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Novel Pathway for Thyroid Hormone Receptor Action through Interaction with *jun* and *fos* Oncogene Activities

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Many essential biological pathways, including cell growth, development, and metabolism, are regulated by thyroid hormones (THs). TH action is mediated by intracellular receptors that belong to a large family of ligand-dependent transcription factors, including the steroid hormone and retinoic acid receptors. So far it has been assumed that TH receptors (TRs) regulate gene transcription only through the classical protein-DNA interaction mechanism. Here we provide evidence for a regulatory pathway that allows cross-talk between TRs and the signal transduction pathway used by many growth factors, oncogenes, and tumor promoters. In transient transfection studies, we observe that the oncogenes c-*jun* and c-*fos* inhibit TR activities, while TRs inhibit induction of the c-*fos* promoter and repress AP-1 site-dependent gene activation. A truncated TR that lacks only 17 amino acids from the carboxy terminus can no longer antagonize AP-1 activity. The cross-regulation between TRs and the signal transduction pathway appears to be based on the ability of TRs to inhibit DNA binding of the transcription factor AP-1 in the presence of THs. The constituents of AP-1, c-Jun, and c-Fos, vice versa, can inhibit TR-induced gene activation in vivo, and c-Jun inhibits TR DNA binding in vitro. This novel regulatory pathway is likely to play a major role in growth control and differentiation by THs.

Thyroid hormones (THs) are important effectors of cell growth, development, tissue differentiation, and metabolism. Disturbance of these and other biological processes often results in neoplastic transformation and cancer development (20, 51). The recent cloning and characterization of TH-specific nuclear receptors (TRs) have greatly increased knowledge of the mechanism(s) by which THs control gene expression. TRs belong to a large family of regulatory proteins that include receptors for steroid hormones and for vitamin D and vitamin A derivatives (3, 13, 23). These receptors function as ligand-activated transcription factors that bind to their cognate DNA sequences near promoters of responsive genes. In the absence of ligand, TRs bind thyronine (T_3)-responsive elements (T_3RE) and function as transcriptional repressors or silencers (9, 22, 55). This is in contrast to the larger glucocorticoid receptor (GR) protein, which is predominantly located in the cell cytoplasm in the absence of hormone and has not yet been shown to possess any ligand-independent regulatory functions in vivo (3, 69).

The TRs are encoded by two different genes, TRα and TRβ, from which multiple isoforms can be generated (4, 30, 54, 65, 67). The two TRβ isoforms, TRβ1 and TRβ2, differ in their amino termini but are both ligand-dependent transcriptional enhancers. At least three distinct isoforms have been isolated from the human and rat TRα genes (4, 36, 38, 44, 45, 47). However, only one of these isoforms, TRα1, is a ligand-dependent activator of transcription, while the other isoforms are receptorlike molecules that do not bind TH and are not transcriptional activators. Although these isoforms appear to have repressor activities (25, 35, 38), their biological functions are not well understood.

The role of TH in cancer development and cellular transformation has been controversial. Positive effects were observed in studies on mammary cancers (62–64, 66), while other data suggested an enhancing effect of TH on cell transformation (24, 40, 41). However, the identification of TRα as a cellular counterpart of the viral *erb*A gene implicates its potential role in tumor development (30, 54, 67). v-*erb*A cooperates with a number of tyrosine-kinase-encoding oncogenes and *ras*-like oncogenes in the process of cellular transformation (17, 32, 33). v-*erb*A potentiates the transforming activity of v-*erb*B (a truncated epidermal growth factor receptor) in chicken embryo fibroblast (18) and is required for v-*erb*B, *ras*, and *src* oncogenes to transform erythrocyte progenitor cells (17, 32, 33). The role of v-*erb*A in the cooperation with these oncogenes during cell transformation is probably due to its ability to interfere with the c-*erb*A-regulated differentiation program of these cells (17, 32, 33, 72). Recent studies suggest that v-*erb*A might have arisen from a dominant negative mutation of c-*erb*A, resulting in the loss of the ligand-dependent transcriptional activation function but maintaining its repressor activity (9, 55). It is possible that the lack of ligand-dependent function, in combination with the constitutive repressor activity of v-*erb*A, plays a crucial part in the events leading to cell transformation. This would also suggest that the normal cellular counterpart of v-*erb*A might participate in the control of cellular transformation and possibly interfere with the action of certain oncogenes, including v-*erb*B, *ras*, and *src*. These oncogenes as well as many growth factors and the tumor promoter tetradecanoyl phorbol acetate (TPA) activate c-Jun or c-Fos, components of the transcription factor AP-1, through the protein kinase C pathway (reviewed in references 8, 34, and 53). AP-1 binds to specific DNA sequences either as c-Jun homodimers or c-Jun/c-Fos heterodimers. Here we report evidence for a regulatory pathway by which TRs can directly interfere with the signal transduction pathway induced by the tumor promoter TPA and counteract AP-1 activity. The interference appears to be due to the direct interaction of TRs with AP-1, the activation product of TPA.

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MATERIALS AND METHODS

Recombinant plasmids. The T_3RE_2-tk-CAT construct was prepared as described previously (22, 73). Briefly, two copies of the synthetic palindromic sequence (TCAGGT CATGACCTGA) were inserted into the *Bam*HI site of pBL-CAT_2 by using *Bgl*II linkers. The AP-1 site present in the vector was deleted by *Dra*II and *Nde*I digestion, filling in, and religation. The −73Col-CAT and pfos-CAT reporter constructs were described by Angel et al. (1) and Schönthal et al. (56), respectively. Coding sequences for TRα and TRβ receptor were inserted into the multiple cloning sites of the eukaryotic expression vector pECE or pBluescript (Stratagene) as described previously (22, 73). To obtain the deletion mutants of TRβ, existing restriction sites (*Bsm*I and *Sma*I) in the TRβ cDNA clone were used to obtain the TRβ truncated mutants, which were cloned into pECE to generate ΔTRβ1 and ΔTRβ2 expression vectors. The c-*jun* and c-*fos* expression vectors have been described elsewhere (70).

