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Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor

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Transcriptional activation by nuclear receptors is achieved through autonomous activation functions (AFs), a constitutive N-terminal AF-1 and a C-terminal, ligand-dependent AF-2 that comprises a motif conserved between nuclear receptors. We have performed an extensive mutational analysis of the putative AF-2 domain of chicken thyroid hormone receptor alpha ($cT_3R\alpha$). We show that the AF-2 region mediates transactivation as well as transcriptional interference (squelching), not only between the thyroid hormone and vitamin (type II) receptors, but also between type II and steroid hormone (type I) receptors. Transcriptional activation and interference require equivalent doses of the cognate ligand, and mutations in the conserved motif that reduce ligand-induced transactivation also impair transcriptional interference. When fused to the Gal4 DNA binding domain, a 35 amino acid long fragment containing the conserved motif is able to transactivate and squelch, albeit in a ligand-independent manner. Our results define the AF-2 of $cT_3R\alpha$ as an autonomous transactivation domain that, in its natural context, is governed by ligand. We propose that AF-2 is probably part of a surface for interaction with either a general transcription factor or a putative bridging factor, that might be utilized by type I and II receptors.

Key words: nuclear receptors/thyroid hormone receptor/transactivation domain/transcriptional interference

Introduction

Nuclear receptors are ligand-inducible transcription factors that modulate the activity of promoters through *cis*-acting sequences called hormone response elements. The superfamily includes receptors for steroid hormones (type I receptors) and for thyroid hormone and vitamins, referred to as type II receptors, as well as orphan receptors whose cognate ligands are as yet unidentified. Type I and II receptors show functionally distinct properties. Firstly, type II receptors are able to bind to their responsive elements in the absence of ligand, whereas ligand is required to dissociate the type I receptor–hsp90 complex and hence governs DNA binding. Secondly, type II receptors bind and transactivate through responsive elements that are composed of half-sites arranged as direct repeats, as opposed to palindromically arranged half-sites invariably separated by three nucleotides for type I receptors. Finally, type II receptors do not bind to their respective binding sites as homodimers but require an auxiliary factor, RXR, for high affinity binding [for reviews see Leid *et al.* (1992a) and Stunnenberg (1993)].

Transcriptional regulation by type I and II receptors is achieved through autonomous transcription activation functions (AFs): a constitutive AF-1 located in the N-terminal part of the receptor and a ligand-dependent AF-2 located in the C-terminal domain (Hollenberg and Evans, 1988; Webster *et al.*, 1988; Tora *et al.*, 1989; Danielian *et al.*, 1992; Nagpal *et al.*, 1992, 1993; Folkers *et al.*, 1993; Saatcioglou *et al.*, 1993b; see Gronemeyer, 1992, for review). Despite intensive studies, it has proven difficult to delineate the partially overlapping functions located in the C-terminal domain of the receptors, i.e. ligand binding, dimerization and transactivation. Furthermore, little is known about the mechanisms by which the ligand-activated, DNA-bound receptors transmit their transactivating 'signal' to the basal transcription machinery and what factors are involved in mediating and receiving this signal.

Transcriptional activators are thought to modulate transcription by promoting or stabilizing the assembly of preinitiation complexes which may involve direct or indirect actions on components of the basal transcription machinery [for reviews see Roeder (1991) and Zawel and Reinberg (1992)]. Direct interactions between the basal factor TFIIB and transactivators such as Gal4-VP16 (Lin and Green, 1991; Lin *et al.*, 1991; Choy and Green, 1993) or the nuclear receptors COUP-TF1 (Ing *et al.*, 1992) or $T_3R\beta$ (Baniahmad *et al.*, 1993) have been described. The constituents of the basal factor TFIID, the TATA binding protein TBP and its associated factors TAFs (Pugh and Tjian, 1990; Zhou *et al.*, 1992) have also been identified as targets for transcriptional signalling (Ingles *et al.*, 1991; Choy and Green, 1993; Goodrich *et al.*, 1993; Hoey *et al.*, 1993; Weinzierl *et al.*, 1993). Experimental evidence further supports the existence of bridging molecules, also termed coactivators, transcription intermediary factors (TIFs) or adaptors, that are thought to mediate the interaction of transactivators with the basal transcription machinery, for example the adenoviral E1A 289R protein (Liu and Green, 1990; Lee *et al.*, 1991; Schöler *et al.*, 1991; Berkenstam *et al.*, 1992; Keaveney *et al.*, 1993). In transcriptional interference (squelching) experiments, the presence of a strong activator suppresses the activity of other related *trans*-acting factors by sequestering putative bridging factors [for reviews see Lewin (1990) and Ptashne and Gann (1990)], or by occupying a surface required to mediate or receive *trans*-acting signals, a phenomenon referred to as surface saturation (Schöler *et al.*, 1991).

Previous studies had suggested that the most C-terminal part of nuclear receptors is involved in ligand-dependent transactivation *in vivo* (Zenke *et al.*, 1990; Danielian *et al.*, 1992; Saatcioglou *et al.*, 1993a) and *in vitro* (Schmitt and Stunnenberg, 1993) and sequence comparison revealed that this region contains a stretch of six amino acids that shows a high degree of conservation between type I and II receptors (Danielian *et al.*, 1992). A drawback of the studies to date is that the identification of the putative AF-2 transactivation domain is based solely on mutations that cause a loss-of-activation phenotype. Transcriptional interference, documented to occur between type I receptors, has only been grossly mapped to the N- and C-terminal halves of the receptors (Meyer *et al.*, 1989), but not delineated to a particular region.

