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DETERMINATION OF THE PROMOTER ELEMENTS THAT MEDIATE REPRESSION OF c-FOS GENE TRANSCRIPTION BY THYROID HORMONE AND RETINOIC ACID RECEPTORS

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SUMMARY. Basal and stimulated activity of the c-*fos* promoter is reduced by triiodothyronine (T3) and retinoic acid (RA) in GH1 cells. We examined the influence of these ligands on the activity of reporter constructs containing the AP-1 site, the serum response element (SRE) and the cyclic AMP responsive element (CRE) of the c-*fos* promoter under control of an heterologous promoter. T3 and RA decreased the response of AP-1 and SRE sequences to phorbol esters, forskolin or serum but they did not reduce basal or forskolin-stimulated activity mediated by the CRE. Therefore, repression of c-*fos* gene expression by T3 and RA receptors appears to be exerted through transcriptional interference with the SRE and the AP-1 binding site of the promoter.

We have previously shown that T3 and, to a lesser extent, RA decrease the expression of the c-*fos* oncogene and its response to the tumor promoter 12-*O* -tetradecanoylphorbol-13-acetate (TPA) and to cyclic AMP (cAMP) in pituitary GH1 cells [1]. This inhibitory effect is reciprocal, and c-Fos blocks transcriptional induction of the growth hormone promoter by T3 and RA receptors [1].

The 5′-flanking region of the c-*fos* gene does not contain consensus hormone response elements that could bind the nuclear T3 and RA receptors and mediate the repression. Therefore, negative regulation by the receptors could be a consequence of transcriptional interference on other regulatory sequences. The c-*fos* promoter contains several elements required for basal and induced expression. The SRE is required for serum and TPA induction and has an adjacent AP-1 binding site, and the CRE is implicated in c-*fos* gene transcription by cAMP [for review see 2,3]. Our results show that both AP-1 and SRE elements are involved in the repression of c-*fos* gene expression by the nuclear receptors. Since these elements mediate the effect of a variety of mitogens, growth factors and oncogenes on the c-*fos* gene as well as in other cell growth-related genes, the interference of the nuclear receptors on the activity of these motifs might have important implications in proliferation, differentiation and transformation of pituitary cells.

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MATERIALS AND METHODS

Plasmids. The plasmid FC4 [4] contains 404 base pairs upstream of the c-*fos* start of transcription linked to the chloramphenicol acetyltransferase (CAT) gene. In the constructs pSRE/AP-1, p4xSRE, p4xAP-1 and p4xCRE tk CAT, these synthetic elements were inserted in front of the thymidine kinase (tk) promoter of pBLCAT2. The expression vectors for the α human retinoic acid receptor (RAR) [5] or the α thyroid hormone receptor (TR) [6] have been previously described.

DNA transfection. GH1 cells were transfected by electroporation [1,7]. The plasmids were mixed with 30-40 million cells and exposed to a high voltage pulse (170-200 V, 960 μF). The total amount of transfected DNA was kept constant by addition of carrier DNA. The cells were split in different culture plates in RPMI medium containing 10% AG1x8 resin-charcoal stripped serum. CAT activity was determined by incubation of the cell extracts with [^{14}C] chloramphenicol. The unreacted and acetylated [^{14}C]chloramphenicol were separated by thin layer chromatography, identified by autoradiography and quantitated. Treatments were performed in duplicate cultures and each experiment was repeated at least 2-3 times with similar relative differences in T3 and RA regulated expression.

DNA binding assays. Gel retardation analysis were carried out with nuclear extracts [8] from GH1 cells. As probes we used the AP-1 site at -304/-283 of the c-*fos* promoter (5′-GGACATCTGCGTCAGCAGGTTT-3′) and the SRE (5′GCTTACACAGGATTGTCCATATT AGGACATCT-3′) at -259/-290. For the binding assays the extracts were incubated on ice for 15 min in a buffer (20 mM Tris HCl pH=7.6, 75 mM KCl, 1mM DTT, 5 μg/ml BSA, 13% glycerol) containing 3 μg poly(dI-dC) and then for 20 min at room temperature with approximately 70.000 cpm double stranded oligonucleotide end-labeled with [^{32}P]ATP. For competition experiments an excess (50-fold) of unlabeled double-stranded oligonucleotides was added to the binding reaction. DNA-protein complexes were resolved on 5% polyacrylamide gels in 0.5% TBE buffer. The gels were then dried and autoradiographed at -70°C.

