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THE THYROID HORMONE RECEPTOR INTERFERES WITH TRANSCRIPTIONAL ACTIVATION VIA THE AP-1 COMPLEX

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The nuclear thyroid hormone (T_3) receptor, encoded by the c-*erbA* genes, represses transcriptional activation by the transcription factor AP-1 in a T_3-dependent fashion. The viral homologue of the T_3 receptor, the v-*erbA* gene product, does not repress AP-1 activity. Inhibition by T_3 involves reduced binding of AP-1 to its cognate DNA target sequence. The reduction in AP-1 binding does not, however, result from competitive binding by the T_3 receptor to the AP-1 response element.

Certain cytokines and phorbol esters stimulate the activity of the transcription factor AP-1, a hetero-dimeric complex consisting of the proteins *jun* and *fos* [1]. Through signalling pathways that transmit stimuli from the cell surface to the nucleus, these mitogens induce an increased binding of the *trans*-acting AP-1 complex to a *cis*-acting promoter sequence, further defined as the AP-1 response element [2,3]. A well-studied example of such genes is the human collagenase gene, the expression level of which is largely determined by AP-1 [2]. In addition to its induction by cytokines, transcription of the collagenase gene, and other genes with AP-1 response elements, have been shown to be regulated by a variety of hormones including estrogens [4], glucocorticoids [5,6], vitamin D [7] and retinoids including vitamin A [7-9]. All these hormones mediate their effect via binding to ligand-dependent transcription factors belonging to the steroid-hormone receptor superfamily [10]. In an effort to find other members of the steroid receptor family that might regulate AP-1 activity we tested the

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nuclear 3,5,3'-triiodo-L-thyronine (T_3) receptor, which is encoded by the c-*erbA* α and ß genes [11,12]. Previous studies have shown that the T_3 receptor can act both as an activator of transcription, as is the case for the growth hormone gene [13] and as a repressor of transcription as observed for the thyroid stimulating hormone (TSH) ß gene [14]. This regulation of transcription is due to interaction of the T_3 receptor with its cognate *cis*-acting promoter sequences. The viral homologue of c-*erbA*, the v-*erbA* oncogene product, is known to act as a T_3-independent repressor of T_3-responsive genes [15-17]. As for repression by the T_3 receptor, repression by v-*erbA* involves binding to specific target sites on the DNA.

In this paper we present evidence for the T_3-dependent inhibition of AP-1 by the c-*erbA* α and ß gene products. The repression of AP-1 dependent transcription does not involve binding of the T_3 receptor to a *cis*-acting promoter sequence, and therefore represents a different mechanism of T_3-dependent gene regulation.

MATERIALS AND METHODS

Plasmid constructs

All plasmid constructs which were used in this study have been described previously. The expression vectors for *jun*, *fos*, neo, and adenovirus (AD) E1a are all under control of a Rous sarcoma virus (RSV) promoter [18]. The expression vector for the v-*erbA* gene was driven by a Simian virus 40 (SV40) early promoter [17] and expression of the c-*erbA* α and ß genes are under control by a cytomegalovirus (CMV) promoter [19]. The reporter gene construct COLL-AP1-TATA-CAT consists of the chloramphenicol acetyl transferase (CAT) gene fused to a minimal promoter containing the collagenase AP-1 target sequence (-73/-65) 5'-ATGAGTCAG-3' [5].

Tissue culture and DNA transfections

HeLa tk^- cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Transient transfections of HeLa tk^- cell monolayers in 10 cm ϕ dishes were performed using the DEAE-dextran method [18]. For each transfection we used 4 µg each of the vectors expressing *jun*, *fos* and adenovirus E1a [18], v-*erbA* [17] and rat c-*erbA*-α and c-*erbA*-ß [19]. When one or more of the expression vectors were omitted from the experiment we used the same amount of pRSVneo [18] to keep the concentration of DNA constant. From the reporter gene constructs 2 µg was added in each experiment. After 8 hours of exposure of the HeLa cells to the precipitate they were induced with or without 100 nM T_3 in medium containing 10% charcoal-treated hormone-depleted fetal calf serum [20]. Forty hours later, protein extracts were prepared and tested for CAT-activity [18]. To increase endogeneous AP-1 activity 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) was added during the transfection when indicated, at a concentration of 50 µg/L, for a period of 90 min. [2].