Proteins and antibodies. The cDNAs for TRα, TRβ, c-Fos and c-Jun were cloned into pBluescript (Stratagene) or a comparable vector (pGem4z; Promega). They were transcribed by using the T7, T3, or SP6 RNA polymerase and translated in rabbit reticulocyte lysate (Promega) as described previously (52, 73). The relative amount of the translated proteins was determined by separating [^{35}S]methionine-labeled proteins on sodium dodecyl sulfate (SDS)-polyacrylamide gels, quantitating the amount of incorporated radioactivity, and normalizing it relative to the content of methionine in each protein. Flag-TRα was constructed by ligating a double-stranded oligonucleotide containing an ATG codon and the Flag sequence (Arg Tyr Lys Asp Asp Asp Asp Lys) to the N terminus of TRα (25, 27). The fusion product was cloned into pBluescript. In vitro-synthesized Flag-TRα was checked for correct size and antigenic specificity by immunoprecipitation with anti-Flag antibody (obtained from M. Leahy, Immunex, Seattle, Wash.) on SDS-polyacrylamide gels. Flag-TRβ, FlagΔTRβ1, and FlagΔTR β2 were constructed as described for FlagTRα. In vitro-synthesized ^{35}S-labeled Flag-fusion proteins were checked for correct size and antigenic specificity by immunoprecipitation with anti-Flag monoclonal antibody (M2, obtained from M. Leahy, Immunex) followed by SDS-polyacrylamide gel electrophoresis (PAGE). The bacterially expressed c-Jun protein and anti-Jun antibody have been described elsewhere (70).

Indirect immunofluorescence analysis of receptor expression. CV-1 cells (1×10^6 to 2×10^6) were transiently transfected as described below with expression plasmids encoding the Flag version of TRβ, ΔTRβ1, or ΔTRβ2. Cells were harvested 48 h after transfection, washed in phosphate-buffered saline (PBS), resuspended in 70% ethanol, and incubated on ice for 1 h. After washing of the fixed cells in PBS, staining for indirect immunofluorescence was performed essentially as described elsewhere (29) with monoclonal anti-Flag antibody M2 (Immunex) at 10 μg/ml as a first antibody and anti-mouse G-F(ab')$_2$-fluorescein isothiocyanate conjugate (Sigma) as a second antibody. Direct examination and photography of the stained cells were done on a Nikon fluorescence microscope. Flow cytometric analysis was performed on a fluorescence-activated cell sorter (FACS) (FMF; The Salk Institute, La Jolla, Calif.); approximately 35,000 single cell events (as predicted by size) were analyzed per sample.

Gel retardation assay. Gel retardation assays using in vitro-synthesized proteins were performed as described previously (22, 52, 73). Typically, 1 to 5 μl of a reticulocyte lysate containing a known amount (in counts per minute) of in vitro-synthesized protein was incubated with ^{32}P-labeled DNA probe in 20 μl of DNA binding buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 10% glycerol, and 1 μg of poly(dI-dC). When an AP-1 binding-site dimer (5'-AGCTTGGTGACTCATCCG GATCCGGATGAGTCACCAAGCT-3') or the collagenase promoter fragment was used as a probe, the binding reaction was performed at 4°C for 30 min. When binding of TR to T_3RE was analyzed, the binding reaction was performed at 25°C for 20 min. To obtain T_3RE dimer, a perfect palindromic T_3RE oligonucleotide flanked by *Bgl*II adaptor sequences was synthesized on an Applied Biosystem DNA synthesizer. The T_3RE monomer was ligated, and double-stranded T_3RE dimer was purified by PAGE (73). The collagenase promoter DNA fragment (−73 to +63) was generated by digesting −73Col-CAT with *Hin*dIII and *Bam*HI. The binding reactions were analyzed on a 5% polyacrylamide gel containing 0.5× Tris-borate-EDTA buffer at 4°C. To analyze protein-protein interactions, proteins were preincubated at 37°C for 15 min before the DNA binding assay was performed. Each binding reaction mixture was adjusted to equal amounts of reticulocyte lysate by adding the appropriate amounts of unprogrammed lysate. When antibodies were used, 1 μl of the antiserum was incubated with the specific translation mixture at room temperature for 45 min before the experiments described above were performed.

Tissue culture, transient transfection, and CAT assay. HeLa and CV-1 cells were grown in DME medium supplemented with 10% calf serum. GC cells were maintained in DME medium with 5% horse serum and 2.5% fetal calf serum. A modified calcium phosphate precipitation procedure was used for transient transfection as described previously (52). Typically, 2 μg of reporter construct, 3 μg of β-galactosidase (β-gal) expression vector (pCH110; Pharmacia), and various expression vectors for receptors, c-Jun, or c-Fos were mixed with carrier DNA (pBluescript) to 20 μg of total DNA per plate. After transfection, cells were grown in DME medium supplemented with the appropriate percentage of serum and treated with TH or TPA as described in the figure legends. β-Gal activity was measured as described elsewhere (52). CAT activity was determined by using ^{3}H-acetyl coenzyme A as the substrate (52). To normalize for transfection efficiency, CAT activities were corrected for β-gal activity.

RESULTS

c-*jun* and c-*fos* inhibit TR activities. TRs and the nuclear proto-oncogenes *fos* and *jun* are important mediators of cell growth and differentiation. To study a possible link between the proto-oncogenes and TR activities, we investigated whether c-*jun* and c-*fos* expression could affect TR activity. Transfection of c-*jun* and c-*fos* expression vectors into CV-1 cells had no effect on the basal level activity of a TR-responsive reporter gene (T_3RE_2-tk-CAT). However, when c-*jun* or c-*fos* expression vectors were cotransfected together with TRα or TRβ, they strongly inhibited the T_3-induced activity of the reporter gene (Fig. 1). The degree of inhibition was dependent on the amount of c-*jun* or c-*fos* expression vector used. However, a slight enhancement of TRβ activity was consistently observed at low c-*jun* concentrations, while c-*fos* was an efficient inhibitor even at low concentrations. In addition, a clear increase in repression of

