbol esters and growth factors, is involved in modulation of the RA response. Expression of nur77 enhances ligand-independent transactivation of RA response elements (RAREs) and desensitizes their RA responsiveness. Conversely, expression of COUP-TF sensitizes RA responsiveness of RAREs by repressing their basal transactivation activity. Unlike the effect of COUP-TFs, the function of nur77 does not require direct binding of nur77 to the RAREs, but is through interaction between nur77 and COUP-TFs. The interaction occurs in solution and results in inhibition of COUP-TF RARE binding and transcriptional activity. Unlike other nuclear receptors, a large portion of the carboxy-terminal end of nur77 is not required for its interaction with COUP-TF. In human lung cancer cell lines, COUP-TF is highly expressed in RA-sensitive cell lines while nur77 expression is associated with RA resistance. Stable expression of COUP-TF in nur77-positive, RA-resistant lung cancer cells enhances the inducibility of RARβ gene expression and growth inhibition by RA. These observations demonstrate that a dynamic equilibrium between orphan receptors nur77 and COUP-TF, through their heterodimerization that regulates COUP-TF RARE binding, is critical for RA responsiveness of human lung cancer cells.

Keywords: lung cancer/orphan receptors/receptor dimerization/retinoic acid receptors/retinoic acid sensitivity

Introduction

Retinoic acid (RA) and its natural and synthetic vitamin A derivatives, retinoids, are known to regulate a broad range of biological processes, and are used currently in the treatment of epithelial cancer and promyelocytic leukemia (Gudas *et al.*, 1994; Hong and Itri, 1994). However, retinoid resistance associated with many different types of cancer has prevented retinoids from further application (Warrell *et al.*, 1993; Hong and Itri, 1994). The effects of retinoids are mediated mainly by two classes of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily that also includes a number of orphan receptors whose ligands and function remain to be determined. They modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs). Some of the target genes are RARs themselves, including the RARβ gene where a RARE (βRARE) was identified in its promoter region, which mediates up-regulation of RARβ by RA in many different cell types (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990).

Although the expression of RARs and RXRs is essential for the RA response, we and others recently have demonstrated that it is not sufficient to render RA target genes responsive to RA (van der Leede *et al.*, 1993; Zhang *et al.*, 1994; Kim *et al.*, 1995). In lung cancer cell lines, RARs and RXRs are well expressed, but the majority of the cell lines are RA resistant, and RA-responsive genes, such as the RARβ gene, could not be induced by RA (Zhang *et al.*, 1994). The loss of RARβ inducibility by RA is particularly interesting since we have observed recently that up-regulation of the RARβ gene by RA correlates with RA-induced growth inhibition in breast cancer cell lines (Liu *et al.*, 1996) and in lung cancer cell lines (Zhang *et al.*, 1996). In RA-sensitive cancer cell lines, expression of RARβ is strongly induced by RA. In contrast, RA had little effect on RARβ expression in RA-resistant cancer cell lines. *In vivo*, up-regulation of RARβ is associated with clinical response in patients with pre-malignant oral lesions (Lotan *et al.*, 1995). In the course of investigating the mechanism by which RARβ is not induced by RA, we found that the expression of transfected RARs and/or RXRs could not restore RA responsiveness of the βRARE in certain lung cancer cell lines (Zhang *et al.*, 1994). These observations suggest that sensitivity to RA of lung cancer cells is also influenced by factors other than RARs and RXRs. One of the factors known to regulate the RA response is COUP-TF. COUP-TF is encoded by two distinct genes, COUP-TFI (ear-3) (Miyajima *et al.*, 1988; Wang *et al.*, 1989) and COUP-TFII (ARP-1) (Ladias and Karathanasis, 1991), that are orphan members of the nuclear receptor superfamily (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). Several studies recently have demonstrated that COUP-TF can repress transcription induced by a number of nuclear receptors including RARs, thyroid hormone receptors (TRs) and vitamin D receptor (VDR)

(Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992), probably due to its competition for DNA binding of the receptors. The binding specificity of COUP-TFs exhibits a strong preference for those bound by retinoid receptors, suggesting that COUP-TFs are probably involved in the regulation of RA target genes.

Nur77 (also known as NGFI-B and TR3) (Chang and Kokontis, 1988; Hazel *et al.*, 1988; Milbrandt, 1988) is another orphan member of the nuclear receptor superfamily. It is induced rapidly by a variety of growth stimuli, including growth factors and phorbol esters (Hazel *et al.*, 1988; Milbrandt, 1988; Williams and Lau, 1993; Lim *et al.*, 1995). How nur77 functions to mediate the growth signaling remains largely unknown. Nur77 binds to its recognition element (NBRE) as a monomer (Wilson *et al.*, 1991). The NBRE consists of the half-site binding motif (AGGTCA) of RAR/TR/VDR with two additional adenine nucleotides at its 5′ end (AAAGGTCA) (Wilson *et al.*, 1991). Interestingly, such sequences are found in the RARβ gene promoter and are located within the βRARE (Perlmann and Jansson, 1995). Investigation of the binding of nur77 on the βRARE demonstrates that nur77 can bind to the βRARE as a heterodimer with RXR (Forman *et al.*, 1995; Perlmann and Jansson, 1995). These observations suggest that nur77 may be involved in the regulation of RARβ gene expression and may function to mediate the interaction between retinoid and growth signalings (reviewed in Leblanc and Stunnenberg, 1995).

In the course of investigating the effect of nur77 on RA-induced RARβ gene expression, we found that nur77 could significantly enhance the transactivation activity of RAREs in a RA and RARE binding-independent manner. By using a variety of approaches, we demonstrate that the effect of nur77 is due to inhibition of COUP-TF RARE binding through direct protein–protein interaction. Transient transfection analysis reveals that COUP-TF RARE binding functions to sensitize the RA responsiveness of RAREs and, conversely, that nur77 desensitizes RAREs through its ability to inhibit COUP-TF RARE binding. In human lung cancer cell lines, loss of RA sensitivity is associated with overexpression of nur77 and/or low expression levels of COUP-TF, and can be restored by introduction and expression of COUP-TF. These results reveals a novel regulatory mechanism established through heterodimerization of orphan receptors nur77 and COUP-TF, that is expected to play an important role in the regulation of retinoid sensitivity of lung cancer cells, and in the cross-talk between growth factors and vitamin A signal transduction pathways in the cells.

Results

Nur77 enhances RARE activity in an RA-independent manner

We have shown recently that induction of RARβ by RA mediates the growth inhibitory effects of retinoids in human breast cancer and lung cancer cells (Liu *et al.*, 1996; Zhang *et al.*, 1996). RA-induced RARβ expression is mediated mainly by the βRARE in its promoter. To investigate the effect of nur77 on the transactivation of the βRARE, the βRARE-tk-CAT that contains the βRARE cloned into $pBLCAT_2$ (Hoffmann *et al.*, 1990) was used as a reporter gene and was transiently transfected into CV-1 cells. When nur77 expression vector was co-transfected, both all-*trans* RA- and 9-*cis* RA-induced reporter gene activities were enhanced in a concentration-dependent manner (Figure 1). Co-transfection of 200 ng of nur77 expression vector resulted in an ~2-fold increase of the reporter activity when cells were treated with all-*trans* RA. Surprisingly, the basal transcription of the reporter was even greatly increased, with ~5-fold enhancement. The effect of nur77 was specific to the βRARE because addition of nur77 did not show any activity on the parental $pBLCAT_2$ reporter. To investigate the possibility that the nur77 response is due to the presence of an NBRE within the βRARE, we changed two adenines in the spacing region of the βRARE to mutate the NBRE (Figure 1A). The mutations introduced do not affect the consensus half-site binding motifs of RAR/RXR heterodimers. The resulting element (ΔβRARE) was cloned into $pBLCAT_2$ and used as a reporter. When the reporter was analyzed, we observed a similar increase in its basal transcription by co-transfection of nur77 expression vector (Figure 1). This observation suggests that the presence of an NBRE in the βRARE is not essential for the enhancing effect of nur77. To determine whether the enhancing effects of nur77 could be extended to other hormone response elements, reporter constructs containing the tk promoter linked to either TREpal, ApoAI-RARE, CRBPI-RARE, lactoferrin-RARE, CRBPII-RARE, a thyroid hormone-specific response element (MHC-TRE) or a CAT reporter containing the RARβ gene promoter from –60 to +70, including the βRARE (Hoffmann *et al.*, 1990), were transfected into CV-1 cells with or without nur77 expression vector. Similarly to the effect on the βRARE, various degrees of enhancement by nur77 were observed with all the reporter constructs except the reporter containing MHC-TRE (Table I), suggesting that the effect of nur77 may be specific to RAREs. Thus, nur77 can enhance the transactivation of various RAREs in an RA-independent manner.

The effect of nur77 on RAREs does not require direct nur77–RARE interaction

To investigate whether the enhancement of RARE activity by nur77 is due to its binding to the elements, gel retardation assays were performed. When the βRARE was used as a probe, nur77 alone did not exhibit clear binding (Figure 2A). However, a strong complex was formed when nur77 was mixed with RXR. The complex could be upshifted by anti-nur77 antibody and abolished by anti-RXR antibody, demonstrating that the complex represents RXR/nur77 heterodimers. When the ΔβRARE was used as a probe, we did not see any binding of the RXR/nur77 heterodimers (Figure 2B). As a control, RXR/RAR heterodimers formed a strong complex with the element. These data indicate that the integrity of the NBRE within the βRARE is required for efficient RXR/nur77 binding. We also analyzed the binding of nur77 to other RAREs, such as TREpal, CRBPI-RARE, CRBPII-RARE and ApoAI-RARE, and we did not detect any binding of nur77 to these elements either in the presence or absence of RXR or RAR, except a weak RXR/nur77 heterodimer binding to the CRBPII-RARE (data not shown). Together, these results indicate that nur77 enhances the activities of different RAREs via a mechanism that is unlikely to involve a direct nur77/RARE interaction.