RESULTS

In agreement with our previous observations, incubation with T3 caused a decrease of c-*fos* promoter activity and strongly repressed the induction caused by TPA and forskolin (Fig.1A). RA did not affect the activity of the c-*fos* promoter. A direct proof of the implication of the SRE and

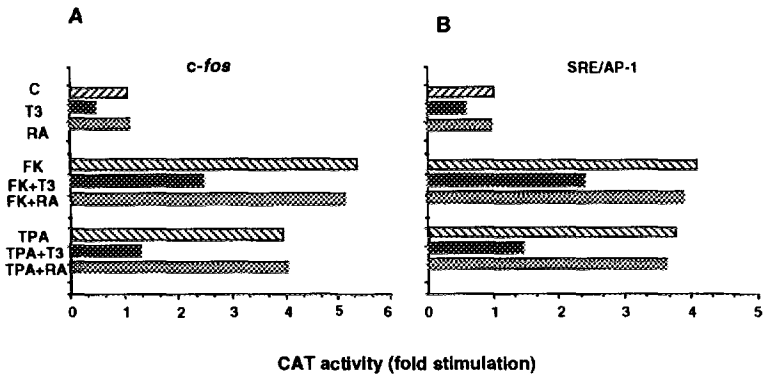

Figure 1. *Influence of T3 and RA on the activity of regulatory elements of the c-fos promoter.* In *panel* A GH1 cells were transfected with 10 μg of the c-*fos* promoter plasmid FC4. CAT activity was determined in control cells (C) and in cells incubated with 1nM T3 or 1 μM RA for 48 hours and/or with 100 nM TPA or 15 μM forskolin (Fk) for the last 8 hours. In *Panel* B the cells were transfected with a construct containing the SRE/AP-1 sequences of the c-*fos* promoter under control of an heterologous promoter. The data represent the mean of duplicate transfections with less than 5% variation.

AP-1 motifs on the transcriptional repression caused by T3 was obtained with a plasmid containing these sequences of the c-*fos* promoter under control of the thymidine kinase (tk) promoter. Figure 1B shows that the activity of this plasmid was increased by both TPA and forskolin, and that the repression caused by T3 was very similar to that found with the c-*fos* promoter. The effect of T3 was not longer observed when the SRE and the AP-1 binding sites were mutated (not illustrated).

To analyze separately the influence of these elements on the inhibitory response, GH1 cells were transfected with CAT plasmids containing tandem repeats of the AP-1 binding site (p4xAP-1 tk CAT) or the serum response element (p4xSRE tk CAT). Figure 2A shows that the AP-1 element conferred responsiveness to both TPA and forskolin, and that T3 reduced basal and induced CAT levels. As shown in Figure 2B, T3 did not significantly decrease the basal activity of p4xSRE tk CAT, but strongly inhibited the induction caused by TPA. The SRE does not contribute to the effect of cAMP, since incubation with forskolin did not stimulate activity of p4xSRE tk CAT. Addition of 20% fetal calf serum increased CAT levels by about 3-fold, and this response was reduced to almost basal levels by T3 (Table 1). As can be observed in Fig.2C the activity of the p4xCRE tk CAT construct was significantly stimulated by forskolin, but not by TPA, and this stimulation was not affected in the presence of T3 or RA.

To analyze whether the inhibitory effect of T3 on constructs containing the SRE and AP-1 sites reflects a decrease in the abundance of nuclear proteins that bind to these sites, gel retardation assays were performed with AP-1 and SRE motifs. As shown in Fig.3 (left panel) the intensity and mobility of the SRE-protein complexes remained unaltered after incubation with T3 or RA. However, T3 significantly decreased the abundance of the retarded bands formed with the AP-1 binding site (right panel) in agreement with our previous observations [1]. Additionally, the mobility shift produced by extracts of GH1 cells with a CRE oligonucleotide was not significantly altered by treatment with T3 or RA (not illustrated).