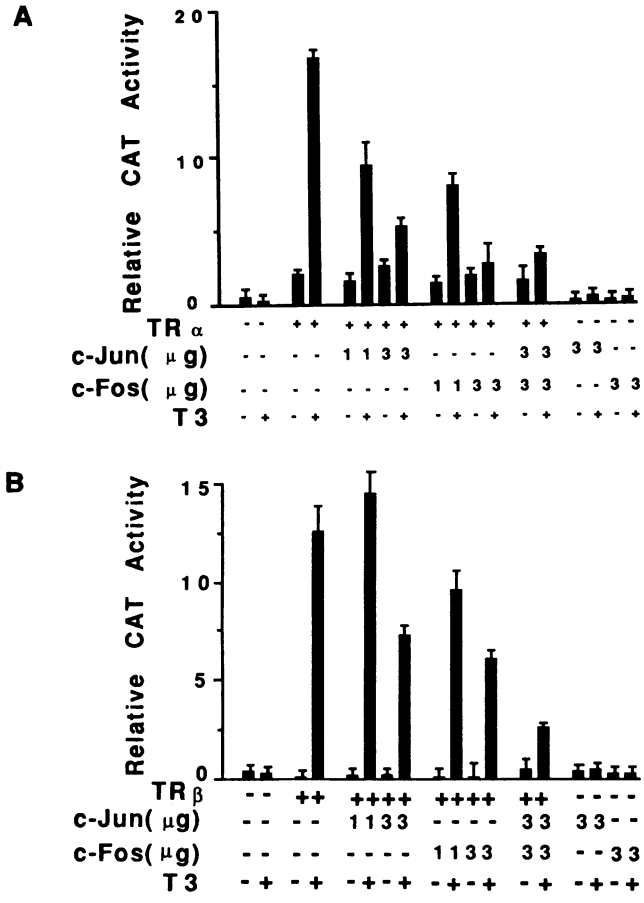

FIG. 1. Inhibition of TR activity by c-*jun* and c-*fos*. CV-1 cells were plated at 1.5×10^6 to 2.0×10^6 per dish 16 to 24 h prior to transfection in DME medium supplemented with 10% fetal calf serum. A modified calcium phosphate precipitation procedure was used for transient transfection as described previously (52). Two micrograms of reporter construct T_3RE_2-tk-CAT (4, 16), 3 μg of β-gal expression vector (pCH110; Pharmacia), 3 μg of TRα or TRβ expression vector (4), and the indicated amounts c-*fos* and c-*jun* expression vectors (70) were mixed with carrier DNA (Bluescript) to 20 μg of total DNA per plate. Cells were grown in the absence or presence of 10^{-7} M T_3. β-Gal was measured as described previously (52). CAT activity was determined by using ^{3}H-acetyl coenzyme A as the substrate (52). To normalize for transfection efficiency, CAT activities were corrected for β-gal activity. Experiments were repeated at least three times. (A) Inhibition of TRα activity by c-*jun* and c-*fos*. (B) Inhibition of TRβ activity by c-*jun* and c-*fos*.

TRβ activity was observed when c-*jun* and c-*fos* were transfected together (Fig. 1B).

TRβ inhibits induction of the c-*fos* promoter. The ability of c-*jun* and c-*fos* to interfere with TR-mediated TH induction led us to question whether TRs can also influence proto-oncogene activity. As one test system, a c-*fos* promoter reporter construct (pfos-CAT) (56) was chosen. The c-*fos* promoter can be activated by the tumor promoter TPA (5, 14, 15, 19, 61), which activates endogenous AP-1 activity via protein kinase C. We therefore investigated whether TRs would also inhibit TPA-induced *fos* promoter activity. When pfos-CAT was cotransfected into CV-1 cells together with a TRβ expression vector, we observed a strong reduction of TPA-induced Fos-CAT activity by coexpression of TRβ. This inhibition was T_3 dependent (Fig. 2). Cotransfection of

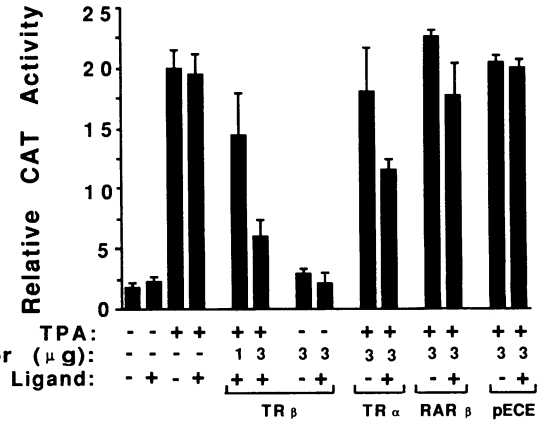

FIG. 2. Inhibition of TPA-induced c-*fos* promoter activity. CV-1 cells were transfected with 2 μg of pfos-CAT, 3 μg of β-gal, and the indicated amounts of receptor expression vectors in the presence or absence of respective ligands (10^{-7} M T_3, 10^{-7} M retinoic acid). After transfection, cells were grown in 0.5% fetal calf serum with or without TPA (100 ng/ml) for 24 h before harvesting.

a TRα expression vector also led to a reduction of Fos-CAT activity in the presence of T_3. However, TRα was consistently a less effective repressor than TRβ, despite being approximately equally efficient in transcriptional activation (73). The empty expression vector (pECE) had no effect (Fig. 2). Somewhat surprisingly, a retinoic acid receptor (RARβ) showed no repressing activity in CV-1 cells in the presence of retinoic acid, while we consistently observed a slight enhancing effect of RARβ on Fos-CAT in the absence of ligand (Fig. 2). We have, however, found that RARβ is a potent TPA response inhibitor in HeLa cells (71), indicating that cell-type-specific factors may modulate the anti-AP-1 activities of receptors. Thus, TRs can negatively regulate the induction of the c-*fos* promoter by TPA.

TRs repress AP-1 site-dependent gene activation. The Fos promoter contains a serum response element and an AP-1 site, which both control its activity (5, 14, 15, 19, 50, 61). To investigate the effect of TR on gene activation by TPA in a simpler model system, expression vectors for TRs were cotransfected into HeLa and CV-1 cells along with a collagenase promoter-reporter construct (−73Col-CAT). This collagenase promoter fragment lacks a serum response element but carries a TPA-responsive element (1, 54) that can be activated via the protein kinase C pathway by AP-1, resulting in a strong induction of the reporter gene in the presence of TPA (Fig. 3A). Deletion of the AP-1 site leads to a loss of the TPA response (56). In the absence of T_3, expression of TRα or TRβ did not negatively interfere with TPA-induced activity of the reporter gene. However, the addition of T_3 to the culture medium led to a drastic decrease of the TPA-induced CAT activity (Fig. 3A). Inhibition of the TPA-induced reporter gene activity correlated positively with the concentration of the receptor expression vector used (Fig. 3A).