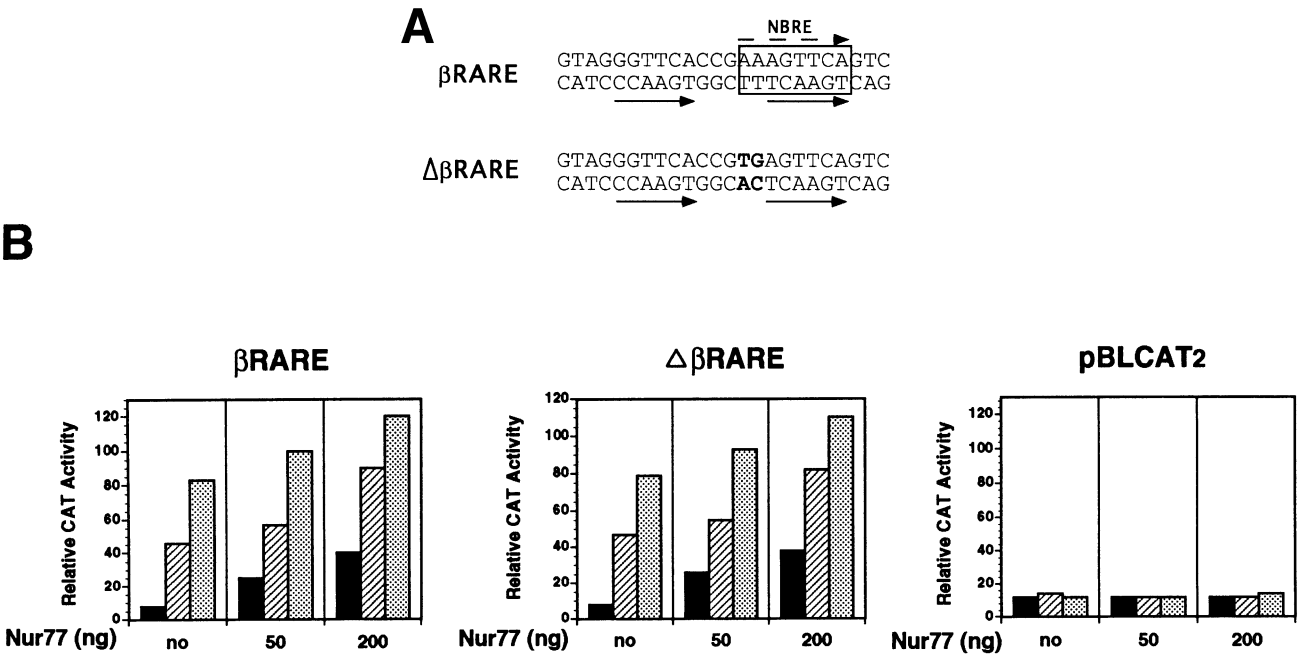

Fig. 1. RA-independent enhancement of RARE activities by nur77. (**A**) Sequence comparison of βRARE and ΔβRARE. Arrows indicate receptor-binding core motifs. The nur77 binding site (NBRE) is boxed and is also indicated by the dashed arrow. Two nucleotides (in bold) of the NBRE were mutated in the ΔβRARE. (**B**) Nur77 promotes βRARE and ΔβRARE activities. CV-1 cells were transfected with 100 ng of the indicated CAT reporter gene together with the indicated amounts of nur77 expression vector. Cells were treated with either all-*trans* RA (striped bar), 9-*cis* RA (dotted bar) or no hormone (filled bar), and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. Data shown represent the means of three independent experiments.

Table I. Nur77 activities on various RAREs and TRE

Reporter	Fold induction	
	–Ligand	+Ligand
RARβ promoter	4.5	4.1
TREpal	4.6	2.9
ApoAI-RARE	3.5	3.0
CRBPI-RARE	7.9	4.1
CRBPII-RARE	4.8	4.0
Lactoferrin-RARE	8.3	6.6
MHC-TRE	1.1	1.2

CV-1 cells were transfected with 100 ng of CAT reporter genes containing the indicated RARE or TRE together with 200 ng of nur77 expression vector. Cells were treated with either all-*trans* RA (10^{-7} M) (for RAREs), thyroid hormone (10^{-7} M) (for MHC-TRE) or no hormone, and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. Fold induction represents the ratio between relative CAT activity before and after transfection of nur77.

Nur77 inhibits COUP-TF DNA binding

The above data suggest that nur77 may function to repress the activity of an inhibitor on RAREs, thereby alleviating its inhibition. We then investigated the possibility that nur77 affects the binding of COUP-TFs that are known to bind to and restrict transcription of various RAREs (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We first examined the effect of nur77 on COUP-TF binding to the βRARE. COUP-TFI or COUP-TFII formed a strong complex with the βRARE (Figure 3A). However, when nur77 protein was added, the COUP-TF–RARE binding complex was inhibited. The inhibition was very efficient in that a 1 M excess amount of nur77 significantly inhibited the COUP-TF binding and was also specific, as a similar amount of RAR had no effect on the binding (Figure 3A). This result suggests that nur77 may interact with COUP-TF, resulting in formation of nur77/COUP-TF heterodimers that cannot bind to the βRARE. We next investigated whether this interaction could affect nur77/RXR heterodimer binding to the βRARE. When COUP-TFI or COUP-TFII was incubated with nur77 and RXR, the binding of RXR/nur77 to the βRARE was also inhibited efficiently (Figure 3B). A 2 M excess amount of COUP-TFI or COUP-TFII was sufficient to inhibit nur77/RXR heterodimer binding. When a larger amount of COUP-TFI or COUP-TFII was used, the nur77/RXR heterodimer binding was completely inhibited and binding of COUP-TF appeared. Thus, nur77 and COUP-TF can inhibit each other's DNA binding to the βRARE. Nur77 could also inhibit COUP-TF binding to other RAREs, such as TREpal, CRBPI-RARE, CRBPII-RARE, and ApoAI-RARE, although the efficiency of inhibition varied among these elements (data not shown). Together, these data demonstrate that inhibition of COUP-TF DNA binding by nur77 is probably responsible for its enhancement of transactivation activity of RAREs. This is supported by our observation that COUP-TF could not bind to the MHC-TRE (data not shown) which did not show any response to nur77 (Table I).

Interaction of nur77 and COUP-TF in solution

To provide evidence that inhibition of COUP-TF DNA binding by nur77 on RAREs was due to a direct interaction of nur77 and COUP-TF in solution, we first performed an immunoco-precipitation assay using anti-nur77 antibody (Figure 4A). When ^{35}S-labeled COUP-TFI or COUP-TFII

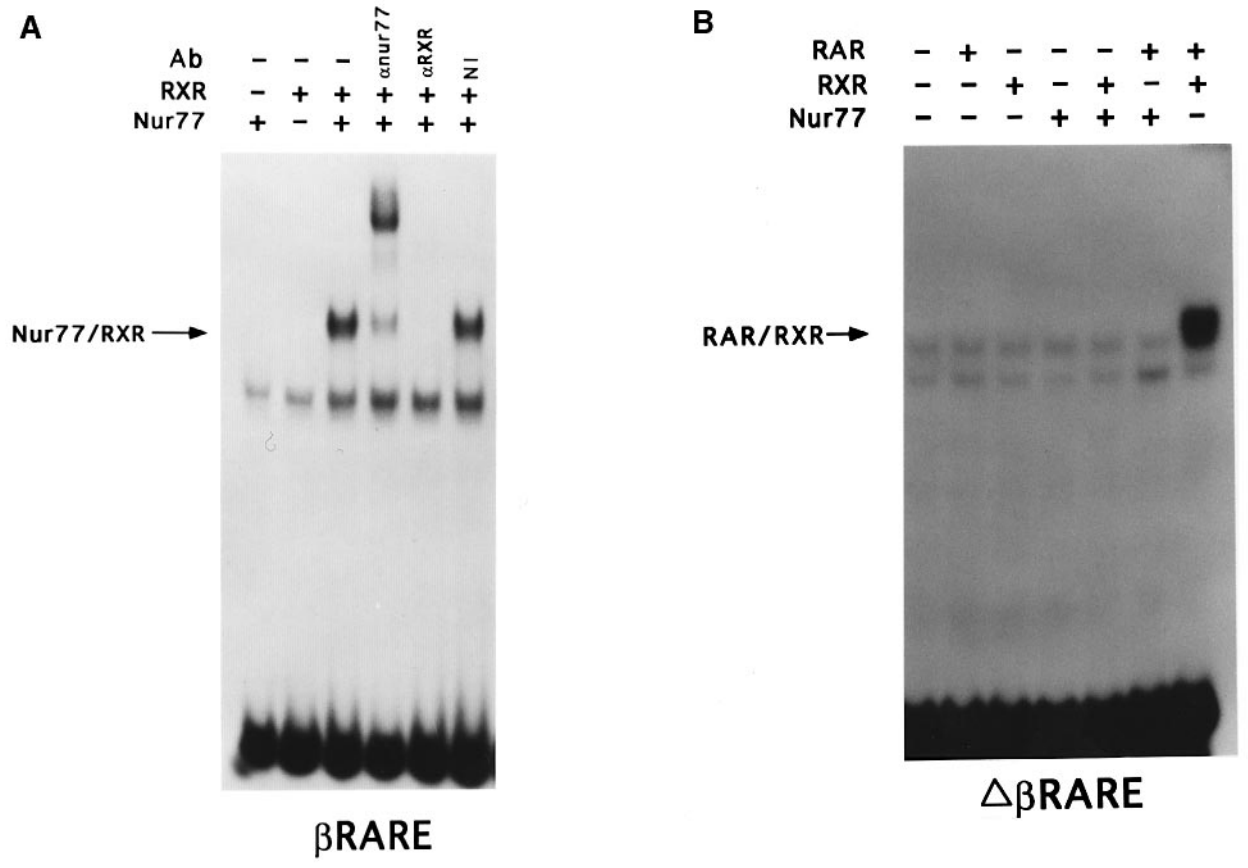

Fig. 2. Binding of nur77 to βRAREs. (**A**) Nur77 forms heterodimers with RXR on the βRARE. Equal amounts of *in vitro* synthesized nur77 and RXR were incubated alone or together at room temperature for 10 min. The reaction mixtures were then incubated with ^{32}P-labeled βRARE and analyzed by gel retardation assay. When antibody (Ab) was used, it was incubated with receptor protein for 30 min at room temperature before performing the gel retardation assay. (**B**) Analysis of nur77 binding to ΔβRARE in the presence or absence of RAR or RXR. An equal amount of nur77 was incubated alone or together with RAR or RXR prior to performing the gel retardation assay using the indicated ΔβRARE as a probe. For comparison, the binding of RAR/RXR heterodimers is shown.