Here, we have analyzed the ligand-dependent transactivation function in the C-terminal domain of the chicken $T_3R\alpha$. Mutational analysis shows that this region is involved in ligand-induced transcriptional activation as well as interference. We document squelching experiments among type II receptors and between type II and type I receptors. In addition, we show that a region of 35 amino acids comprising the conserved motif of AF-2 acts as an autonomous ligand-independent transactivation domain when fused to the Gal4 DNA binding domain (DBD). Our results define the AF-2 of $cT_3R\alpha$ as an autonomous transactivation domain and suggest that it mediates interactions with a putative coactivator or basal transcription factor that is commonly utilized by both type I and II receptors.

Results

A conserved region of the E-domain of $T_3R\alpha$ is involved in ligand-dependent transactivation

The 12 C-terminal amino acids of $cT_3R\alpha$ have previously been implicated in transactivation (Zenke *et al.*, 1990; Saatcioglou *et al.*, 1993a) and are postulated to adopt an amphipathic α-helical conformation (Zenke *et al.*, 1990). We have introduced a series of point mutations affecting either the hydrophobic or acidic amino acids and have tested their effects on transactivation in transient transfection experiments (Figure 1A). Two different T_3-responsive reporter constructs were used, containing either three copies of the artificial palindromic TRE_{pal} [$(T_3RE_{pal})_3$-TK-Luc] or a single copy of a direct repeat with a four nucleotide spacing, T_3RE_{MoMLV}-TK-Luc (Vivanco Ruiz *et al.*, 1991) (Figure 1B). As expected, the wild-type $T_3R\alpha$ causes a strong activation of both reporters whereas construct C1, which carries a C-terminal deletion as is found in v-erbA (Muñoz *et al.*, 1988), is unable to activate transcription (Figure 1B), even at T_3 concentrations of up to 10^{-5} M (data not shown). A thyroid receptor with the Glu residue at position 401 changed to either a Gln or Lys (E401/Q and E401/K, respectively) loses the ability to activate transcription. In contrast, changing the adjacent

A

B

	Fold activation		Fold suppression	K_d (nM)
	$(TRE_{pal})_3$-TK	TRE_{MoMLV}-TK		
pSG-5	1.0	1.6	0.9	–
$CT_3R\alpha$	30.1	12.3	14.1	0.2
C1	0.5	1.2	0.9	5.4
F399/A	21.4	13.5	6.5	1.3
E401/Q	0.6	0.9	1.0	0.1
E401/K	0.6	1.0	1.0	3.3
F403/A	2.4	3.4	5.6	0.6
F403/Y	12.2	5.7	5.6	1.7
F399,F403/A	0.8	1.3	1.3	2.7
E404,D405/A	22.7	8.2	9.7	0.1
E404,D405/K	27.8	5.2	11.1	0.1

Fig. 1. Mutagenesis of the C-terminal transactivation domain of $cT_3R\alpha$. (**A**) Amino acid sequence of the C-terminal region of the E domain of the $cT_3R\alpha$. The ninth heptad repeat of the putative dimerization domain, the conserved region in which point mutations have been introduced, the v-erbA specific deletion and the borders of the two Gal4-$cT_3R\alpha$ chimeras referred to in the text (amino acids 374 and 396) are indicated. (**B**) Characterization of $cT_3R\alpha$ E-domain mutants. The table summarizes the transactivation, transcriptional suppression and T_3 binding properties of the different $cT_3R\alpha$ mutants. Transactivation was assayed in transient transfection experiments with the luciferase reporters $(T_3RE_{pal})_3$-TK-Luc and T_3RE_{MoMLV}-TK-Luc in P19 EC cells. Cells were transfected with 5 μg of reporter, 0.5 μg of internal control plasmid and 0.5 μg of pSG-based expression vectors for each $cT_3R\alpha$ mutant, and processed as indicated in Materials and methods. The table shows the fold induction by 10^{-7} M T_3 over the corrected luciferase activity value obtained by co-transfection of empty pSG-5 vector in the absence of T_3. Suppression of the activation of the $RAR\beta_2$ promoter by RA was also assayed by transient transfection in P19 EC cells, with the RA-responsive reporter plasmid R140-Luc (5 μg), along with internal control plasmid (0.5 μg), 0.5 μg of expression vector for hRXRα, and 1 μg of the expression plasmids for each of the indicated $cT_3R\alpha$ mutants. The fold suppression was calculated as the ratio between the corrected luciferase activity obtained in the presence of 10^{-6} M RA alone and that obtained in the presence of RA plus 10^{-7} M T_3. Dissociation constants (K_d) for T_3 of the different $cT_3R\alpha$ mutants are also shown. Values were calculated from ligand binding experiments using nuclear extracts from COS-7 cells transiently transfected with expression vectors for the different $cT_3R\alpha$ mutants (see Materials and methods).

pair of acidic amino acids E404,D405 to either Ala or Lys (E404,D405/A and E404,D405/K, respectively) resulted in a very marginal loss of transactivation. A receptor mutant with a Phe to Ala change at position 403 (F403/A) was defective when assayed on the $(T_3RE_{pal})_3$ reporter, but retained considerable activity when assayed on the T_3RE_{MoMLV} reporter. A more conservative change of the Phe to Tyr (F403/Y) retained in part the transcriptional activity. However, mutation of the upstream Phe residue to Ala (F399/A) did not have a large effect on the level of transcription whereas a double mutant F399,F403 to Ala resulted in a transcriptionally inactive receptor.