The studies described above suggested that the inhibition of TPA activity by TRs may be mediated by the interaction of TRs with c-Jun and c-Fos, the constituents of AP-1. We therefore questioned whether the activities of c-Jun and c-Fos could be directly inhibited by TRs. In Fig. 3B, we show the induction of the −73Col-CAT reporter construct by c-Jun or a combination of c-Jun and c-Fos and the dose-dependent inhibition of this activation by TRβ and TRα

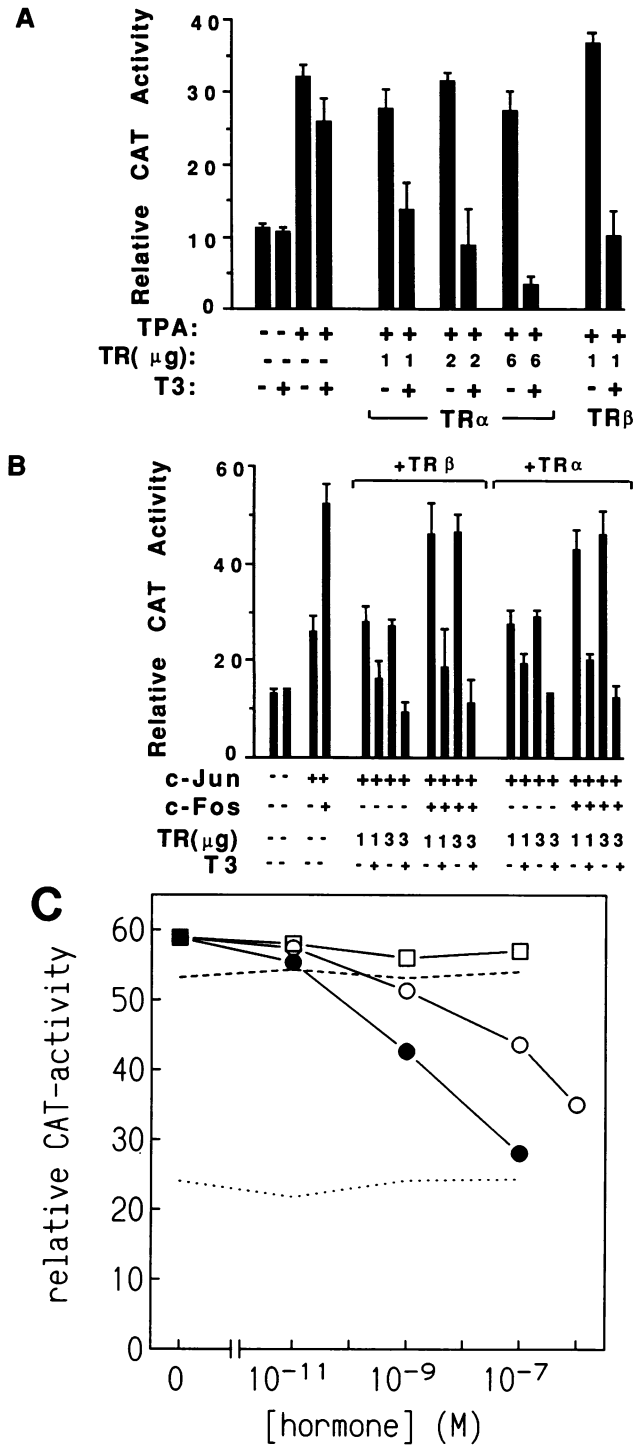

FIG. 3. Inhibition of AP-1-induced collagenase promoter activity. (A) Inhibition of TPA activity by TRs. HeLa cells were grown in DME medium supplemented with 10% calf serum. At 24 h after transfection with 2 μg of −73Col-CAT reporter and the indicated amount of TR expression vectors, cells were transferred into DME medium containing 0.5% calf serum and 100 ng of TPA per ml with or without 10^{-7} M T_3 for 24 h before harvesting. CAT and β-gal activities were determined as described for Fig. 1. (B) Inhibition of c-Jun, and c-Jun/c-Fos activity by TRs. CV-1 cells were cotransfected with 2 μg of −73Col-CAT reporter, 3 μg of c-*jun* or c-*jun*/c-*fos* (2 μg of each) expression vectors, and the indicated amounts of TR expression vectors and grown in the presence or ab-sence of T_3 (10^{-7} M). CAT assays were performed as described for Fig. 1. (C) Ligand-dependent inhibition of c-Jun/c-Fos activity on collagenase promoter by TRβ. CV-1 cells were cotransfected with −73Col-CAT reporter, c-*jun*, and c-*fos* expression vectors (2 μg of each) together with 3 μg of TRβ expression vector (——) and grown in the presence of the indicated concentrations of T_3 (●), T_4 (○), or reverse T_3 (□). The basal level CAT activity (. . . .) and c-Jun/c-Fos-induced CAT activity (– – –) in the absence of TRβ are indicated. CAT assays were performed as described for Fig. 1.

in the presence of hormone. These results suggest that the inhibition of c-Jun and c-Fos activity is most likely to account for the repressive effects of TRs on TPA activity. This repressive effect was observed only in the presence hormone. Half-maximal repression was observed at 10^{-9} M T_3. Thyroxine (T_4) could also stimulate the anti-AP-1 activity of TRs, but a more than 10-fold-higher concentration was required. Similar inhibitory effects were observed on −73Col-CAT activity induced by c-Jun alone (data not shown). Reverse T_3 (a weak agonist) showed no effect (Fig. 3C). Cotransfection of an empty expression vector (pECE) together with different ligands also had no effect on c-Jun/c-Fos activity (data not shown). Thus, the activity of the tumor promoter TPA, c-Jun, and c-Fos, all of which function through the AP-1 site in the −73Col-CAT construct, can be antagonized by TR expression in the presence of active THs.

Mutual endogenous AP-1/TR antagonism in GC cells. The mutual antagonism observed between AP-1 and TR activities is reminiscent of the mutual antagonism observed between AP-1 and GRs (11, 31, 42, 47, 70) and may suggest direct interaction between nuclear receptors and Jun and/or Fos proteins. However, one important question that remains to be answered is whether this mutual antagonism can indeed function at physiological concentrations of both transcription factors. We examined TPA-TR antagonism in GC cells. These pituitary-derived cells showed a strong T_3-dependent induction of the T_3RE_2-CAT reporter in the absence of cotransfected TR (Fig. 4A). In addition, TPA led to a marked induction of the Col-CAT reporter in these cells (Fig. 4B). At the same time, TPA also inhibited T_3-induced reporter activity (Fig. 4A). Inhibition of T_3 activity was also observed by cotransfection of either c-*jun* or c-*fos* expression vectors or both. Conversely, T_3 was able to markedly inhibit activation of the Col-CAT reporter by TPA in a concentration-dependent manner, while no significant T_3 effect was observed on the basal level activity of this reporter. Our data thus clearly demonstrate that endogenous AP-1 and TR activities can inhibit each other in the same cell type.

A functional ligand/activation domain is necessary for TRβ-TPA antagonism. To delineate the regions of TRs that are necessary for the anti-AP-1 activity, a series of TRβ deletion mutants was constructed; Fig. 5A depicts two of these mutants. The mutants were analyzed for their anti-AP-1 activity. Deletion of only 17 amino acids from the carboxy terminus of TRβ (ΔTRβ1) resulted in a loss of anti-AP-1 activity (Fig. 5B). Deletion of larger regions of the carboxy terminus did not revert the loss of anti-AP-1 activities. In all cases, loss of AP-1 antagonism was coupled with loss of ligand-dependent gene induction activity (Fig. 5C).