was mixed with nur77, each was precipitated by anti-nur77 antibody. The co-precipitation of COUP-TFI or COUP-TFII by anti-nur77 antibody was specific because neither could be precipitated by non-specific pre-immune serum. In addition, incubation of anti-nur77 antibody with peptide used to generate anti-nur77 antibody prevented its precipitation. To study the interaction further, we cloned nur77 cDNA into pGEX-2T expression vector and expressed a GST–nur77 fusion protein in bacteria. The fusion protein was immobilized on glutathione-Sepharose beads, and mixed with either ^{35}S-labeled COUP-TFI or COUP-TFII protein. For comparison, labeled RARα and RXRα were used. As shown in Figure 4B, the labeled COUP-TFI or COUP-TFII protein bound specifically to nur77-immobilized Sepharose beads but not to the control beads, demonstrating the specific interaction between nur77 and COUP-TF in solution. Under the conditions used, we did not observe a clear binding of RARα or RXRα to the nur77-immobilized Sepharose beads, suggesting that interaction between nur77 and COUP-TF in solution is much stronger than nur77–RXR interaction. To study whether nur77 and COUP-TF could interact *in vivo*, we cloned nur77 in-frame into the yeast expression vector pGAD424 that contains the Gal4 activation domain, and COUP-TF into the yeast expression vectors pGBT9 that contains the Gal4 DNA-binding domain (DBD). The resulting vectors, pGBT9/nur77 and pGAD424/COUP-TF, were analyzed for their interaction *in vivo* by the yeast two-hybrid system (Bartel *et al.*, 1993). Transformation of either COUP-TF and empty vector pGAD424, or nur77 and empty vector pGBT9 into Y190 yeast cells could not activate the *LacZ* reporter containing the Gal4-binding site. However, when COUP-TF was transformed together with nur77 the reporter was strongly activated (Figure 5). Thus, nur77 and COUP-TF can interact in intact cells. Together, these data demonstrate that nur77 can inhibit COUP-TF DNA binding through a direct protein–protein interaction.

Nur77 regions required for interaction with COUP-TF

Nuclear receptors are characterized by a highly conserved DBD, a well conserved ligand-binding domain (LBD) located at the carboxy-terminal half of the receptor. In addition to ligand binding, the C-terminal region is critical in receptor homo- and heterodimerization (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). To determine whether a similar domain of nur77 is employed in the interaction with COUP-TF, two deletion mutants of nur77, Δnur77-1 and Δnur77-2, were constructed (Figure 6A) and tested for their interaction with COUP-TF by the yeast two-hybrid assay (Figure 6B). In Δnur77-1, a segment

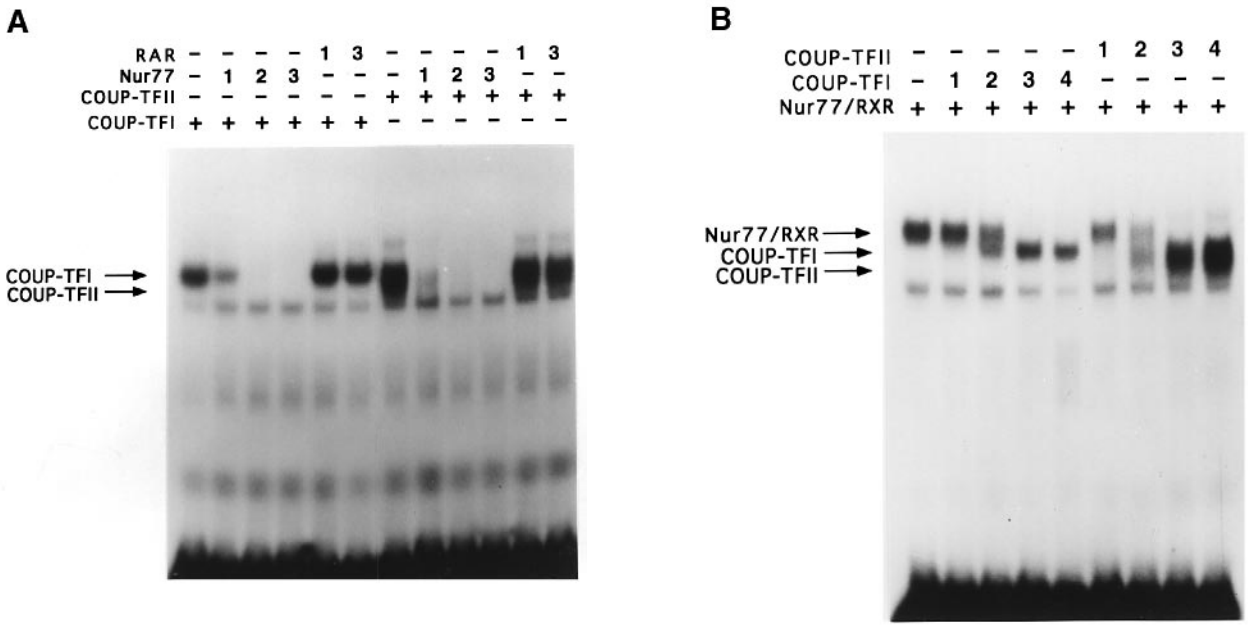

Fig. 3. Mutual inhibition of nur77 and COUP-TF DNA binding. (**A**) Inhibition of COUP-TF DNA binding on the βRARE by nur77. *In vitro* synthesized COUP-TF was pre-incubated with the indicated molar excess of nur77. Unprogramed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. Following this pre-incubation, the reaction mixtures were incubated with ^{32}P-labeled βRARE and analyzed by the gel retardation assay. For the control, COUP-TF was also pre-incubated with the indicated molar excess of *in vitro* synthesized RARα protein. (**B**) Inhibition of nur77/RXR heterodimer binding on the βRARE by COUP-TFs. *In vitro* synthesized nur77 protein was pre-incubated with RXRα in the presence or absence of the indicated molar excess of COUP-TFI or COUP-TFII, and analyzed by gel retardation assay using the βRARE as a probe. Unprogramed reticulocyte lysate was used to maintain an equal protein concentration in each reaction.

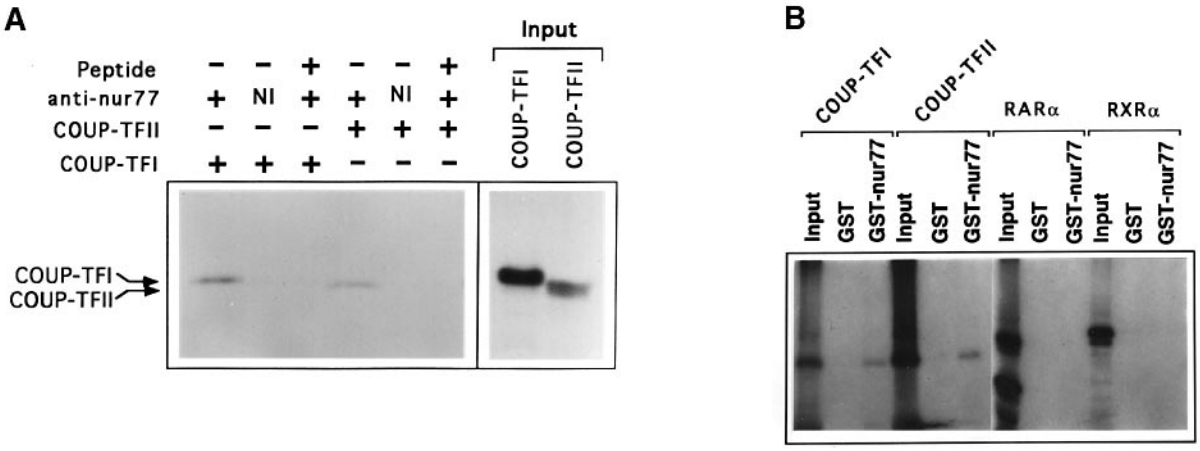

Fig. 4. Direct interaction of nur77 and COUP-TF in solution. (**A**) Analysis of nur77–COUP-TF interaction by the immunocoprecipitation assay. ^{35}S-Labeled *in vitro* synthesized COUP-TFI or COUP-TFII was incubated with *in vitro* synthesized nur77. After incubation, either anti-nur77 antibody or non-specific pre-immune serum (NI) was added. In the control, anti-nur77 antibody was pre-incubated with a peptide from which the antibody was generated. The immune complexes were washed, boiled in SDS sample buffer and separated on a 10% SDS–PAGE. The inputs of the labeled COUP-TFI and COUP-TFII are shown for comparison. (**B**) Analysis of nur77–COUP-TF interaction by the GST pull down assay. To analyze the interaction between nur77 and COUP-TFs further, nur77 protein was synthesized in bacteria using the pGEX-2T expression vector (Pharmacia). The GST/nur77 fusion protein was immobilized on glutathione–Sepharose beads. As a control, the same amount of GST was also immobilized on the beads. ^{35}S-Labeled COUP-TFI, COUP-TFII, RARα or RXRα was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS–PAGE. The input proteins are shown for comparison.