To establish whether the mutations that diminish or abolish T_3-dependent transactivation solely affected the putative transactivation function AF-2 or the ability of the mutant receptors to bind T_3 and, consequently, to transactivate, we determined the K_d for T_3 binding (Figure 1B). Different mutant phenotypes were obtained; firstly, mutant receptors that are not significantly affected either in their ability to bind T_3 or transactivate, i.e. E404,D405 to Ala or Lys; secondly, mutations that significantly diminish the affinity of the receptor for ligand, but have a minor effect on transactivation, such as F403/Y and F399/A; a number of mutants affect both properties

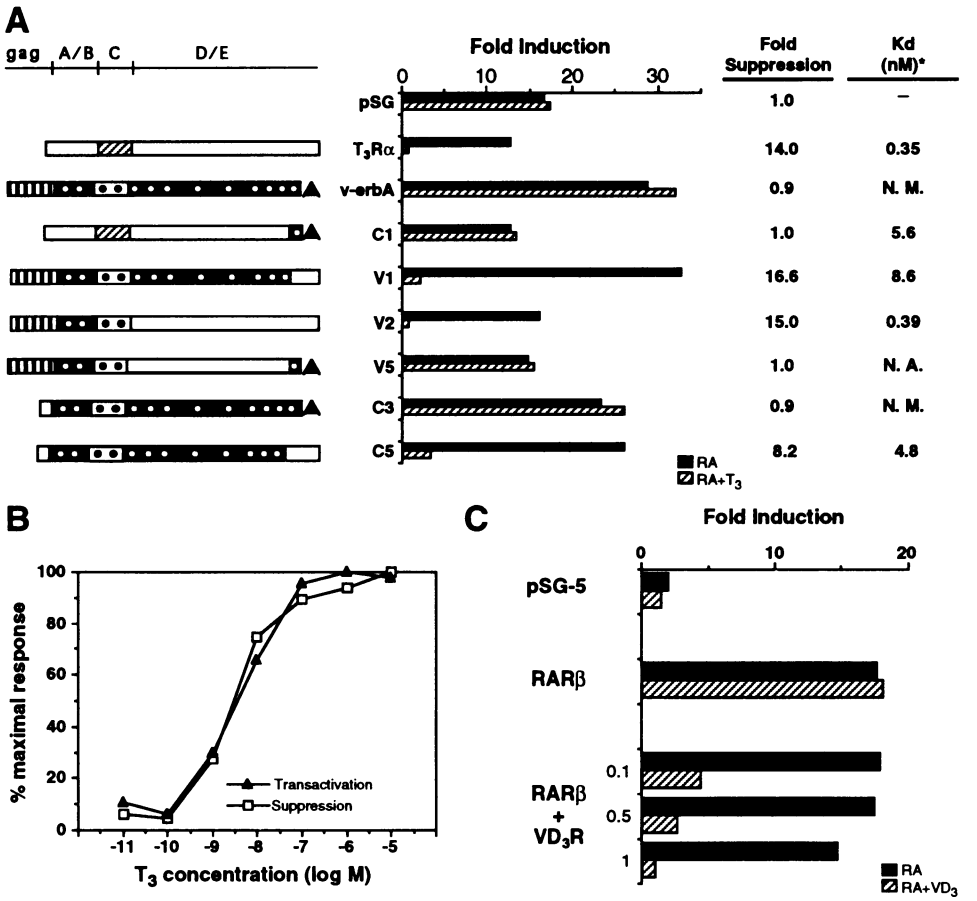

Fig. 2. Transcriptional suppression among type II receptors. (**A**) Localization of the domain of $cT_3R\alpha$ involved in transcriptional suppression. Chimeras between c $T_3R\alpha$ and v-erbA are schematically depicted in the left part, with v-erbA-derived sequences shown as solid boxes and $cT_3R\alpha$-derived sequences as empty boxes. The vertically hatched box indicates the gag region of v-erbA, the dots indicate point mutations and the C-terminal v-erbA-specific deletion is shown as a solid triangle. These chimeric constructs were assayed in transient transfection experiments for their ability to suppress RA-induced activation of the $RAR\beta_2$ promoter in RAC65 cells. RAC65 cells were transfected with the reporter plasmid R140-Luc, internal control plasmid, 0.5 μg of expression vector for hRARβ, and 1 μg of expression plasmids for the indicated chimeric proteins, as described in Materials and methods. Fold induction by 10^{-6} M RA (solid bars) or RA plus 10^{-7} M T_3 (hatched bars) is relative to the corrected luciferase activity value obtained by co-transfection of the parental expression vector pSG-5 in the absence of ligands. Fold suppression values indicated on the right side of the figure were calculated as described in the legend to Figure 1B. In addition, the dissociation constants for T_3 calculated for the different chimeras (Muñoz *et al.*, 1988) are shown in the right part of the figure (N.M.: binding not measurable; N.A.: data not available). (**B**) T_3 concentration dependence curves for hormone-induced transactivation by $cT_3R\alpha$ (solid triangles) and ligand-induced suppression by $cT_3R\alpha$ of the activation of the $RAR\beta_2$ promoter by RA (empty squares). Both transactivation and suppression were assayed in transient transfection experiments in RAC65 EC cells. For transactivation, reporter plasmid $(T_3RE_{pal})_3$-TK-Luc was co-transfected along with internal control plasmid and 0.5 μg of $cT_3R\alpha$ expression plasmid as described in the legend to Figure 1B. Fresh medium containing the indicated concentrations of T_3 was added after transfection. For suppression, RAC65 cells were transfected with R140-Luc reporter plasmid, internal control plasmid, and expression vectors for hRARβ (0.5 μg) and $cT_3R\alpha$ (1 μg) as described in the legend to Figure 2A. Fresh medium containing 10^{-6} M RA plus the indicated concentrations of T_3 was added after transfection. The plot shows the percentage of maximal response at the indicated T_3 concentrations obtained for T_3-induced activation of the $(T_3RE_{pal})_3$-TK-Luc reporter (solid triangles) and for T_3-induced suppression of the activation of the R140-Luc reporter by RA (empty squares). (**C**) Ligand-activated hVD_3R suppresses activation of the $RAR\beta_2$ promoter by RA. RAC65 EC cells were transfected with R140-Luc reporter plasmid, internal control plasmid and, when indicated, expression plasmids for hRARβ (0.5 μg) and hVD_3R (the amounts indicated in the figure). After transfection, cells were incubated in medium containing solvent, 10^{-6} M RA (solid bars) or 10^{-6} M RA plus 10^{-6} M 1,25-dihydroxy-vitamin D_3 (hatched bars). Fold inductions were calculated as described in the legend to Figure 2A.