The loss of anti-AP-1 activity observed with these TRβ deletion mutants could be due to reduced expression levels of the mutant proteins. To compare the levels of mutant and wild-type receptor expression, receptor derivatives that carried an eight-amino-acid immunogenic sequence (Flag) at the amino-terminal end were analyzed (Fig. 6A to E). Expression levels of transfected Flag derivatives of TRβ, ΔTRβ1,

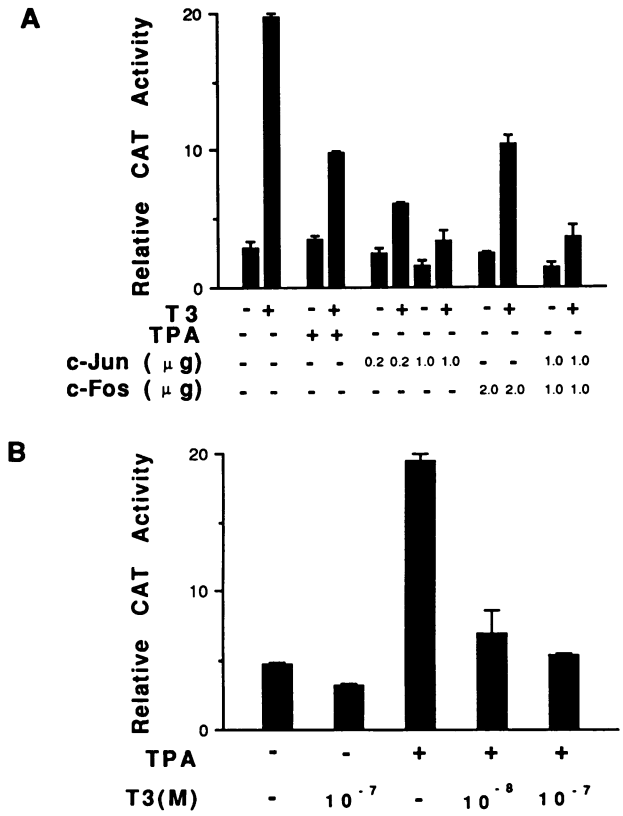

FIG. 4. Mutual inhibition of endogenous AP-1 and TR activities. (A) Inhibition of endogenous TR activity by endogenous and exogenous AP-1 activity. GC cells were grown in DME medium supplemented with 5% horse serum and 2.5% fetal calf serum. At 24 h after transfection with 5 μg of the reporter construct T_3RE_2-tk-CAT and the indicated amounts of cotransfected c-*fos* and/or c-*jun* vectors, cells were transferred to fresh medium supplemented with 5% horse serum and 2.5% fetal calf serum containing 10^{-7} M T_3 or 100 ng of TPA per ml where indicated. Cells were harvested 24 h later, and CAT and β-gal activities were determined as described in Materials and Methods. (B) Inhibition of endogenous AP-1 activity by endogenous TR. GC cells were grown as described above and transfected with 3 μg of −73Col-CAT reporter construct. At 24 h after transfection, cells were transferred to DME supplemented with 0.33% horse serum and 0.17% fetal calf serum with or without TPA (100 ng/ml) or T_3 as indicated. CAT and β-gal activities were determined 24 h later.

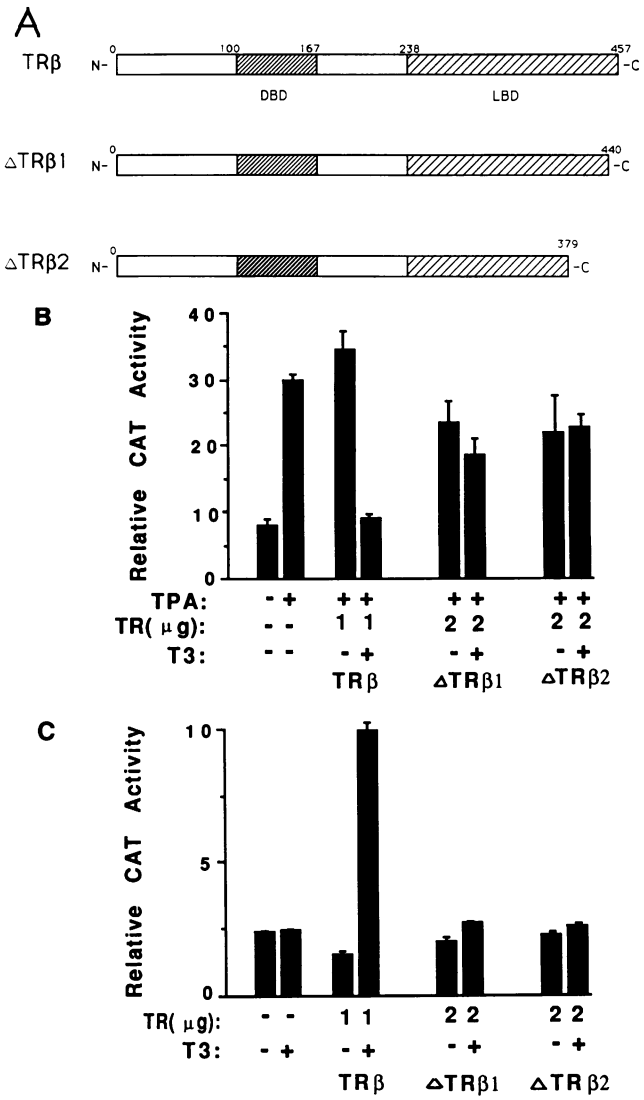

FIG. 5. Evidence that the C terminus of TRβ is required for anti-AP-1 effect. (A) Schematic representation of TRβ deletion mutants. Deletions of the TRβ C terminus were generated by using existing restriction sites on TRβ (73). The DNA binding domain and ligand binding domain of the receptor are indicated. Amino acid numbers are indicated above the bars. (B) Inhibitory activity of TRβ mutants. HeLa cells were cotransfected with −73Col-CAT reporter and the indicated amounts of TRβ mutant expression vectors. After transfection, the cells were treated with TPA in the presence or absence of 10^{-7} M T_3. CAT assays were performed as described for Fig. 1. The inhibitory activity of wild-type TRβ is shown for comparison. (C) Transcriptional activity of TRβ mutants. CV-1 cells were cotransfected with 2 μg of T_3RE_2-CAT reporter and the indicated amounts of TRβ and TRβ mutant expression vectors in the presence or absence of 10^{-7} M T_3. CAT assays were performed as described for Fig. 1.

and ΔTRβ2 were similar, as judged by indirect immunofluorescence microscopy and FACS analysis: approximately 1% of the cells were stained with comparable fluorescence intensities for all constructs. The staining was predominantly in the nucleus.