of amino acids from 168 to 468 was removed, whereas in Δnur77-2, 126 amino acids were deleted from the C-terminal end of nur77. Both mutants were cloned in-frame into pGAD424. The resulting vectors, pGAD424-Δnur77-1 and pGAD424-Δnur77-2, were analyzed for their interaction with COUP-TF. Co-expression of Δnur77-1 and COUP-TF did not show any activation of the reporter, suggesting that the deleted region is required for nur77 to interact with COUP-TF. Unexpectedly, the expression of Δnur77-2 together with COUP-TF strongly activated the reporter to a degree similar to that observed with wild-type nur77. Further deletion of 28 amino acids from the

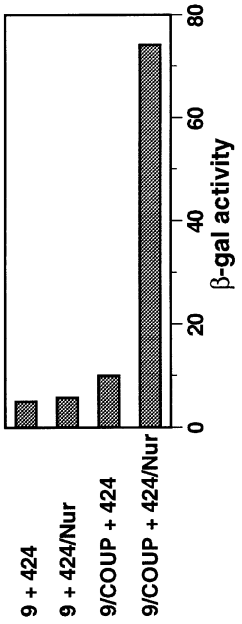

Fig. 5. Nur77 interacts with COUP-TF in yeast. The nur77 and COUP-TFI cDNAs were cloned into the yeast expression vectors pGAD424 (424) and pGBT9 (9), respectively. The resulting expression vectors, 424/nur and 9/COUP, were introduced into Y190 yeast cells as indicated. β-Galactosidase activity was assayed from a yeast strain Y190 containing the LacZ reporter plasmid to study the *in vivo* interaction. The β-galactosidase activity measured with the indicated combinations of yeast expression vectors is shown for comparison.

C-terminal end of Δnur77-2 did not affect its interaction with COUP-TF (data not shown). These data demonstrate that a putative domain is utilized by nur77 to interact with COUP-TF. We also analyzed the domain requirement of COUP-TF. In contrast to nur77, deletion of a region encompassing the DBD (ΔCOUP-TF-1) or 116 amino acids from the C-terminal end (ΔCOUP-TF-2) completely abolished its interaction with nur77, suggesting that both regions are required.

To investigate the interaction of the mutants further, a gel retardation assay was conducted by using the βRARE as a probe (Figure 6C). Similarly to what was observed with wild-type nur77 (Figure 3A), Δnur77-2 effectively inhibited the binding of COUP-TF to the βRARE. In contrast, Δnur77-1 did not show any effect. When the effect of COUP-TF mutants on nur77/RXR heterodimer binding was analyzed, we did not observe any inhibition of nur77/RXR binding. To study whether Δnur77-2 retained its ability to enhance the basal activity of RAREs, Δnur77-2 cloned into the pECE expression vector was transfected into CV-1 cells together with βRARE-tk-CAT (Figure 6D). Like wild-type nur77, Δnur77-2 could also enhance the reporter activity in a RA-independent manner. Similar results were obtained when reporters containing other RAREs were used (data not shown). These gel retardation and transfection data are consistent with the yeast two-hybrid results and suggest that the interaction between nur77 and COUP-TF is mediated by a mechanism that is different from that employed by many other nuclear receptors.

Antagonistic effect of nur77 and COUP-TF on modulating the RA sensitivity of RAREs

The previous demonstration that COUP-TF can inhibit RA-induced activity was based mainly on transient co-transfection assays where COUP-TF might be over-expressed (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We then examined the effect of various concentrations of COUP-TF on the βRARE activity. Co-transfection of larger amounts of COUP-TF expression vector with the βRARE-tk-CAT reporter almost completely inhibited RA-induced reporter activity, consistent with previous results (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). However, at low concentrations (1, 5 or 10 ng), COUP-TF either did not affect or even slightly enhanced the RA-induced βRARE activity (Figure 7A). At these concentrations, COUP-TF significantly inhibited the basal activity of the reporter, resulting in an increase of RA-dependent fold induction of the βRARE activity (Figure 7B). Without co-transfection of COUP-TF, a 4-fold induction by RA was seen. However, when 10 ng of COUP-TF expression vector was co-transfected, we observed a 14-fold induction of reporter activity in response to RA. These data are in agreement with observations made previously on the peroxisome proliferator responsive element (PPRE) (Baes *et al.*, 1995) and ApoAI-RARE (Widom *et al.*, 1992), where co-transfection of COUP-TF enhanced the hormonal sensitivity of both responsive elements. Thus, COUP-TF, at appropriate concentrations that are likely to occur in most cells, can enhance the RA sensitivity of the βRARE. To analyze the effect of nur77 on COUP-TF activity, we co-transfected nur77 expression vector together with COUP-TF. As shown in Figure 7A, the inhibition of basal transcription of the βRARE by COUP-TF was alleviated completely when nur77 was co-transfected, resulting in a decrease in RA-dependent fold induction of the βRARE activity (Figure 7B). These data, together with the results shown in Figure 1, demonstrate that nur77 can desensitize the RA responsiveness of RAREs by antagonizing COUP-TF transcriptional activity.

Expression of nur77 and COUP-TF in human lung cancer cell lines

The above observations prompted us to investigate whether expression levels of nur77 and COUP-TF correlate with RA sensitivities observed in various lung cancer cell lines (Figure 7). These cancer cell lines displayed various degrees of RA sensitivity in inducing βRARE despite the fact that RARs and RXRs are well expressed (Zhang *et al.*, 1994; data not shown). Although the degree of RA induction of βRARE activity may also depend on levels of retinoid receptors expressed in the cell lines, we found a perfect correlation between COUP-TF expression and RA induction of βRARE activity (Figure 8). COUP-TF was well expressed in Calu-6, H460, H596, SK-MES-1 and H661 lung cancer cell lines, in which βRARE activity was highly induced by RA. In contrast, COUP-TF transcripts were not detected in other cancer cell lines in which the βRARE was not induced by RA. These observations suggest that the expression of COUP-TF in these cancer cell lines does not repress RA-induced transactivation activity but is required for RA-dependent transactivation