simultaneously such as F403/A, E401/K, C1, and the double mutant F399,F403/A; finally, one mutant, E401/Q, shows wild-type T_3 binding ability but is transcriptionally inactive even at 10^{-7} M T_3. The results show that although the T_3 binding and transactivation functions are intertwined, their amino acid requirements are different.

The transactivation domain AF-2 mediates T_3-dependent transcriptional interference

We have previously reported that ectopic expression of $cT_3R\alpha$ in embryonal carcinoma (EC) cells results in suppression of retinoic acid (RA)-dependent activation of the $RAR\beta_2$ promoter (Barettino *et al.*, 1993). In the absence of T_3, this suppression is due to sequestration of the common dimerization partner, the retinoid X receptor (RXR), and thus can be alleviated by co-transfection of this receptor. However, a ligand-dependent repression of RAR-mediated transactivation by $T_3R\alpha$ becomes evident after T_3 administration (Barettino *et al.*, 1993). We refer to this ligand-dependent inhibition of transcriptional activity as transcriptional interference or suppression. Transcriptional interference is not related to the phenomenon of silencing that has been documented for unliganded T_3R and its oncogenic variant v-erbA (Baniahmad *et al.*, 1990).

Co-expression of $cT_3R\alpha$ leads to a block in the activation of the RA-responsive $RAR\beta_2$-promoter reporter construct, R140-Luc, after simultaneous administration of RA and T_3 hormone (Figure 2A; see also Barettino *et al.*, 1993). The dose-dependence curves for T_3-induced transactivation for wild-type $T_3R\alpha$ through the T_3RE_{pal} or T_3RE_{MoMLV} elements and suppression of the $RAR\beta_2$ promoter reporter coincide (Figure 2B and data not shown). Co-expression of v-erbA does not result in suppression of the $RAR\beta_2$ promoter in either the absence (Barettino *et al.*, 1993) or presence of T_3 (Figure 2A). On the contrary, we routinely observe a modest enhancement of RA-dependent transactivation upon co-transfection of v-erbA and its derivatives, the basis of which is unknown. We made use of the discrepancy between v-erbA and $cT_3R\alpha$ in their ability to suppress RA-dependent transactivation, to map the region that is required for transcriptional interference. For this purpose, v-erbA/$cT_3R\alpha$ chimeras (C- and V-series; Muñoz *et al.*, 1988; Zenke *et al.*, 1990) were tested (Figure 2A). Construct C1, an otherwise wild-type $T_3R\alpha$ containing the C-terminus of v-erbA, has lost the ability to suppress RA-dependent activation of the $RAR\beta_2$ promoter. Conversely, the V1 chimera, in which the C-terminal putative transactivation domain of wild-type $cT_3R\alpha$ has been introduced in the v-erbA background, gains the ability to suppress the $RAR\beta_2$ promoter. The differences in the affinities of the chimeric receptors for T_3 do not correlate with their abilities to suppress. For example, the chimeric receptors C5 and V1 that have low affinities for the ligand cause suppression, whereas C1, which has an affinity for T_3 comparable with those of C5 and V1, does not suppress even in the presence of up to 10^{-7} M T_3 (Figure 2A). Transcriptional interference is not restricted to chicken $T_3R\alpha$, and suppression of the activation of the $RAR\beta_2$ promoter can also be obtained with human $T_3R\beta$ (data not shown) and the more distantly related receptor for vitamin D_3 (Figure 2C).