The carboxy-terminal regions of TRs encode complex functions, including ligand binding, dimerization/oligomerization, and transcriptional activation (16). It has recently been shown that deletion of carboxy-terminal regions of TRβ result in reduced affinity of these proteins for the T_3RE as a result of impairment of the dimerization/oligomerization function (73). Zenke et al. (72) showed that a region comparable to that deleted in ΔTRβ1 is essential for transcriptional activation of TRα. The data presented here suggest to us that in addition, this region, either directly or via the dimerization function of the intact receptor, is essential for the anti-AP-1 activity of TRβ. It is interesting that the extreme C-terminal region of c-*erb*A is deleted in v-*erb*A (30, 54, 65, 67). We infer from this that v-*erb*A is likely to lack the anti-AP-1 activity.

AP-1 but not TRs bind the AP-1 site. Overlapping hormone receptor response elements and AP-1 sites have been reported for several promoters (46, 58). We therefore examined the possibility that TRs can interfere with AP-1 activity by binding to the AP-1 site in the collagenase promoter,

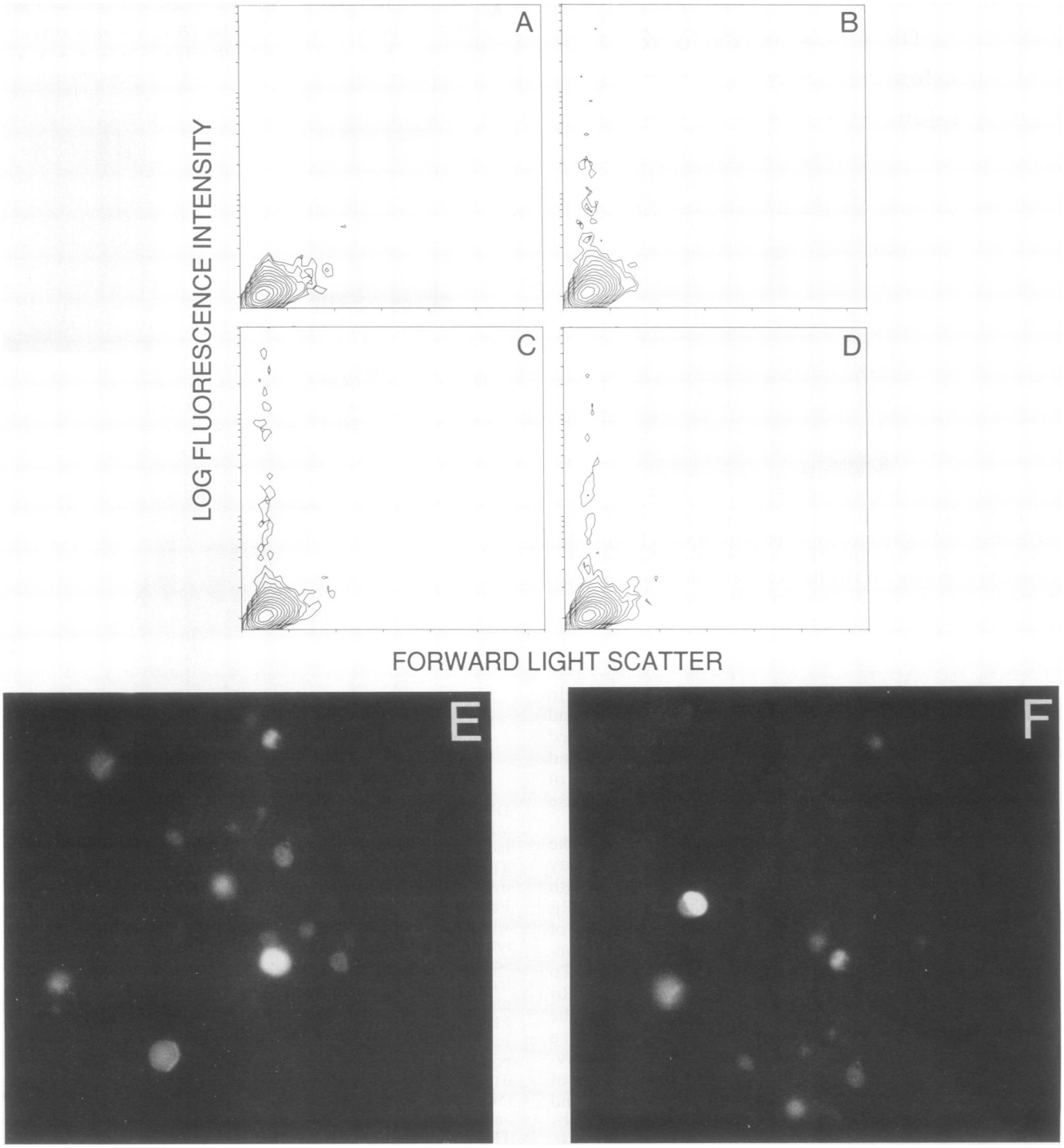

FIG. 6. Immunofluorescence microscopy and FACS analysis, revealing comparable expression levels of TRβ, ΔTRβ1, and ΔTRβ2. CV-1 cells were transiently transfected with expression vectors for the Flag derivative of TRβ or the deletion mutants. Cells were stained 48 h after transfection as described in Materials and Methods. (A) Mock-transfected cells; (B and E) ΔTRβ1; (C) ΔTRβ2; (D and F) TRβ. FACS analysis of cells transfected with either one of the three constructs yielded a population of approximately 1% positive cells displaying a similar distribution of fluorescence intensities. (The homogeneity of the forward light scatter indicates homogeneity in size of the stained particles [cells].) The results from microscopic examinations (ES) supported these data.

thereby repressing AP-1 activity by a simple competitive DNA binding mechanism as has been suggested for other receptors (46, 58). When in vitro-synthesized TRα and TRβ proteins were incubated with a ^{32}P-labeled DNA fragment of the collagenase promoter, no binding could be detected with use of the gel shift technique (Fig. 7, lanes 5 to 8). The failure to observe binding was not due to insufficient amounts or inactive receptor proteins used, since the same protein