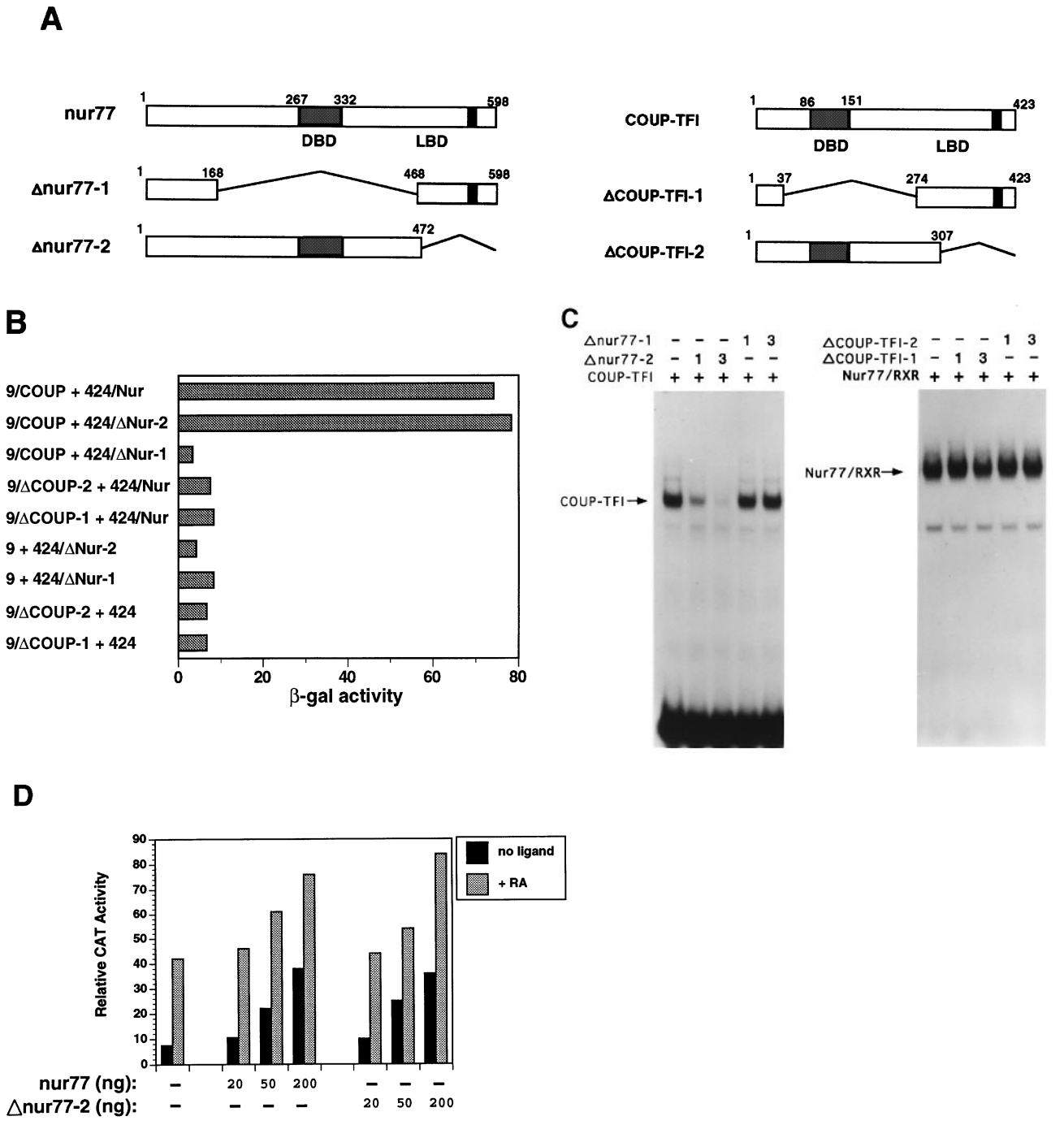

Fig. 6. Domain requirements for nur77–COUP-TF interaction. (**A**) Schematic representation of the nur77 and COUP-TF deletion mutants. The DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated. Amino acid numbers are indicated above the bar. (**B**) Δnur77-2 interacts with COUP-TF in yeast. Δnur77-1 and Δnur77-2 were closed into the pGAD424 vector in-frame and ΔCOUP-TF-1 and ΔCOUP-TF-2 were cloned into pGBT9 in-frame. The resulting expression vectors, 424/Δnur-1, 424/Δnur-2, 9/ΔCOUP-1 and 9/ΔCOUP-2, were introduced into yeast Y190 cells as indicated. b-Gal activity was measured in yeast cells. For comparison, the interaction between wild-type nur77 and COUP-TF is shown. (**C**) Δnur77-2 inhibits COUP-TF DNA binding. To investigate the interaction between nur77 and COUP-TF further, nur77 deletion mutants were synthesized by *in vivo* transcription–translation, and analyzed for their effect on COUP-TF binding to the βRARE by gel retardation. Similarily, the effect of COUP-TF1 deletion mutants on nur77/RXR binding was analyzed. (**D**) Δnur77-2 enhances βRARE activity. CV-1 cells were transfected with 100 ng of βRARE-tk-CAT together with the indicated amounts of Δnur77-2 expression vector. Cells were treated with or without 10^{-7} all-*trans* RA and assayed for CAT activity. The effect of the wild-type nur77 is shown for comparison.

of the βRARE. Hence, COUP-TF may sensitize βRARE responsiveness to RA through its binding to the element. When the expression of nur77 was analyzed, we found that it was highly expressed in RA-resistant H520 and H292 lung cancer cell lines. Although high levels of nur77 were also observed in RA-sensitive H661 and H460 cell lines, these cell lines expressed significant amounts of COUP-TF, that may counteract the effect of nur77. Under the conditions used, we did not detect expression of nur77 in the RA-resistant H441 cell line. It is likely that factors

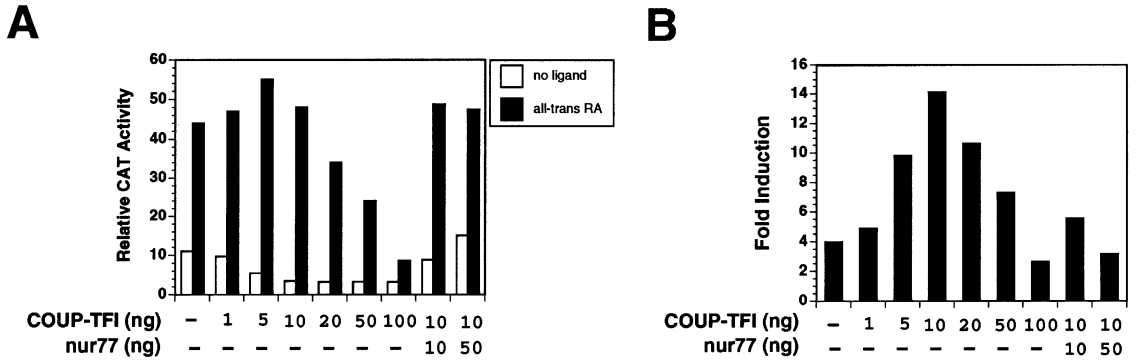

Fig. 7. Modulation of RA sensitivity of βRARE by COUP-TF and nur77. (**A**) βRARE-tk-CAT was co-transfected with the indicated amounts of nur77 and/or COUP-TF into CV-1 cells. Cells were treated with or without 10^{-7} M all-*trans* RA, and 24 h later assayed for CAT activity. Data shown represent the means of three independent experiments. (**B**) The same data were plotted to indicate the fold activation by RA.

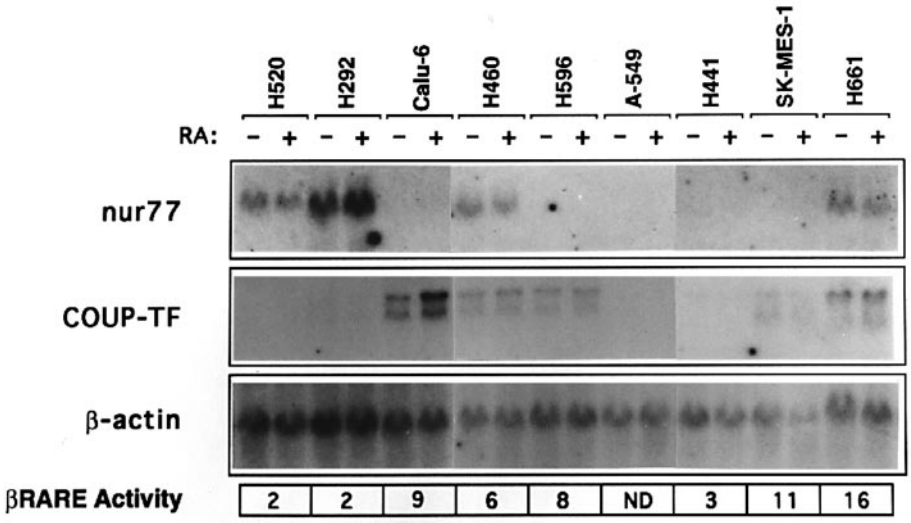

Fig. 8. Expression of COUP-TF and nur77 and RA-dependent βRARE activity in human lung cancer cell lines. Total RNAs were prepared from the indicated human lung cancer cell lines treated with or without 10^{-6} M all-*trans* RA for 24 h and analyzed for the expression of COUP-TF and nur77. As a control, the expression of β-actin is shown. βRARE activity represents the fold induction by all-*trans* RA as determined by transient transfection assay using the βRARE-tk-CAT as a reporter.

other than nur77 may be responsible for RA resistance in these cells.

Dynamic balance of nur77 and COUP-TF regulates RA sensitivity in human lung cancer cell lines

In our previous studies, we observed that RARβ was differentially expressed in several human lung cancer cell lines (Zhang *et al.*, 1994). RARβ was not expressed in Calu-6 lung cancer cells but its expression was greatly induced by RA treatment. In contrast, RARβ was highly expressed in H292 lung cancer cells but in an RA-independent manner (Figure 9A). COUP-TF is expressed in RA-sensitive Calu-6 but not in RA-resistant H292 cells, whereas nur77 is expressed in H292 but not in Calu-6 cells. This suggests that relative expression levels of COUP-TF and nur77 may affect expression of the RARβ gene. We therefore analyzed whether co-transfection of nur77 or COUP-TF affects the RA sensitivity of the βRARE in Calu-6 and H292 cells. We first investigated the effect of nur77 in RA-sensitive Calu-6 cells (Figure 9B). When nur77 expression vector was co-transfected together with the βRARE-tk-CAT into the cells, we observed an increase in basal activity and a decrease in RA-dependent fold induction of the reporter. Co-transfection of 50 ng of nur77 expression vector reduced RA-dependent βRARE activity from 7-fold to 2-fold. This result suggests that the high sensitivity of Calu-6 cells to RA may be due to a low expression level of nur77. We next analyzed the effect of COUP-TF on RA-resistant H292 cells (Figure 9C). Co-transfection of COUP-TF expression vector with the βRARE-tk-CAT into the cells reduced basal reporter activity while RA-induced activity was not clearly affected. In the absence of COUP-TF, we did not see a clear effect of RA on βRARE activity. However, when 20 ng of COUP-TF expression vector was co-transfected, we found a 3-fold induction of the βRARE activity by RA. This data demonstrates that loss of RA sensitivity in H292 cells may be due to a low level of COUP-TF in the cells. In addition, nur77 expressed in H292 cells may further inhibit the COUP-TF effect. Thus, a dynamic balance of nur77 and COUP-TF is important in regulating the RA sensitivity of the βRARE in these cancer cells and overexpression of nur77 and/or lack of COUP-TF may be responsible for RA resistance in H292 cells.