Finally, we tested the ability of the $cT_3R\alpha$ mutants in the C-terminal region to suppress the induction of the $RAR\beta_2$ promoter (Figure 1B). The results reveal a good correlation between the ability of the mutant receptors to activate a T_3-responsive promoter and to interfere with RAR-dependent transactivation. For example, the mutant receptors C1, E401/Q, E401/K and F399,F403/A are severely impaired in their ability to transactivate and to suppress.

Taken together, the results show that both transcriptional activation and interference map to the C-terminal part of $cT_3R\alpha$. Furthermore, the amino acid requirements for T_3-dependent activation and interference of transcription are very similar if not identical.

Transcriptional interference between type I and II receptors

The C-terminal AF-2 region is not only conserved among type II receptors but is also found in the E-domain of type I receptors and has been shown to be involved in ligand-induced transactivation by the glucocorticoid receptor (GR) and the estrogen receptor (ER) (Danielian *et al.*, 1992). Mutations in the hydrophobic residues of GR and ER are more deleterious to transactivation than are changes in the acidic residues (Danielian *et al.*, 1992). We have tested the abilities of wild-type rat GR and a derivative with mutations introduced in the C-terminal transactivation domain (M770,L771/A; Schmitt and Stunnenberg, 1993) to suppress the activation of $RAR\beta_2$ promoter by RA. As shown in Figure 3A, transfection of rat GR into P19 EC cells leads to suppression of the activation of $RAR\beta_2$ promoter upon simultaneous addition of RA and dexamethasone. However, co-transfection of mutant M770,L771/A, which is inactive in transactivation *in vivo* (Danielian *et al.*, 1992) and *in vitro* (Schmitt and Stunnenberg, 1993), does not cause suppression (Figure 3A). The results obtained with a derivative of the mouse ER (MOR121–599) and the mutant L543,L544/A (Danielian *et al.*, 1992) were similar to those obtained with GR (data not shown). Thus, ligand-activated type I receptors are able to interfere with transactivation by type II receptors notwithstanding their functional divergence.

Next, we tested whether type II receptors can repress ligand-dependent activation by type I receptors. Figure 3B shows that transactivation of $(GRE)_2$-TK-CAT (J.Schmitt and H.G.Stunnenberg, unpublished) by rGR is suppressed only partially (~45% residual activity) by co-transfected $cT_3R\alpha$ in a T_3-dependent manner. As the GR contains two independent activation functions, a N-terminal constitutive AF-1 and a C-terminal ligand-dependent AF-2 (Hollenberg and Evans, 1988; Webster *et al.*, 1988), we surmised that the ligand-dependent AF-2 of $cT_3R\alpha$ interfered only with ligand-dependent transactivation mediated by the AF-2, but not by the AF-1, of GR. To test this hypothesis, we used a chimeric transactivator with the EF domain of human GR fused to the Gal4 DBD (amino acids 1–147) and the reporter plasmid 5 × GalRE (Smith and Bohmann, 1992). As shown in Figure 3C, co-transfection of $cT_3R\alpha$ and Gal4-GR(EF) leads to a full repression of the 5 × GalRE reporter after simultaneous addition of dexamethasone and T_3. Low amounts of $cT_3R\alpha$ expression vector were sufficient to repress transactivation by Gal4-GR(EF) to basal levels. Suppression was not observed following co-transfection of the transcriptionally inactive