Protein extracts

For bandshift experiments HeLa tk^- cells were induced with 50 µg/L 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) for 90 min. Proteins were harvested in a buffer containing 25 mM Hepes-NaOH [pH 7.9], 50 mM NaCl, 1.5 mM EDTA, 1.5 mM DTT and 1 mM phenyl-methyl sulfonyl fluoride (PMSF). V_3 extract of vaccinia

virus expressing chicken c-erbA-α was obtained from infected HeLa cells [17,23,24]. Nuclear extract from these cells was isolated in a buffer containing 20 mM Hepes-NaOH [pH 7.9], 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF. Recombinant c-*erbA*-lac Z fusion protein was produced in *E.coli*, using the pEX expression vector, and was purified as described previously [25]. The bacterial protein extract contained approximately 25% receptor protein [26], either full length chicken c-erbA-α [11], or rat c-erbA-ß [19] from which the 30 N-terminal amino-acids had been deleted [27]. As a negative control we used an *E.coli* extract, in which the Lac Z gene of the pEX plasmid without insert was expressed [26].

Avidin-biotin complex-DNA (ABCD)-assay

The double stranded oligonucleotides used in this study contained a 5' overhang at both ends to allow incorporation of biotin-11-dUTP [22]. Full length biotinylated probes were purified by nondenaturing polyacrylamide gel electrophoresis and quantitated by fluorometry [28]. The ABCD-assay was performed as previously described [21,22]. In short, 20 nM [^{125}I]-T_3 was incubated with a protein extract (containing the T_3 receptor) for 20 min at room temperature in a volume of 48 µl buffer H (20 mM Hepes-NaOH [pH 7.8], 50 mM KCl, 1 mM ß-mercaptoethanol, 20% glycerol, 200 µg/ml poly[d(I.C).d(I.C)]) and competitor DNA as mentioned in the figure legends. This was followed by the addition of 2 µl biotinylated DNA solution and a 40 min incubation at room temperature. Hormone-receptor-DNA complexes were precipitated by addition of 20 µl of streptavidin-agarose slurry, washed three times with 1 ml of ice-cold buffer H, and assayed for [^{125}I] activity.

Oligonucleotides

DNA probes containing *cis*-acting sequences were obtained by annealing the following sense and antisense oligonucleotide sequence. The human collagenase AP-1 element [18] AGCATGAGTCAGACAC. An optimized palindromic TRE sequence (PAL) [21] TCAGGTCATGACCTGA. Rat growth hormone (rGH) TRE [13] CGGTAAGATCAGGGACGTGACCGCAGG. Rat α-myosin heavy chain (MHC) TRE [22] TTGGCTCTGGAGGTGACAGGAGGACAGC. A negative control (AD5) has no homology with AP-1 or the T_3 receptor binding sites [21]. GCGGTGTACACAGGAAGTGACAATTTTCGC. Biotinylated oligonucleotide probes were prepared as previously described [21,22] and purified by non-denaturing polyacrylamide gel electrophoresis.

Gel retardation assay

A double-stranded oligonucleotide corresponding to the collagenase promoter sequence from -76/-61 was labeled at the 5'-overhang with [α-^{32}P]dATP using the Klenow fragment of *E.coli* DNA polymerase I. We incubated 10 fmol (approximately 10.000 cpm) of the resulting probe with various amounts of proteins and/or non-radioactive competitor DNA (as indicated in the Figure legends) for 30 min. at room temperature. The incubation was carried out in a 50 µl volume in the presence of 25 mM Hepes-NaOH [pH 7.6], 10% glycerol, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 µM $ZnCl_2$, 1 mM DTT, 1 µM T_3, 200 µM PMSF, 10 µM leupeptin and 1 µg/50 µl poly[d(I.C).d(I.C)].