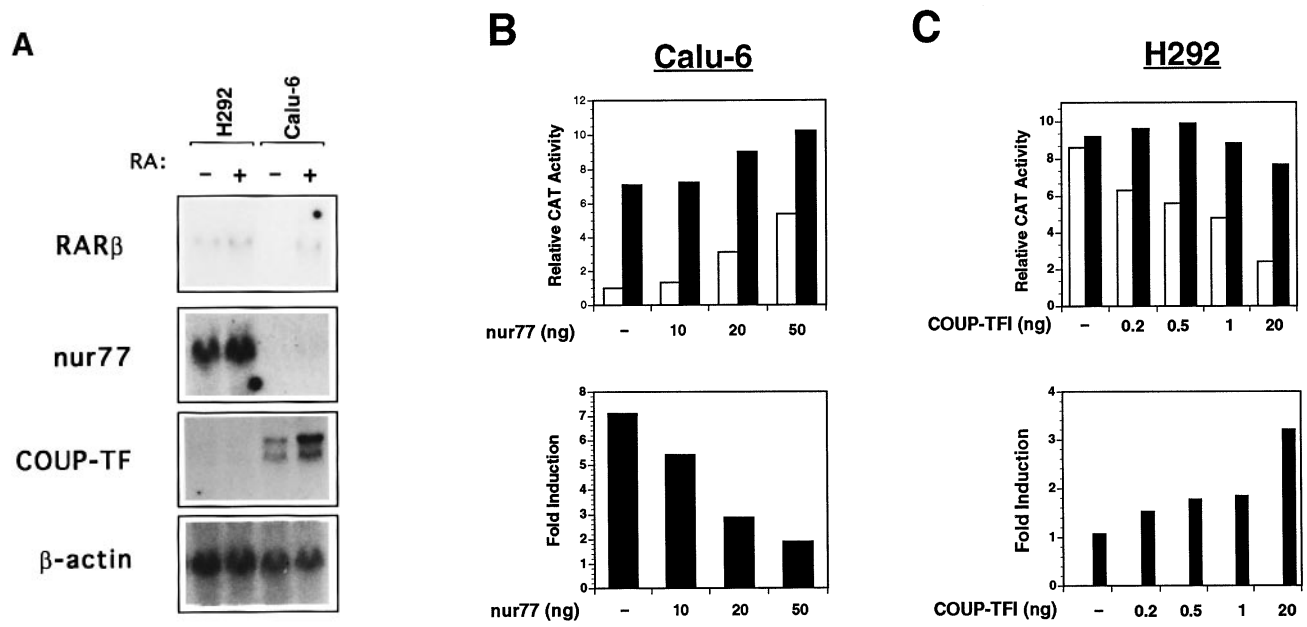

Fig. 9. Modulation of RA sensitivity by COUP-TF and nur77 in human lung cancer cell lines. (**A**) Effect of RA in inducing RARβ expression in Calu-6 and H292 cell lines. Total RNAs were prepared from Calu-6 or H292 lung cancer cells treated with or without 10^{-6} M all-*trans* RA for 24 h and analyzed for the expression of RARβ. For comparison, expression of nur77 and COUP-TF is shown. The expression of b-actin is used as a control. (**B**) Nur77 decreases RA sensitivity in Calu-6 cells. βRARE-tk-CAT was co-transfected with the indicated amounts of COUP-TFI into Calu-6 cells. The cells were treated with (filled bars) or without (empty bars) 10^{-7} M all-*trans* RA for 24 h, and assayed for CAT activity. Data shown represent the means of two experiments. (**C**) COUP-TF enhances RA sensitivity in H292 cells, βRARE-tk-CAT was co-transfected with the indicated amounts of COUP-TFI into H292 cells. The cells were then treated with (filled bars) or without (empty bars) 10^{-7} M all-*trans* RA for 24 h and assayed for CAT activity. Data shown are representative of four independent experiments.

COUP-TF or COUP-TF-like protein forms a major complex with βRARE in RA-resistant lung cancer cells

The above data suggest that COUP-TF may enhance RA sensitivity through its binding to RAREs. To test directly that COUP-TF expressed in RA-sensitive Calu-6 lung cancer cells binds to RARE, nuclear proteins were prepared from Calu-6 cells and RA-resistant H292 cells and analyzed for their RARE binding by gel retardation using the βRARE as a probe. As shown in Figure 10, in addition to several weak complexes, a strong βRARE-binding complex (indicated by the arrow) was observed with nuclear proteins from Calu-6 but not from H292 cells. To determine whether COUP-TF contributed to the βRARE binding, nuclear proteins from Calu-6 cells were incubated with anti-COUP-TF antibody prior to the gel retardation assay. Interestingly, the major βRARE-binding complex was completely upshifted by the anti-COUP-TF antibody, while binding of other weak binding complexes was not affected. Similar results were obtained when CRBPI-RARE was used as a probe (data not shown). Thus, these data clearly demonstrate that the binding of COUP-TF to the βRARE contributes to its effect on the RA sensitivity of the RARE in these lung cancer cell lines.

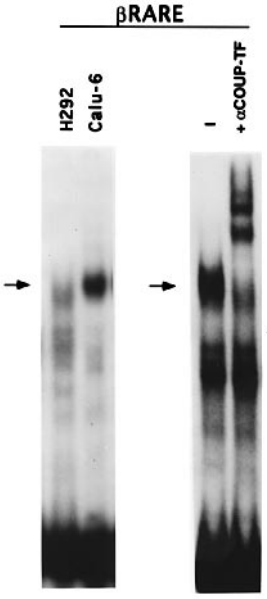

Fig. 10. COUP-TF or COUP-TF-like protein contributes to the βRARE binding activity in an RA-sensitive, COUP-TF-positive lung cancer cell line. Nuclear proteins were prepared from COUP-TF-positive Calu-6 and -negative H292 cells, and analyzed for their DNA binding activity using βRARE as a probe. Nuclear proteins from Calu-6 cells were also analyzed for the effect of anti-COUP-TF antibody. The arrow indicates the binding complex(es) present in Calu-6 but not in H292 cells.

Stable expression of COUP-TF restores RA sensitivity in RA-resistant human lung cancer cells

The observations that nur77 and COUP-TF are differentially expressed in RA-sensitive Calu-6 and RA-resistant H292 cells (Figure 8) and that they can antagonize each other's transcriptional activity (Figure 9) suggest the possibility that constitutive expression of RARβ in H292 cells may be due to overexpression of nur77 and lack of COUP-TF in the cells. To test whether expression of COUP-TF could antagonize the effect of nur77 and

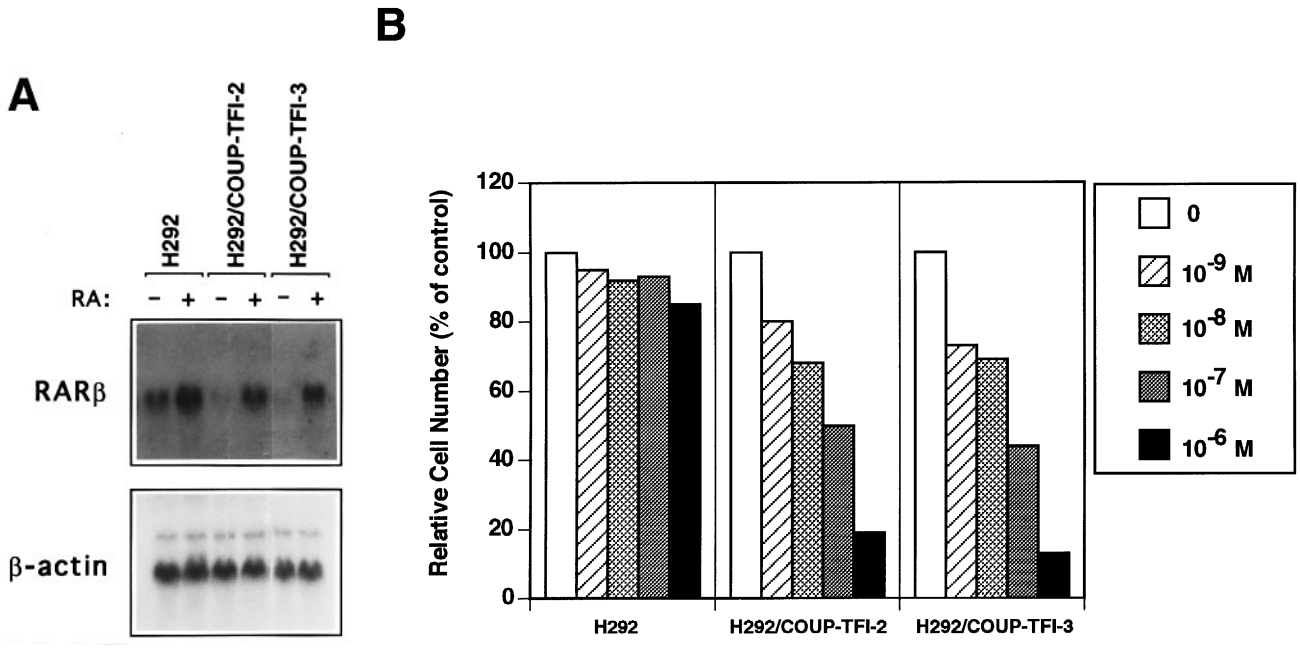

Fig. 11. Stable expression of COUP-TF in RA-resistant H292 cells restores their RA sensitivity. (**A**) Expression of the RARβ gene in H292 and stable clones. Total RNAs were prepared from Calu-6 and H292 human lung cancer cell lines treated with or without 10^{-6} M all-*trans* RA for 24 h and analyzed for the expression of RARβ. In the control, the expression of β-actin is shown. (**B**) RA-induced growth inhibition in H292 and H292 stable clones that expressed transfected COUP-TF. Cells were seeded at 1000 cells per well in a 96-well plate and treated with the indicated concentrations of all-*trans* RA for 6 days. The numbers of viable cells were determined by the MTT assay.

sensitize RARβ expression responsiveness to RA in H292 cells, we stably expressed COUP-TF in the cells. Two stable clones (H292/COUP-TFI-2 and H292/COUP-TFI-3) that expressed COUP-TF were subjected to analysis of their RARβ gene expression in the absence or presence of RA. In the absence of RA, the level of RARβ expression in these stable clones was largely reduced (Figure 11A), consistent with our transient transfection results (Figure 9C). Surprisingly, when the stable clones were exposed to RA, the reduced level of RARβ was significantly enhanced to the level observed in parental H292 cells (Figure 11A). This, again, is consistent with our transient transfection data (Figure 9C), and provides strong evidence that appropriate levels of COUP-TF expression do not inhibit RA-induced βRARE activity. RA did not significantly inhibit the growth of parental H292 cells (Figure 11B). However, it could now strongly inhibit the growth of the stable clones, with ~85% inhibition observed when the cells were treated with 10^{-6} M RA for 6 days (Figure 11B). Thus, the expression of COUP-TF could sensitize RA responsiveness of RARβ expression and growth inhibition in RA-resistant H292 lung cancer cells by reducing the basal activity of the βRARE in the absence of RA.