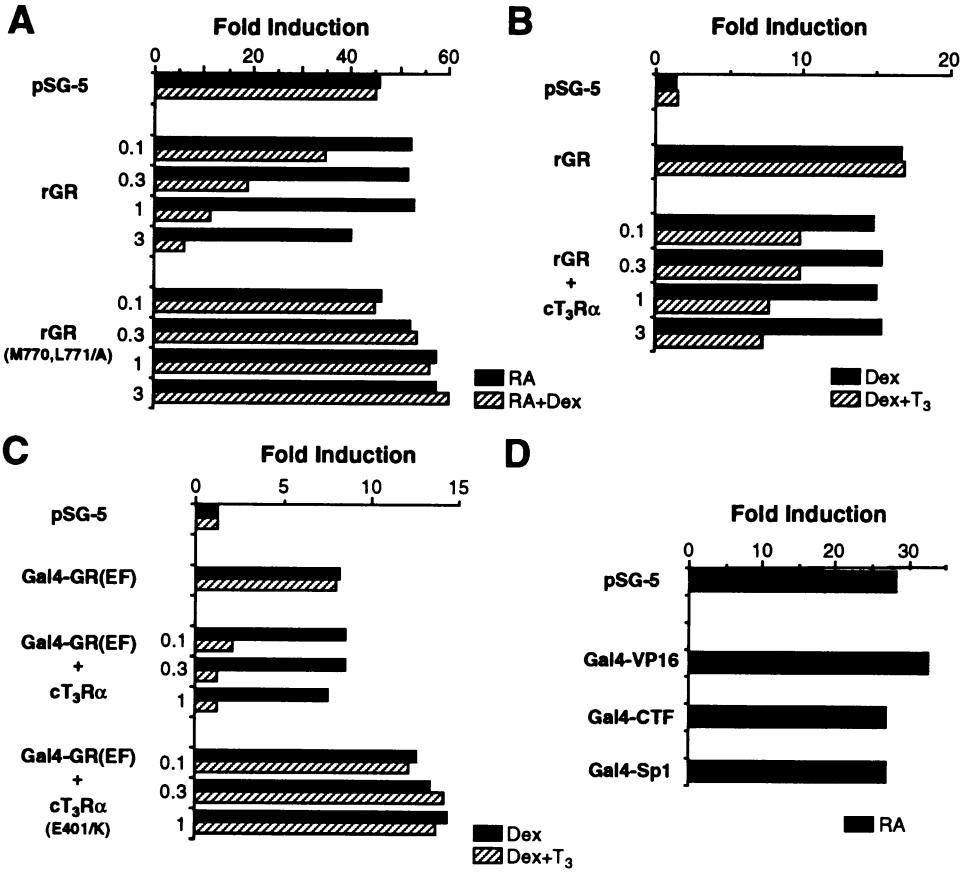

Fig. 3. Transcriptional interference between type I and type II receptor. (**A**) Ligand-activated rGR suppresses RA-induced activation of the $RAR\beta_2$ promoter. P19 EC cells were transfected with R140-Luc reporter plasmid, internal control plasmid and the indicated amounts (μg/dish) of rGR or rGR(M770,L771/A) mutant expression plasmids. After transfection, fresh medium containing solvent, 10^{-6} M RA (solid bars) or 10^{-6} M RA plus 10^{-7} M dexamethasone (hatched bars) was added. Fold inductions were calculated as described in the legend to Figure 2A. (**B**) Ligand-activated $cT_3R\alpha$ only partially suppresses hormone-induced transactivation by rGR. P19 EC cells were transfected with $(GRE)_2$-TK-CAT reporter plasmid, internal control plasmid, rGR expression plasmid (0.5 μg) as indicated and the amounts (μg/dish) of $cT_3R\alpha$ expression plasmid shown in the figure. After transfection, fresh medium containing solvent, 10^{-7} M dexamethasone (solid bars) or 10^{-7} M dexamethasone plus 10^{-7} M T_3 (hatched bars) was added. Fold inductions were calculated as described in the legend to Figure 2A. (**C**) Ligand-activated $cT_3R\alpha$ fully suppresses hormone-induced transactivation mediated by the AF-2 of GR. P19 EC cells were transfected with 5 × GalRE reporter plasmid, internal control plasmid, Gal4-GR(EF) expression plasmid (0.5 μg) when indicated, and the amounts (μg/dish) of $cT_3R\alpha$ or $cT_3R\alpha$(E401/K) mutant expression plasmids shown in the figure. After transfection, fresh medium containing solvent, 10^{-7} M dexamethasone (solid bars) or 10^{-7} M dexamethasone plus 10^{-7} M T_3 (hatched bars) was added. Fold inductions were calculated as described in the legend to Figure 2A. (**D**) Expression of unrelated transactivation domains does not affect transactivation by ligand-activated RAR. P19 EC cells were transfected with R140-Luc reporter plasmid, internal control plasmid and 3 μg of Gal4-VP16, Gal4-CTF or Gal4-Sp1 expression plasmids. After transfection, fresh medium containing solvent or 10^{-6} M RA (solid bars) was added. Fold inductions were calculated as described in the legend to Figure 2A.

mutant $cT_3R\alpha$(E401/K). Transfection of unrelated transcriptional activation domains, like the glutamine-rich domain of Sp1, the proline-rich domain of CTF/NF1 and the acidic activation domain of VP16, fused to Gal4 DBD did not significantly affect induction of the $RAR\beta_2$ promoter by RA in P19 cells (Figure 3D), supporting the idea that *trans*-repression is a specific effect mediated by a particular type of transactivation domain.

The C-terminal 35 amino acids of the $cT_3R\alpha$ constitute an autonomous activation domain

To test if the transactivation domain encompassing the conserved motif can function independently of the ligand binding/dimerization domain, we fused different parts of the E-region of $cT_3R\alpha$ to the Gal4 DBD (amino acids 1–147). Transient transfection experiments were performed using plasmid 5 × Gal4RE as a reporter (Figure 4A). Fusion of the last 13 residues of the $cT_3R\alpha$ to the Gal4 DBD, yielding Gal4-T_3R(396–408), results in a protein with no transcriptional activity, either in the presence or the absence of T_3 (Figure 4A). However, fusion of the 35 most C-terminal residues of $cT_3R\alpha$ to Gal4 DBD yielding Gal4-T_3R(374–408), results in a protein with considerable transactivation activity albeit in the absence of T_3, as the addition of hormone does not lead to a further increase in transactivation activity (Figure 4A). The level of transcription obtained with Gal4-T_3R(374–408) is about half of that obtained with a Gal4 fusion containing the intact DE domain of $T_3R\alpha$, Gal4-DE-($T_3R\alpha$) (Baniahmad *et al.*, 1992). The latter chimeric transactivator includes the complete ligand binding domain and hence its activity remains under the control of the hormone (Figure 4A).