RESULTS

Thyroid hormone inhibits the AP-1 response

To investigate the regulation by the thyroid hormone (T_3) receptor of transcriptional activation by AP-1, we performed transient transfection experiments in

HeLa cells. These cells can be transfected with high efficiency, do not express endogenous T_3 receptor and show a strong increase in AP-1 activity after treatment with the phorbol ester 12-O-tetradecanolphorbol-13-acetate (TPA) [1-3]. The transcriptional effect of the AP-1 site alone has been investigated with the plasmid COLL-AP1-TATA-CAT. This construct consists of the chloramphenicol acetyltransferase (CAT) reporter gene fused to a minimal promotor, which only contains the collagenase AP-1 element upstream of a TATA-box. This construct is highly activated after TPA treatment or upon co-transfection of plasmids expressing *jun* and *fos* (Fig. 1A,B). Co-transfection of the adenovirus E1a gene, which is known to repress AP-1 activity [18], inhibited the CAT-expression (Fig. 1A). These data confirm that the COLL-AP1-TATA-CAT reporter gene can serve to demonstrate activation and inhibition of the AP-1 response in HeLa cells. Using a RSV-CAT construct in similar co-transfections, it was shown that variation in transfection efficiency in these experiments was too small (two-fold) to bias the data from the COLL-AP1-TATA-CAT construct (Fig. 1B).

The activity of the COLL-AP1-TATA-CAT construct could be inhibited strongly, if expression vectors encoding the T_3 receptor were co-transfected. This inhibitory effect of the T_3 receptor could be observed both in HeLa cells overexpressing *jun* and *fos* from co-transfected plasmids, and in HeLa cells stimulated with TPA (Fig. 1A,C). The degree of inhibition is largely dependent on thyroid hormone, although *c-erbA-α* also causes some inhibition of reporter gene expression in cells not treated with hormone. This partial inhibition is probably due to trace amounts of T_3 which are still present in the charcoal-treated serum [20]. Since the viral counterpart of the T_3 receptor, the v-*erbA* gene, is known to repress gene activity [15-17], we tested its effect on AP-1 dependent gene expression. Co-transfection of a v-*erbA* expression vector did not, however, inhibit the expression of the COLL-AP1-TATA-CAT construct (Fig. 1D) This observation is in line with our conclusion that the T_3 receptor represses AP-1 activity only when occupied by T_3, because the viral homologue of the T_3 receptor has lost its ability to bind T_3.

T_3 receptor inhibits binding of AP-1 to DNA

We investigated whether the T_3 receptor can interfere with the binding of AP-1 to its target site on the DNA. Therefore, we studied AP-1 binding in the presence or absence of T_3 receptor in gel retardation experiments. As was also reported previously [18], a small DNA probe containing the collagenase AP-1 response element can form a specific complex with AP-1 from HeLa extracts. (Fig. 2, lanes 1 and 2). When we