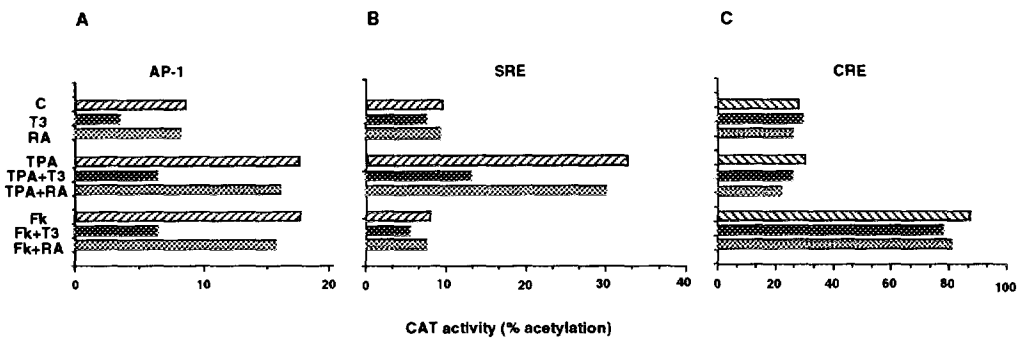

Figure 2. *Regulation of the activity of AP-1, SRE and CRE elements by T3 and RA.* GH1 cells were transfected with plasmids containing tandem repeats of the AP-1 (left *panel*), SRE (middle *panel*) or CRE (right *panel*) motifs. After transfection the cells were incubated in the presence or absence of 1 nM T3 or 1μM RA for 48 h and/or with 100 nM TPA or 15 μM forskolin (Fk) for the last 8 hours.

Table 1. Influence of T3 and RA on the response of the SRE to serum

	CAT activity (% acetylation)	
	-FCS	+ FCS
control	8.6 (1)	23.5 (2.7)
T3	8.1 (0.9)	9.4 (1.2)
RA	8.4 (1)	27.3 (3.2)

GH1 cells were transfected with 10 µg of p4xSRE-tk-CAT. After transfection the cells were incubated either in hormone-depleted medium (- FCS) or with 20% fetal calf serum (+ FCS) in the presence or absence of 1 nM T3 or 1µM RA and CAT activity determined 48 h later. The data represent the mean of duplicate transfections that did not vary among them more than 5-10% and the numbers in parenthesis indicate the ratio to the values found in the untreated cells in the absence of fetal calf serum.

Figure 4 shows the influence of an expression vector encoding the RA receptor RARα on the activity of the p4xAP-1 tk CAT construct. Whereas RA did not significantly alter CAT activity in control transfections (see Fig.2A), the retinoid reduced basal and stimulated CAT activity when the reporter plasmid was co-transfected with RARα (Figure 4A). This inhibition was similar to that obtained with T3 and reproduces our previous observations with the c-*fos* promoter [1]. When the thyroid hormone receptor (TRα) was over-expressed (Figure 4B), incubation with its ligand produced a strong inhibition of basal CAT levels, and very effectively blocked the response

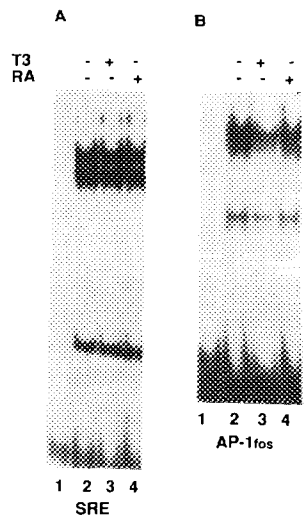

Figure 3. *Mobility shift assay of nuclear extracts with the serum response element and the AP-1 binding site.* The ^{32}P-labeled SRE (left panel) and AP-1 binding site (right panel) of the c-*fos* gene were used for gel retardation with 20 µg of nuclear extracts from control GH1 cells or from cells treated with 1nM T3 or 1 µM RA for 48 h. The first lane in each panel indicates competition of lane 4 with an excess of the corresponding unlabeled oligonucleotide.