To establish whether the ligand-independent transactivation mediated by Gal4-T_3R(374–408) has an amino acid requirement similar to that of ligand-dependent activation, we utilized the mutations in the conserved motif described

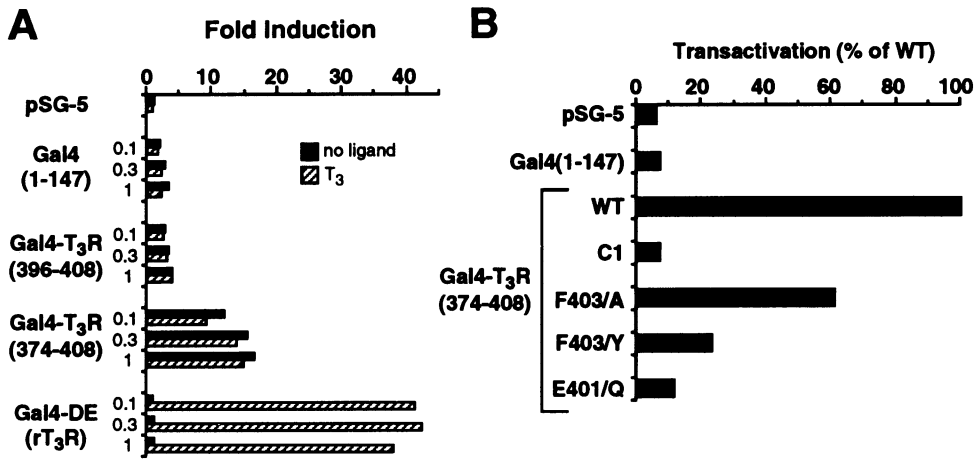

Fig. 4. Transactivation by Gal4-cT$_3$Rα chimeric proteins. (**A**) Transactivation by Gal4-T$_3$R chimeras. P19 EC cells were transfected with reporter plasmid 5 × GalRE, internal control plasmid and the indicated amounts (μg/dish) of Gal4(1–147) or Gal4-T$_3$R chimeras expression plasmids. After transfection, cells were incubated in medium containing solvent (solid bars) or 10^{-7} M T$_3$ (hatched bars). Fold inductions were calculated as described in the legend to Figure 2A. (**B**) Effect of transactivation domain mutations on the transcriptional activity of Gal4-T$_3$R(374–408) fusion protein. P19 EC cells were transfected with reporter plasmid 5 × GalRE, internal control plasmid and 1 μg of expression vectors for Gal4(1–147), wild-type Gal4-T$_3$R(374–408) or the indicated mutants. Transactivation (solid bars) is shown as the percentage of the activity obtained with the wild-type Gal4-T$_3$R(374–408) chimera.

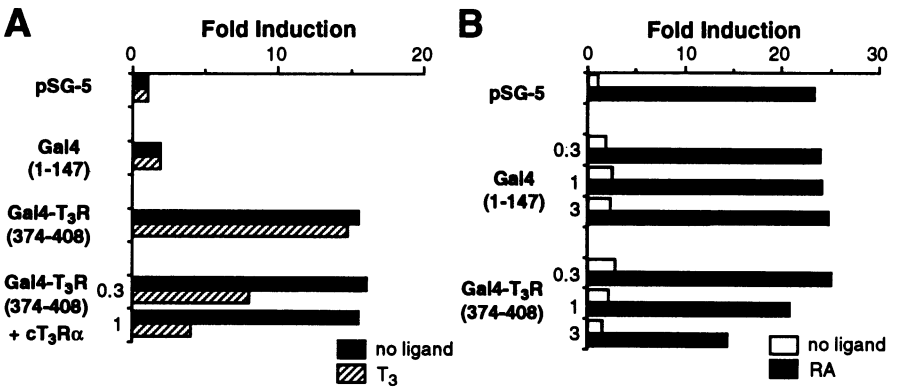

Fig. 5. Transcriptional interference properties of the Gal4-T$_3$R(374–408) chimeric transactivator. (**A**) Ligand-activated cT$_3$Rα suppresses transactivation mediated by the Gal4-T$_3$R(374–408) chimera. P19 EC cells were transfected with reporter plasmid 5 × GalRE, internal control plasmid, 1 μg of Gal4(1–147) or Gal4-T$_3$R(374–408) expression plasmids as shown in the figure and the indicated amounts (μg/dish) of cT$_3$Rα expression plasmid. After transfection, cells were incubated in medium containing solvent (solid bars) or 10^{-7} M T$_3$ (hatched bars). Fold inductions were calculated as described in the legend to Figure 2A. (**B**) The Gal4-T$_3$R(374–408) chimera moderately suppresses RA-induced transactivation of the RARβ$_2$ promoter. P19 EC cells were transfected with R140-Luc reporter plasmid, internal control plasmid and the indicated amounts (μg/dish) of Gal4(1–147) or Gal4-T$_3$R(374–408) expression plasmids. After transfection, fresh medium containing solvent (empty bars) or 10^{-6} M RA (solid bars) was added. Fold inductions were calculated as described in the legend to Figure 2A.

in Figure 1. Gal4-T$_3$R(374–408)-C1 or Gal4-T$_3$R(374–408)-E401/Q are strongly impaired in their ability to transactivate (Figure 4B). Surprisingly, the mutant F403/A which was severely compromised in ligand-dependent transactivation in the context of the full-length receptor, retains considerable activity within the context of the Gal4 fusion, whereas mutant F403/Y, which partially retained transactivation activity in the complete T$_3$Rα, is severely impaired as Gal4-T$_3$R(374–408) fusion (Figure 4B).