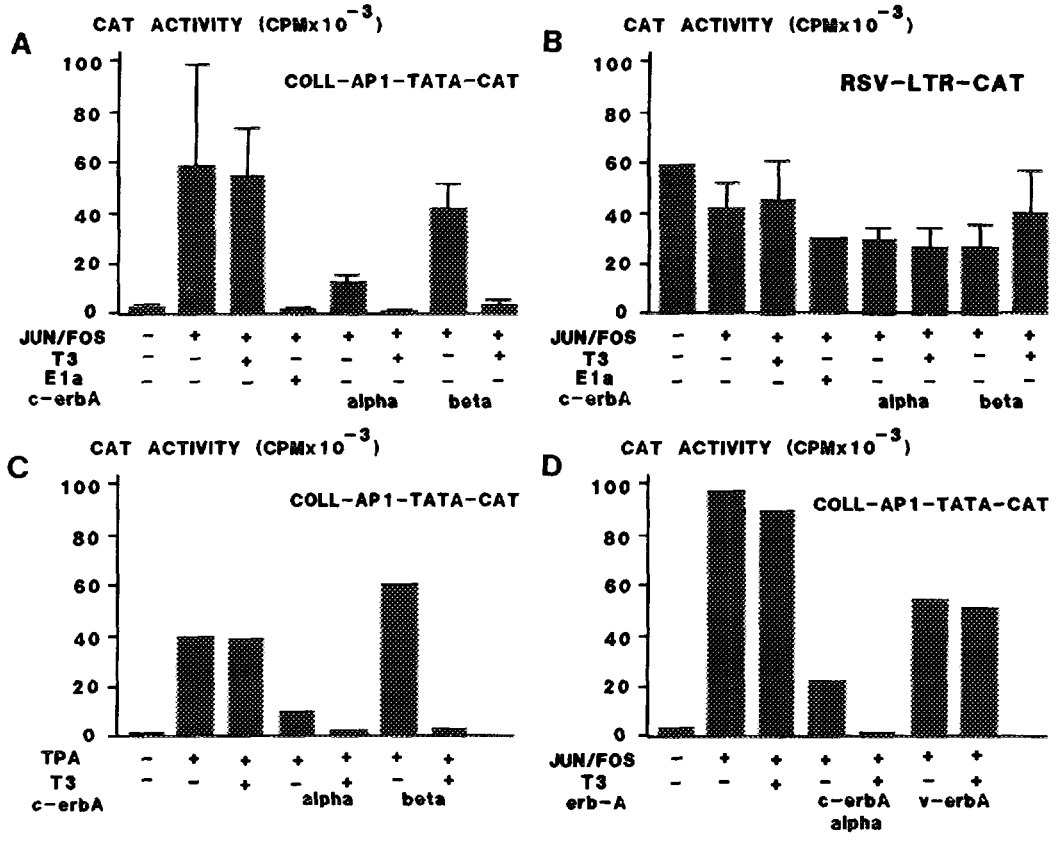

Fig. 1. T_3-dependent repression of the AP-1 induction.
2 μg of reporter plasmid was used to transfect HeLa cells with the DEAE-dextran method, together with 4 μg of the vectors expressing rat c-*erbA* α or ß [17], *jun*, *fos* or E1a [15]. Eight hours after transfection 100 nM T_3 was added when indicated and the incubation was continued for another 40 hours after which CAT-activity was measured. (**A**) Co-transfections with the reporter plasmid COLL-AP1-TATA-CAT (n=3). (**B**) 2 μg of the RSV-LTR-CAT construct was used in a co-transfection experiment (n=3).) (**C**) Co-transfections with COLL-AP1-TATA-CAT, but this time cellular AP-1 levels were increased by TPA induction (n=1). (**D**) The effect of 2μg of a v-*erbA* expression vector [16] was investigated on the reporter gene constructs COLL-AP1-TATA-CAT (n=1).

added an excess amount of unlabeled AP-1 element, we observed, as expected, a decrease in the AP-1 bandshift (lanes 3 and 14). When, alternatively, unlabeled T_3 response element, which has no specific affinity for AP-1, was added, the AP-1 bandshift was only marginally affected (lane 4). In the same experiment we studied the effect of increasing amounts of T_3 receptor on the binding of AP-1 to its target site. The addition of increasing amounts of c-*erbA*-α Lac Z fusionprotein to an incubation mixture, containing a standard amount of AP-1 from the HeLa extract, resulted in a progressive decrease of AP-1 binding to its response element(lanes 6-8).