Discussion

The diverse functions of RA are mediated mainly by RARs and RXRs. However, expression of RARs and RXRs is often not sufficient to render cells RA responsive. Here we provide evidence that orphan receptors COUP-TF and nur77 play a critical role in the regulation of RA responsiveness of various RA target genes through their modulation of RARE binding. COUP-TFs bind to a variety of RAREs and sensitize their RA responsiveness. Conversely, nur77 reduces RA sensitivity of RAREs through heterodimerization with COUP-TF, which results in inhibition of COUP-TF binding to RAREs. These observations reveal a novel mechanism that modulates RA responses through heterodimerization of orphan receptors COUP-TF and nur77.

COUP-TFs function to sensitize the RA responsiveness of RAREs

Results from several previous studies demonstrate that COUP-TFs function to inhibit RA-induced transactivation of RAREs (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We demonstrate here that COUP-TFs may also function to sensitize the RA responsiveness of RA target genes by reducing their basal activity. In transient transfection assays in CV-1 (Figure 7) and in lung cancer cells (Figure 9), expression of appropriate amounts of COUP-TF repressed the basal transcription of the βRARE-tk-CAT reporter while it had no effect on RA-induced reporter activity. This results in an increase of RA sensitivity of the βRARE (Figures 7 and 9). In a previous study, Baes *et al.* (1995) also observed that COUP-TF inhibited the basal level of the PPRE in the absence of exogenously added ligands. These observations suggest that the sensitizing effect of COUP-TF may represent a general regulatory mechanism of COUP-TF functions. The sensitizing effect of COUP-TF on RAREs is probably due to its binding to the elements since COUP-TF binds strongly to RAREs *in vitro* (Figure 3). In addition, in RA-sensitive Calu-6 lung cancer cells, COUP-TF formed a strong complex with the βRARE, while such a complex was not seen in RA-resistant H292 lung cancer cells that constitutively express RARβ (Figure 10). Hence, the binding of COUP-TF to RAREs

may prevent them from binding and activation by certain RA-independent activators, such as MB67 that binds and activates the βRARE in an RA-independent manner (Baes *et al.*, 1994).

The notion that COUP-TF functions to maintain the RA sensitivity of RAREs by binding to the elements would require that the binding of COUP-TF be replaced by retinoid receptors once retinoids are available. This would suggest that retinoid receptors, upon binding to RA, gain affinity for RAREs. We demonstrated previously that binding of RXR homodimers to RAREs was promoted by its ligand 9-*cis* RA *in vitro* (Zhang *et al.*, 1992b). Although RA does not show a clear effect on RAR/RXR heterodimer DNA binding *in vitro* (Zhang *et al.*, 1992a,b), it was observed, by using *in vivo* footprinting, that RAR–RXR heterodimers do not occupy the βRARE in the absence of RA in P19 cells (Dey *et al.*, 1994) and that RAR ligands can promote retinoid receptor βRARE binding *in vivo* (Chen *et al.*, 1996). Thus, it is likely that, *in vivo*, the ligand induces conformational changes of retinoid receptors so that they have a higher affinity for RARE, that would allow them to replace COUP-TF RARE binding, and subsequently RA responses. Whether liganded retinoid receptors are capable of replacing COUP-TF binding on a RARE may also depend on the binding affinity of COUP-TF for the RARE and expression levels of COUP-TF. For example, COUP-TF has a relatively low affinity for the βRARE (Tran *et al.*, 1992) so that the binding of COUP-TFs may be easily replaced by liganded retinoid receptors. In contrast, the affinity of COUP-TFs for some other RAREs, such as TREpal, is much higher and the binding of COUP-TF to these RAREs may not be replaced easily by retinoid receptors even though they are complexed with ligands. This may explain our observations that certain RAREs, such as TREpal, could not be activated by RA-induced endogenous receptors in RA-sensitive Calu-6 cells while βRARE is highly activated in the same cells (data not shown). Similarly, endogenous receptors in CV-1 cells are sufficient to activate βRARE but not TREpal (Zhang *et al.*, 1992b). Hence, COUP-TF may act to sensitize certain RAREs to their RA responsiveness while at the same time functioning as a silencer of other RAREs depending on RARE binding affinity and expression levels.

Nur77 modulates RARE activity through interaction with COUP-TF

Nur77 is an immediate-early protein whose expression is induced rapidly by a variety of growth stimuli (Hazel *et al.*, 1988; Milbrandt, 1988; Williams and Lau, 1993; Lim *et al.*, 1995). However, the function of nur77 and its mechanism of action remain largely unknown. In the present study, we show that nur77 can enhance the transcriptional activity of a variety of RAREs in an RA-independent manner (Figure 1). Enhancement of RARE activity does not require a direct interaction of nur77 with RARE, since nur77, alone or in the presence of RAR or RXR, does not bind to RAREs except to the βRARE (Figure 2 and data not shown). Binding of nur77/RXR heterodimers to the βRARE may be an alternative mechanism to activate the βRARE since the heterodimers can be induced by certain RXR-selective retinoids (Forman *et al.*, 1995; Perlmann and Jansson, 1995). Our DNA binding experiments indicate that the effect of nur77 is mediated largely by its inhibition of COUP-TF RARE binding. A 2 M excess of nur77 almost completely inhibited COUP-TF binding on the βRARE when nur77 was pre-incubated with COUP-TF (Figure 3). However, if COUP-TF is pre-bound to the RARE, it becomes relative refractory to the inhibitory action of nur77 (data not shown). The inhibition of COUP-TF RARE binding activity by nur77 is likely to be mediated by direct interaction between nur77 and COUP-TF in solution, as demonstrated by our immunoco-precipitation (Figure 4A) and GST pull down experiments (Figure 4B). By using the yeast two-hybrid assay, we show that the interaction can occur *in vivo* (Figure 5). In a transient transfection assay, nur77 can counteract the effect of co-transfected COUP-TF in CV-1 cells (Figure 7). These observations clearly demonstrate that nur77 exerts its effect on RAREs through interaction with COUP-TF, forming complexes that do not bind to the RAREs.

We used deletion mutants to identify domains in COUP-TF and nur77 responsible for interaction. One surprising result is that a large portion of the putative LBD of nur77 is not required for the interaction (Figure 6). This is unexpected because the C-terminal half of nuclear receptors is essential for homo- and heterodimerization of many nuclear receptors, such as RARs, RXR, T3R and VDR (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). Our observation would then suggest that the DBD or adjacent sequences is involved in protein–protein interaction or that they are required for other domains in the receptor to achieve the proper conformation for interaction. On the other hand, the A/B region of nur77 is relatively large as compared with other nuclear receptors and may contain sequences responsible for interaction. A detailed analysis will determine the putative domain in nur77 required for interaction with COUP-TF.

Regulation of retinoid sensitivity and RARβ expression in lung cancer cells by COUP-TF and nur77

The observation that expression of COUP-TF is required to maintain RA sensitivity and that nur77 can antagonize the effect of COUP-TF provides a framework for understanding the retinoid sensitivity in cancer cells. This becomes especially apparent since retinoid resistance is observed frequently in various types of cancer cells despite expression of functional retinoid receptors (van der Leede *et al.*, 1993; Zhang *et al.*, 1994; Kim *et al.*, 1995). In human lung cancer cell lines, RARs and RXRs are well expressed, but many of the cell lines show resistance to RA-induced growth inhibition (Zhang *et al.*, 1996) and RARβ expression (Zhang *et al.*, 1994). Our observation that COUP-TF expression is positively correlated with RA sensitivity in lung cancer cell lines (Figure 8) demonstrates that COUP-TF is required for RA sensitivity in the cells. COUP-TF is also highly expressed in RA-sensitive bladder cancer and breast cancer cell lines (data not shown), suggesting that the effect of COUP-TF is not restricted to lung cancer cells. Our studies also reveal that expression of nur77 is associated with retinoid resistance in lung cancer cells (Figure 8). Since nur77 expression is induced rapidly by growth factors and a cAMP-dependent pathway (Hazel *et al.*, 1988; Milbrandt, 1988; Lim *et al.*, 1995), uncontrolled growth signaling in cancer cells may

lead to overexpression of nur77, that in turn may cause retinoid resistance through inhibition of COUP-TF activity. Hence, the studies described here provide an important mechanism by which retinoid sensitivity is regulated in cancer cells.

We have shown recently that up-regulation of RARβ expression by RA correlates with RA-induced growth inhibition in human breast cancer and lung cancer cell lines (Zhang *et al.*, 1996; Liu *et al.*, 1996). In RA-sensitive cancer cell lines, expression of RARβ is strongly induced by RA. In contrast, RA had little effect on RARβ expression in RA-resistant cancer cell lines (Zhang *et al.*, 1996; Liu *et al.*, 1996). *In vivo*, the clinical response of patients with oral dysplasia to RA is associated with inducibility of RARβ (Lotan *et al.*, 1995). The βRARE present in the RARβ promoter mediates the induction of RARβ by RA (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990), and is activated mainly by RAR/RXR heterodimers (Zhang *et al.*, 1992a; Valcarcel *et al.*, 1994). We have observed previously that RARβ cannot be induced by RA in many human lung cancer cell lines even though RAR and RXR are expressed (Zhang *et al.*, 1994). Our present finding that the relative concentrations of COUP-TF and nur77 are involved in the regulation of RARβ inducibility by RA through their modulation of βRARE activity provides an explanation of retinoid refractoriness in inducing RARβ observed in the lung cancer cell lines. COUP-TF is only expressed in lung cancer cell lines in which the βRARE is highly sensitive to RA (Figure 8), suggesting that it is required for maintaining the sensitivity of the βRARE to RA. The effect of COUP-TF is likely to be mediated by its binding to the βRARE, as demonstrated by our finding that COUP-TF expressed in RA-sensitive Calu-6 lung cancer cells formed a strong βRARE-binding complex that was not observed in RA-resistant H292 cells (Figure 10). The observation that RARβ is highly induced by RA in Calu-6 cells (Figure 9A) indicates that the binding of COUP-TF to the βRARE does not interfere with RA-induced retinoid receptor activity. This is also supported by *in vivo* observations (Reuberte *et al.*, 1993; Lutz *et al.*, 1994) that RARβ is expressed in motor neurons at the time when COUP-TF is expressed. Hence, the expression levels we observed in various cancer cell lines do not function to inhibit RA-induced RARβ expression, but repress RARβ expression in the absence of RA. In this study, we also found that nur77 is highly expressed in RA-resistant lung cancer cell lines (Figure 8). Thus, the loss of RA inducibility of RARβ expression in certain lung cancer cell lines, such as H292, is not due to abnormal expression and function of RARs and RXRs that activate the βRARE, but to lack of COUP-TF and/or overexpression of nur77 that modulate basal levels of RARβ expression. Such a loss of RA sensitivity in lung cancer cells can be restored by expression of COUP-TF, as demonstrated by our transient transfection (Figure 9) and stable transfection (Figure 11) of COUP-TF in RA-resistant H292 cells. Interestingly, an increase in RARβ inducibility by stable expression of COUP-TF is also accompanied by an enhancement of growth inhibition by RA (Figure 11). This observation further supports our previous finding that induction of RARβ by RA is involved in RA-induced growth inhibition in breast cancer cell lines (Liu *et al.*, 1996).