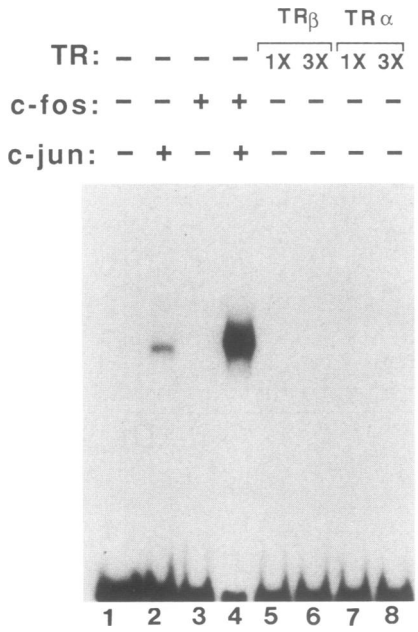

FIG. 7. Binding of AP-1 but not TRs to the AP-1 site. c-Jun, c-Fos, TRα, and TRβ proteins were prepared in vitro. DNA binding activities of equal amounts of in vitro-translated c-Jun and c-Fos (lanes 2 to 4) or TRα and TRβ (lanes 5 to 8) proteins were analyzed by using a ^{32}P-labeled collagenase promoter fragment.

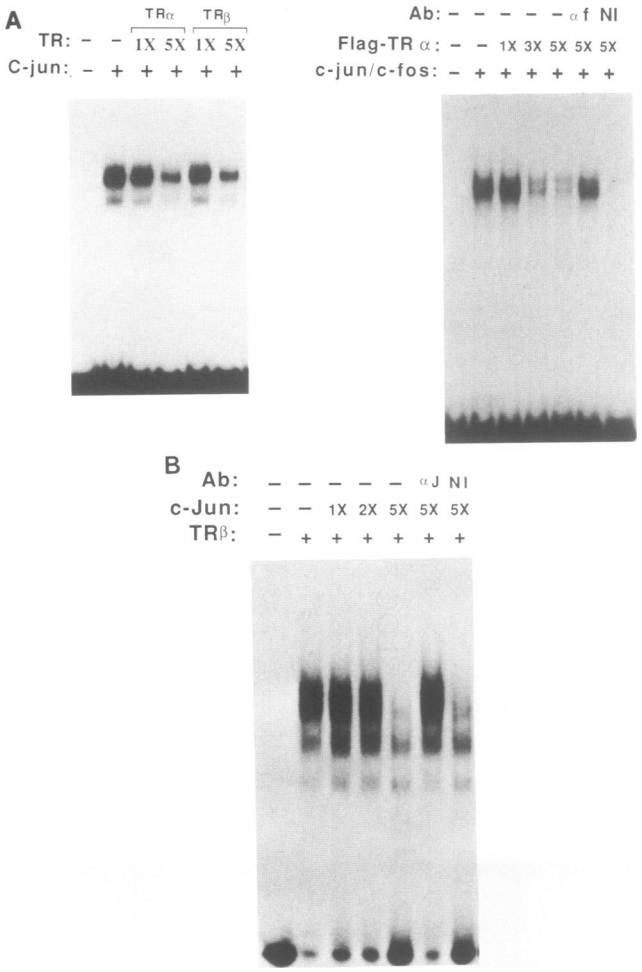

FIG. 8. Evidence that AP-1 and TR antagonize each other's DNA binding. (A) Inhibition of AP-1 DNA binding by TRs. In vitro-translated c-Jun or c-Jun/c-Fos were preincubated with the indicated molar excess of in vitro-synthesized TRα, TRβ, or Flag-TRα at 37°C for 15 min. Unprogrammed reticulocyte lysate was used to maintain equal protein concentrations in each reaction. Following this preincubation, the reaction mixtures were incubated with ^{32}P-labeled synthetic AP-1 binding site and analyzed by the gel retardation assay. When anti-Flag antibody (αf) or preimmune serum (NI) was used, each was incubated with Flag-TRα at room temperature for 45 min prior to mixing with c-Jun and c-Fos proteins. The effect of antibody (Ab) on the interaction between Flag-TRα and c-Jun and c-Fos was analyzed by gel retardation. (B) Inhibition of TRβ DNA binding by c-Jun. Cell-free translated TRβ protein (approximately 10 ng) was incubated at 37°C for 15 min with 0 to 1 μl of bacterially produced (appropriately diluted) c-Jun protein (50 ng/μl [70]) prior to incubation with a T_3RE dimer probe and analysis by gel retardation. The molar excess of c-Jun protein over TRβ protein is indicated. The binding of unprogrammed reticulocyte lysate is shown in the first lane. To examine the specificity of inhibition of TR DNA binding by c-Jun, the bacterial protein was pretreated with anti-c-Jun antibody (αJ) or preimmune serum (NI) prior to mixing with TRβ.

extracts showed strong binding to a T_3RE (Fig. 8B). In vitro-synthesized c-Jun protein and a mixture of c-Jun and c-Fos proteins bound the collagenase promoter as expected (Fig. 7, lanes 2 and 4), while c-Fos protein alone did not bind to the AP-1 site (Fig. 7, lane 3). Thus, the repressive effect of TRs is not due to their direct interaction with the collagenase promoter.

Mutual inhibition of AP-1 and TR DNA binding. The mutual inhibition between TRs and c-Jun or c-Fos activity may be due to the mutual interference of these proteins with each other's DNA binding activity. To test this, TRα or TRβ proteins were preincubated with c-Jun before addition of labeled AP-1 binding site. We observed a concentration-dependent inhibition of c-Jun DNA binding (Fig. 8A). To determine the specificity of inhibition, we used a TRα derivative that contained an eight-amino-acid epitope at the amino-terminal end (Flag-TRα) that is recognized by a specific monoclonal antibody (anti-Flag) (25, 27). The behavior of this TRα derivative was indistinguishable from that of the wild-type receptor in both transcriptional activation and DNA binding activity (data not shown). When Flag-TRα protein was incubated with c-Jun/c-Fos, a concentration-dependent inhibition of binding to the AP-1 DNA site was observed (Fig. 8A). For maximum inhibition, an excess amount of TRα was required. The inhibition was specific in that it could be reversed by preincubation of Flag-TRα with the anti-Flag antibody but not by preimmune serum (Fig. 8A).

Inhibition of c-Jun/c-Fos DNA binding activity by TRs might be due to the formation of a Jun/Fos-TR protein complex, resulting in mutual impairment of DNA binding activity. We therefore investigated the effect of bacterially synthesized c-Jun protein on TRβ DNA binding, using the gel shift assay. c-Jun protein inhibited TRβ binding to T_3RE in a concentration-dependent fashion (Fig. 8B). Preincubation of c-Jun protein with anti-c-Jun antibody, but not with preimmune serum, reversed this inhibition, indicating that the inhibitory effect of c-Jun protein is specific. These DNA binding studies provide further support for a new regulatory

pathway that allows direct cross-talk between TRs and AP-1 via mutual inhibition of DNA binding.