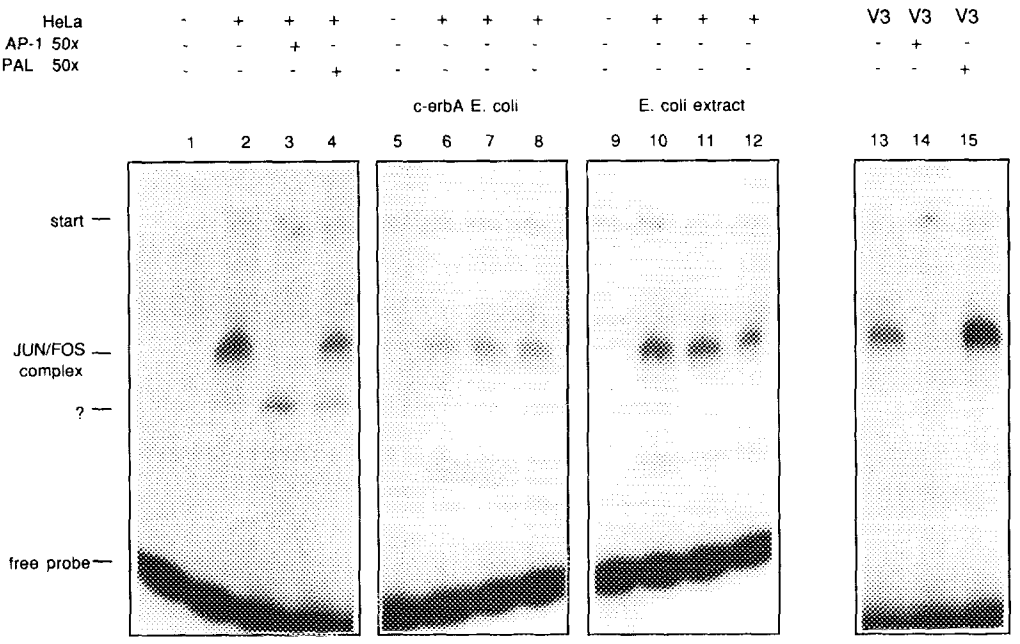

Fig. 2. Gel retardation assay with human collagenase AP-1 element, showing that binding of *jun/fos* to an AP-1 site is inhibited by c-*erbA* proteins. Lane 1, migration of 10 fmol ^{32}P-labelled AP-1 probe alone. Lanes 2-4,6-8,10-12 contain 6 μg HeLa cell extract (HeLa cells were incubated for 90 min. with TPA to increase the levels of *jun/fos* [1]). In lanes 3 and 14 a 50-fold excess of unlabelled AP-1 site and in lanes 4 and 15 a 50-fold excess of unlabelled palindromic T_3 response element (PAL TRE) was added. Lanes 5-8, chicken c-*erbA*-α receptor expressed in *E.coli* (approximately 250 ng c-*erbA*/μg protein extract [25]): 0.8 μg total protein in lanes 5 and 6; 0.4 μg in lane 7 and 0.2 μg in lane 8. Lanes 9-12, *E.coli* extract (without c-*erbA*): 0.8 μg in lanes 9 and 10, 0.4 μg in lane 11 and 0.2 μg in lane 12. The nature of the lower retarded band (?) is unknown. Lanes 13-15 contain 2 μg V_3 extract, which consists of HeLa cell nuclear extract in which chicken c-*erbA*-α protein was over-expressed [16,20,21]. Approximately 20 fmol of T_3 receptor was present in the reaction from the V_3 extract, as calculated from Scatchard analysis [20]. All reaction mixtures contained 1 μM T_3.

A bacterial extract, irrespective of whether it contained the c-*erbA*-α Lac Z fusion protein (lane 5) or not (lane 9) was unable to bind the collagenase AP-1 element. From these results we conclude that the T_3 receptor inhibits binding of AP-1 to the AP-1 response element. In the same experiments we also used extract from HeLa cells in which c-*erbA*-α was overexpressed, (the V_3 extract). The increase in the binding of AP-1 to its cognate DNA binding element upon the addition of unlabeled T_3 response element (compare lanes 13 and 15) suggests that depletion of T_3 receptor from the incubation mixture, by addition of T_3 receptor-binding sequences, abolishes the inhibitory effect of the T_3 receptor on AP-1 binding to the AP-1 response element. These data imply that the T_3 receptor can only interfere with binding of *jun* and *fos* to

their cognate response element, if the this receptor is not bound to its own T_3 response element.

T_3-mediated inhibition does not involve competition for DNA-binding sites

Repression of transcription can be the result of a co-localization of the DNA-binding sites of a stimulatory and an inhibitory transcription factor (e.g. ref. [29]). Such an overlapping response element has recently been described for steroid hormone receptors and AP-1 on the human osteocalcin gene [7]. However, we were unable to detect a direct binding of T_3 receptor to the collagenase AP-1 element using the gel retardation assay (Fig. 2, lane 8), or the more sensitive ABCD assay [21,22] (Fig. 3). Within the same experiment T_3 response elements showed a strong receptor binding, demonstrating that the protein extracts used contained a functional DNA-binding T_3 receptor. Furthermore, DNAse 1 protection assays with T_3 receptor showed no protected footprint on the collagenase AP-1 element, whereas a clear footprint could be observed on T_3 response elements (our additional unpublished data). Therefore, these results indicate that inhibition by the T_3 receptor of the binding of the AP-1 complex to its cognate DNA-binding element does not involve competition for common or overlapping DNA binding sites.