In summary, the studies described here reveal a novel mechanism that regulates RA sensitivity in cancer cells through heterodimerization of nur77 and COUP-TF. Our data demonstrate that a dynamic equilibrium of the two orphan receptors plays a crucial role in the control of inducibility of RARβ expression and growth inhibition by RA in human lung cancer cell lines. Such a mechanism may also be involved in the regulation of the RA sensitivity program during development and in adult life. Since the expression of nur77 is induced by growth signaling (Hazel *et al.*, 1988; Milbrandt, 1988; Lim *et al.*, 1995) while the expression of COUP-TF can be enhanced by RA (Jonk *et al.*, 1994), heterodimerization of nur77 and COUP-TF may mediate 'cross-talk' between growth and vitamin A signalings. Overexpression of nur77 and/or lack of COUP-TF as seen in certain human lung cancer cells may be responsible for RA resistance, and may contribute to cell proliferation and neoplastic transformation by releasing the inhibitory effect of RA on cell growth. Our demonstration that expression of COUP-TF in RA-resistant H292 cells could enhance their RA response provides novel approaches for restoring RA sensitivity in certain RA-resistant cancer cells.

Materials and methods

Cell culture

CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Calu-6 and SK-MES-1 cells were maintained in minimum essential medium (MEM) supplemented with 10% FCS. H292, H520, H460, H596, H441 and H661 cells were grown in RPMI 1640 supplemented with 10% FCS. A-549 cells were maintained in F12 medium supplemented with 10% FCS.

Plasmid constructions

Nur77 expression vectors pECE-nur77, pBluescript-nur77, pGEX-2T-nur77 and pGAD424-nur77 were constructed by cloning the nur77 cDNA (Chang and Kokontis, 1988) fragment into pECE, pBluescript, pGex-2T and yeast vector pGAD424, respectively. The internal *Stu*I fragment was removed from nur77 to generate Δnur77-1. For construction of ΔCOUP-TFI-1, the internal *Nar*I fragment was removed. The deleted COUP-TF was filled in and religated. To obtain the deletion mutants Δnur77-2 and ΔCOUP-TFI-2, the *Pst*I site in nur77 and the *Sal*I site in COUP-TFI were used to delete the C-terminal fragments. The construction of the reporter plasmids βRARE-tk-CAT, TREpal-tk-CAT, CRBPI-RARE-tk-CAT, CRBPII-RARE-tk-CAT, ApoAI-RARE-tk-CAT, lactoferrin-RARE-tk-CAT and MHC-TRE-HC-CAT has been described previously (Tran *et al.*, 1992; Zhang *et al.*, 1992a,b; Lee *et al.*, 1995). The reporter ΔβRARE-tk-CAT was obtained by inserting one copy of mutated βRARE oligonucleotide (TGTAGGGTTCACACTGAGTTCACTCA) (underlining indicates the mutated nucleotides) into the *Bam*HI site of $pBLCAT_2$ (Luckow and Schutz, 1987). The RARβ promoter (*Sma*I–*Eco*RI fragment) reporter has been described (Hoffmann *et al.*, 1990). The construction of COUP-TFI cDNA in the pRc/CMV vector (Invitrogene, San Diego, CA) followed the procedure described previously (Liu *et al.*, 1996).

Preparation of receptor proteins

Receptor proteins were synthesized by an *in vitro* transcription–translation system using rabbit reticulocyte lysate (Promega) as described previously (Zhang *et al.*, 1992a). The relative amount of the translated proteins was determined using [^{35}S]methionine-labeled protein on SDS–PAGE, quantitating the amount of incorporated radioactivity, and normalizing it relative to the content of methionine in each protein.

Transient and stable transfection assay

CV-1 cells were plated at 1×10^5 cells per well in a 24-well plate 16–24 h before transfection as described previously (Zhang *et al.*, 1992a). For Calu-6 and H292 cells, 5×10^5 cells were seeded in six-well culture plates. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (Zhang *et al.*,

1992a). Briefly, 100 ng of reporter plasmid, 150 ng of β-galactosidase expression vector (pCH 110, Pharmacia) and various amounts of nur77 expression vector were mixed with carrier DNA (pBluescript) to 1000 ng of total DNA per well. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. For stable transfection, the pRc/CMV-COUP-TFI recombinant plasmid was stably transfected into H292 cells using the calcium phosphate precipitation method, and screened using G418 (GIBCO BRL, Grand Island, NY) as described (Liu *et al.*, 1996).

Gel retardation assay

The gel retardation assay using *in vitro* synthesized proteins has been described previously (Zhang *et al.*, 1992a). When interaction of nur77 and COUP-TF was studied, they were incubated on ice for 10 min before performing gel retardation in order to prevent the formation of the COUP-TF homodimer. In most cases, co-translation of nur77 and COUP-TF resulted in much more efficient dimerization of the two proteins. When antibodies were used in the gel retardation assay, 1 μl of anti-nur77 (Santa Cruz Biotech., Inc., Santa Cruz, CA) or 1 μl of anti-RXR (Lee *et al.*, 1995) was incubated with receptor protein at room temperature for 30 min prior to performing the gel retardation assay. The oligonucleotides used for the gel retardation assay have been described elsewhere (Tran *et al.*, 1992; Zhang *et al.*, 1992a,b; Lee *et al.*, 1995).

Immunoprecipitation assay

For the immunoprecipitation assay (Zhang *et al.*, 1992a), 5 μl of reticulocyte lysate containing *in vitro* translated ^{35}S-labeled COUP-TFI or COUP-TFII were incubated with 20 μl of *in vitro* translated nur77 in 100 μl of buffer containing 50 mM KCl and 10% glycerol for 15 min on ice. The reactions were then incubated with 5 ml of anti-nur77 antibody or non-specific pre-immune serum for 2 h on ice. When the peptide from which anti-nur77 antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA) was generated was used, anti-nur77 antibody was incubated with 5 μl of peptide at room temperature for 30 min before adding to the reaction mixtures. Immunocomplexes were precipitated by adding 40 μl of protein A–Sepharose slurry and mixing continuously in the cold room for 1 h. The complexes were then washed five times with RIPA buffer, resuspended in SDS sample buffer containing 15% β-mercaptoethanol, boiled and resolved by SDS–PAGE.

GST pull down assay

To prepare GST–nur77 fusion protein, the nur77 cDNA was cloned in-frame into the expression vector pGEX-2T (Pharmacia). The fusion protein was expressed in bacteria using the procedure provided by the manufacturer, and was analyzed by gel retardation assay and Western blot (data not shown). To analyze the interaction between nur77 and COUP-TF, the fusion protein was immobilized on glutathione–Sepharose beads. For control, the vector protein (GST) prepared under the same conditions was also immobilized. The beads were pre-incubated with bovine serum albumin (1 mg/ml) at room temperature for 5 min. ^{35}S-Labeled *in vitro* synthesized receptor proteins (2–5 μl, depending on translation efficiency) were then added to the beads. The beads were then rocked continuously for 1 h at 4°C in a final volume of 200 μl in EBC buffer (140 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 μm sodium orthovanadate and 50 mM Tris, pH 8.0). After washing five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% NP-40), the bound proteins were analyzed by SDS–PAGE.

Two-hybrid assay

For the yeast two-hybrid assay, the yeast two-hybrid system from Clontech Inc. (Palo Alto, CA) was used. Nur77 cDNA and deletion mutants were cloned into the yeast expression vector pGAD424 to generate an in-frame fusion with the Gal4 activation domain. COUP-TF cDNA and deletion mutants were cloned into pGBT-9 to produce an in-frame fusion with the Gal4 DBD. The yeast reporter strain Y190 containing a LacZ reporter plasmid with the Gal4 binding site was used for transformation. β-Galactosidase activity was determined following the conditions provided by the manufacturer to assess the interaction between nur77 and COUP-TF.

MTT assay

To determine the effect of all-*trans* RA on the viability of the stable transfectants, cells were seeded at 1000 cells per well in a 96-well plate, and treated with various concentrations of all-*trans* RA for 6 days. Media were changed every 48 h. The number of viable cells was determined by MTT assay as described previously (Liu *et al.*, 1996).

Northern blot

For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride ultracentrifugation method as described (Zhang *et al.*, 1994). Thirty mg of total RNAs from different cell lines treated with or without 10^{-6} M all-*trans* RA were analyzed by Northern blot. RARβ, COUP-TFI or nur77 cDNA were used as probes. To determine that equal amounts of RNA were used, the expression of β-actin was studied.

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