DISCUSSION

It has been proposed that GR, RAR, and the vitamin D_3 receptor can antagonize AP-1 activity by interacting with AP-1 site-overlapping response elements, involving a mechanism of competitive DNA binding (11, 46, 58). We show here, using in vitro DNA binding assays, that TRs and AP-1 can antagonize each other's activity by a mechanism that does not require protein-DNA interaction but rather appears to result from the mutual interference of these proteins with each other's DNA binding activity which may be due to direct protein-protein interaction, as has been suggested by others for the GR/AP-1 (11, 31, 70) and RAR/AP-1 (71) antagonism. The in vitro inhibition of AP-1 DNA binding shown here requires excess amounts of TR, which may suggest that it is a weak interaction. Although direct evidence for protein-protein interaction has been reported in the cases of GR (11, 70) and RAR (71), other efforts (including our own efforts to show direct TR/AP-1 interaction) have thus far been unsuccessful (57). These data, together with the cell-type-specific effect of RARβ shown in Fig. 2, suggest to us that the interaction between receptor and AP-1 may require additional auxiliary protein(s) or that, for instance, the phosphorylation status of the interactants is important. Although the exact mechanism for TR/AP-1 interaction is unclear, we have shown that deletion of 17 amino acids from the TRβ carboxy-terminal region prevented the receptor from interfering with AP-1 activity. This region contains many hydrophobic amino acids and has been suggested to form an α-helical structure (72). We have reported elsewhere that the carboxy-terminal regions can mediate TRβ receptor subunit interaction (73). It remains to be shown whether the putative α-helical structures are directly involved in the AP-1 interaction or whether they are required for overall receptor stability.

Implication of antioncogene activity. While TRs regulate many important biological programs, only a limited number of genes have been described that are positively regulated by TRs in the presence of TH through the classical protein-DNA interaction mechanism. Negative regulation of several genes by the TR-T_3 complex has been proposed to involve interaction with so-called negative response elements (2, 6, 7, 10, 68). However, these elements, contrary to the positive T_3REs, have not yet been shown convincingly to function in a position-independent manner.

In this study, we have carried out a series of transfection experiments and in vitro DNA binding assays that demonstrate the existence of a novel pathway by which TRs at physiological levels can regulate the activity of oncogenes and the tumor promoter TPA. Growth factors, TPA, *ras*, and many other oncogenes function via a signal transduction pathway that leads to c-Jun/c-Fos activation (34, 53). Our finding that TRs can interfere with the expression of genes regulated through the signal transduction pathway may therefore represent a major regulatory pathway of TR action. Conversely, TR activity can also be regulated by c-Jun/c-Fos and also by the *ras* oncogene product (72a) in a manner similar to that observed for GR (26). The observation of mutual inhibition between TR and the AP-1 complex provides another example that the membrane signaling pathway and nuclear receptor signaling pathway can cross-talk to regulate gene transcription in response to extracellular stimuli. Such interaction might play a crucial role in controlling cell growth and differentiation. AP-1, when stimulated by a number of mitogenic signals, regulates many genes which are involved in cell growth and differentiation (8, 34, 53). Inhibition of AP-1 activity by the TR-T_3 complex can provide cells with a complex control mechanism by which to determine a particular physiological state. The cross-talk between TRs and the signal transduction pathway reported here appears to be a general mechanism that is shared by other nuclear receptors, as was first demonstrated for GR (11, 31, 42, 57, 70). Recent data from our laboratory and others suggest that RARs can also inhibit AP-1 activity (48, 71).

Nuclear receptors have so far received limited attention as antioncogenes. However, the data reported here, in combination with recent data on anti-AP-1 activity of GRs and RAR, suggest a prominent role for nuclear receptors as antioncogenes. Not only does AP-1 mediate mitogenic signals, but its deregulated expression also contributes to cellular transformation (39, 43, 59, 60). The inhibition of AP-1 by TRs might serve as an important regulatory pathway in suppressing in appropriate cellular proliferation initiated by oncoproteins. It is noteworthy that while AP-1 activity can be directly affected by TRs in the presence of TH, we also show that *fos* promoter activity is downregulated by TRs. Thus, a more pronounced downregulation of AP-1 activity can be achieved and at least one of the AP-1 components, c-Fos, may be completely eliminated. This type of a signal-enhancing mechanism could lead to a major switch in a cell differentiation program. This view is consistent with a recent report showing that ligand-activated TRs and RARs are able to antagonize the activities of cellular *erb*B1 and *erb*B2 genes (28).

The ability of TRs to antagonize oncogene activity at various points in the chain of events leading to neoplastic cell proliferation makes it likely that cells in which TRs are downregulated will be more susceptible to growth factor response, tumor promoter, and oncogene activity. Mutation in TRs can therefore increase oncogene effectiveness. A striking example is the v-ErbA protein, which, unlike active TRs, cooperates with a number of oncogenes (17, 32, 33). It becomes an oncoprotein through mutations that effect its ligand and gene activation functions (9, 55, 72). Moreover, the extreme C-terminal region of c-ErbA, which is required for its anti-AP-1 activity, is deleted in v-ErbA (30, 54, 65, 67). Since oncogene inhibition by TRs is ligand dependent, v-ErbA and the receptorlike TRα carboxy-terminal variants may not show anti-AP-1 activity, while their reported repressor activity on active receptors (9, 25, 35, 38, 55) may in addition serve to enhance oncogene activities. Furthermore, it will be important to investigate whether TRs or other nuclear receptors that have anti-AP-1 activity are downregulated or mutated in neoplastic cells. In this context, it is of interest to note that many small lung cell tumors carry a deletion in chromosome 3 which may overlap with the TRβ gene (12, 37).

In conclusion, we have provided evidence for a novel regulatory function of TRs. This function is not mediated by the classical receptor-DNA interaction but is based on cross-talk between the signal transduction pathway and nuclear TRs. Taken together, our recent findings and those of others (11, 31, 42, 48, 57, 70, 71) suggest that this is a general pathway for nuclear receptors. This pathway is not limited to direct c-Jun/c-Fos–receptor antagonism, but may allow the interaction of many oncogenes and growth factors with nuclear receptors through the signal transduction pathway. The interaction of these pathways could provide a

molecular basis to explain the beneficial effects of many steroid hormones, THs, and derivatives of vitamins A and D on cancer development.

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