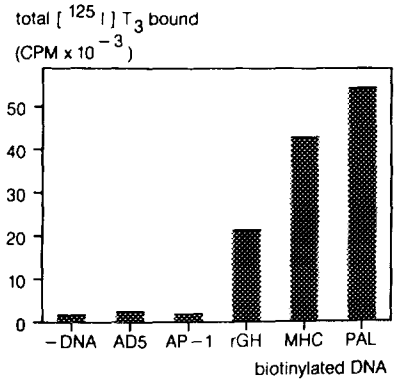

Fig. 3. Binding of T_3 receptors to oligonucleotide probes containing biotin-11-dUTP. Nuclear protein extract (5 µg) contained 40 fmol of specific T_3 receptor-binding activity from a chicken α-c-*erbA* protein (V3), expressed by a vaccinia virus vector in HeLa cells. This T_3 receptor extract was labeled with ^{125}I-T_3 and incubated with 1000 fmol biotinylated TRE sequence in a final reaction volume of 50 µl. After incubation the [^{125}IT_3/receptor/DNA] complex was preciptated with streptavidin-agarose as previously described for this avidin biotin complex DNA (ABCD) assay [19-21]. Results are the mean of two experiments with less than 10% variation.

DISCUSSION

The thyroid hormone (T_3) receptor, encoded by the c-*erbA* genes, is able to regulate gene expression in different ways. In its function as a *trans*-acting transcription factor it binds a *cis*-acting T_3 response element on a promoter and regulates the expression of the gene involved. This regulatory mechanism can result in activation [13] or inhibition [14] of gene expression and has been observed for many other related receptors like those for glucocorticoid hormone. Our data provide evidence for another mechanism by which the T_3 receptor is able to regulate gene expression. The transcription of AP-1 responsive genes can be inhibited by the T_3 receptor, but only if the receptor is occupied by T_3. This specific form of inhibition does not involve binding of the T_3 receptor to AP-1 responsive elements. Furthermore, since the presence of excess T_3 responsive elements abolishes the inhibitory effect of the T_3 receptor on AP-1 responsive elements, this form of inhibition appears to require T_3 receptors that are not bound to their own response elements. Since the activity of bacterially synthesized T_3 receptor and of T_3 receptor obtained from a vaccinia virus expression system in HeLa cells are equivalent (Fig. 2), it is unlikely that posttranslational modifications of the T_3 receptor, like phosphorylation, play a role in the inactivation of AP-1.

The presented data indicate a novel pathway for T_3-dependent regulation of transcription by the T_3 receptor. In contrast with previously described mechanisms for T_3 receptor action, this transcriptional inhibition is not due to direct binding of the T_3 receptor to the AP-1 response element, but involves direct or indirect contact between the T_3 receptor and AP-1 proteins. During the preparation of this manuscript, two publications [30,31] appeared that report experiments leading to the same conclusion, viz. that T_3 receptors can antagonize the transcriptional activity of AP-1 by interfering with its DNA-binding activity.

The observation that the v-*erbA* oncogene product, which is the viral homologue of c-*erbA* and which can no longer bind T_3 [23], fails to inhibit the AP-1 response (this study, 30, 31], suggests that the carboxyterminal ligand binding domain of the T_3 receptor is required for the protein-protein interactions that inactivate AP-1. The glucocorticoid hormone receptor is also able to inhibit the AP-1 response [5-7]. Interestingly, the inhibition by the glucocorticoid receptor is reported to involve a physical interaction with AP-1, resulting in a transcriptionally inactive protein complex [5-7]. The carboxyterminal ligand binding domain is involved in the formation of these complexes as well [32], suggesting the possibility of a functional interaction of the

signal transduction pathway of cytokines (acting via *jun* and *fos*) and ligand-dependent transcription factors of the steroid receptor superfamily in general. Furthermore, the studies on the functional antagonism between the glucocorticoid receptor and the AP-1 transcription factor show that the interference with transcriptional activation is mutual [5,6,32]. This regulatory network therefore appears to open up possibilities to manipulate e.g. the bioavailability of T_3 receptors for their cognate response elements by increasing the concentration of ligand-occupied glucocorticoid receptors and, thus, depleting free AP-1 transcription factor.

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