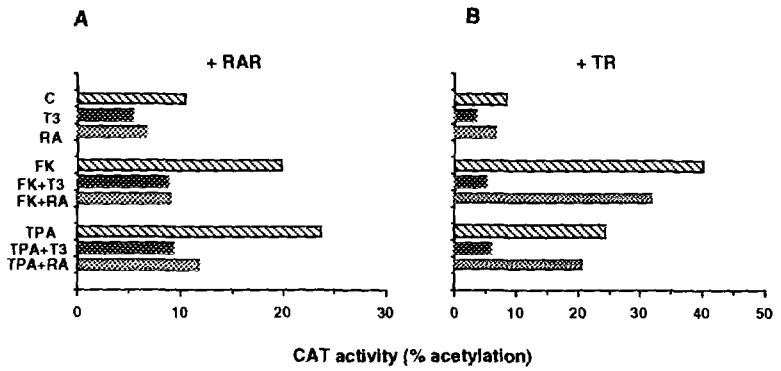

Figure 4. *Transcriptional repression of AP-1 sites by T3 and RA.* CAT activity was determined in GH1 cells co-transfected with 10 μg of the 4xAP1-tk-CAT construct plus 10 μg of an expression vector for either the thyroid hormone receptor TR (right *panel*) or the retinoic acid receptor RAR (left *panel*). The cells were treated as in Figures 1 and 2 with T3, RA, forskolin (Fk) or TPA.

to both TPA and forskolin through the AP-1 binding site. Even after over-expression of TRα or RARα the response to forskolin was unaltered in GH1 cells transfected with p4xCRE tk CAT (not illustrated).

DISCUSSION

In the present study we have examined which are the elements of the c-*fos* promoter that mediate negative regulation by T3 and RA in GH1 cells. Our data show that T3 represses stimulation of constructs containing the SRE/AP-1, SRE or AP-1 motifs of the c-*fos* promoter.

The T3 receptors interfere with AP-1 mediated transactivation and decrease the abundance of AP-1 complexes in a ligand-dependent manner. In GH1 cells the endogenous RA receptors do not decrease AP-1 activity. However, RA inhibits basal and stimulated AP-1 activity after transfection with an expression vector for RAR. The effect of T3 and RA on the AP-1 containing constructs was identical to that previously observed by us with the natural c-*fos* promoter [1]. Negative regulation of gene expression by transcriptional interference of the nuclear receptors with the AP-1 complex has been observed in several instances and it has been suggested that it could involve either competition for binding to DNA or direct protein to protein interaction [9-15].

Transcriptional interference of ligands of the nuclear receptor superfamily with the SRE had not been previously reported. Our data show that T3 strongly decreases the response of the SRE to TPA or serum, both potent stimulators of c-*fos* gene expression [2,3]. These results suggest that this element is implicated in the decreased response of the oncogene to different mitogens in T3-treated cells. The induction of the SRE appears to be mediated by binding of phosphorylated TCF proteins to the serum response factor $p67^{SRF}$ [16,17]. Since T3 does not change the pattern or abundance of proteins bound to this DNA element, there is the possibility that the activity of these factors could be altered by the treatment with the hormone.

The transcriptional actions of cAMP are normally mediated by CRE sequences, but the nucleotide can also act via AP-1 sites [18]. Our data show that the inhibitory effect of T3 or RA on the response of the c-*fos* promoter to cAMP is not mediated by CRE sequences but rather by AP-1 binding sites since T3 and RA showed no repression on forskolin-induced activity of a CRE-containing construct.

From these studies it is clear that the final cellular response to multiple regulatory signals (hormones, growth factors and oncogenes) which converge on the c-*fos* oncogene is modulated by T3 and RA receptors. On the other hand, the response to thyroid hormones and retinoids will depend on the activity of other nuclear factors such as the AP-1 complex or the proteins that bind to the serum response element. These interactions provide not only complexity, but also flexibility increasing the spectrum of the transcriptional responses.

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