We tested if ligand-activated nuclear receptors can reduce transactivation mediated by Gal4-T$_3$R(374–408) chimeric activator, and if, conversely, expression of Gal4-T$_3$R(374–408) chimera can block transcriptional activation by ligand-activated receptors. Transactivation of the 5 × GalRE reporter by Gal4-T$_3$R(374–408) chimera is not affected by co-expression of cT$_3$Rα in the absence of ligand. However, efficient suppression of transactivation by Gal4-T$_3$R(374–408) can be observed upon addition of ligand (Figure 5A), corroborating the notion that the activation function present in Gal4-T$_3$R(374–408) chimera is similar if not identical to the ligand-dependent AF-2 of cT$_3$Rα. Conversely, the Gal4-T$_3$R(374–408) chimera is moderately capable of interfering with transactivation elicited by the ligand-activated AF-2 of RAR. Co-transfection of Gal4-T$_3$R(374–408) in P19 EC cells leads to partial suppression of the activation of the RARβ$_2$ promoter reporter by RA (Figure 5B).

Discussion

We have performed a functional analysis of the extreme C-terminal region of cT$_3$Rα. Three different properties of wild-type and mutant receptors were assessed: ligand binding, transactivation through T$_3$RE-containing pro-

Fig. 6. Sequence alignment of the putative transactivation domain of different nuclear receptors. The sequences of chicken (c), human (h), mouse (m), rat (r), bovine (b), *Xenopus* (x), or *Drosophila* (d) proteins are shown. The receptors are arranged in three different groups. The first group includes type II thyroid/vitamin receptors (cT_3Rα, Sap *et al.*, 1986; hT_3Rβ, Weinberger *et al.*, 1986; hVDR, Baker *et al.*, 1988; hRARα, Giguere *et al.*, 1987; hRARβ, De Thé *et al.*, 1987; hRARγ, Krust *et al.*, 1989; mRXRα, β, γ, Leid *et al.*, 1992b; xPPARα, β, γ, Dreyer *et al.*, 1992; dUSP, Oro *et al.*, 1990; dEcR, Koelle *et al.*, 1991; the second group encompasses type I steroid receptors (hER, Green *et al.*, 1986; mGR, Miesfeld *et al.*, 1986; hPR, Kastner *et al.*, 1990; hAR, Lubahn *et al.*, 1988; hMR, Arrixa *et al.*, 1987); the third group includes mainly orphan receptors and *Drosophila* proteins (bAd4BP, Honda *et al.*, 1993; mLRH, J.D.Tugwood, I.Issemann and S.Green, unpublished; mSF1, Ikeda *et al.*, 1993; rHNF-4, Sladek *et al.*, 1990; dFtz-F1β, Ohno and Petkovich, 1992; hERR-1 and hERR-2, Giguere *et al.*, 1988; dE78a, Stone and Thummel, 1993; dDH3, M.R.Koelle, W.A.Seagraves and D.S.Hogness, unpublished; hRZRα and rRZRβ, Becker-André *et al.*, 1993; xONR1, Smith *et al.*, 1993; hMB67, Baes *et al.*, 1994; hARP-1, Ladias and Karathanasis, 1991; hEAR-2, Miyajima *et al.*, 1988; hCOUP-TF, Wang *et al.*, 1989; dSvp, Mlodzik *et al.*, 1990; dT11, Pignoni *et al.*, 1990; rNGFI-B, Milbrandt, 1988). Amino acids numbers are indicated on the left side, and the C-terminus of the protein is indicated with an asterisk. The conserved glutamic acid (or aspartic acid in the COUP-TF group) residue is shown in bold on a shaded box, the conserved hydrophobic amino acids are shown boxed, and the conserved basic residue is shown in bold. In the consensus, Φ represents a hydrophobic residue (A, F, I, L, M, V, W), B a basic residue (H, K, R) and X a non-conserved residue. Sequence alignment was made by MegAlign computer program (DNASTAR), using the Clustal method.

moters and interference with ligand-dependent transcription mediated by a heterologous member of the receptor superfamily.

Transactivation

The glutamic acid at position 401 in cT_3Rα appears to be essential for T_3-dependent transactivation, as changing this residue to either Gln or Lys leads to a loss of transactivation. E401 is part of an amino acid sequence conserved between type I and II receptors (Danielian *et al.*, 1992; see also Figure 6) and has previously been implicated in transactivation (Zenke *et al.*, 1990; Danielian *et al.*, 1992; Saatcioglou *et al.*, 1993a). The mutant receptor E401/Q displays T_3 binding properties indistinguishable from the wild-type cT_3Rα whereas the mutant receptor E401/K shows a significantly reduced affinity for ligand. The inability of the E401/Q and E401/K mutant receptors to transactivate cannot be explained by the net loss of negative charge (or introduction of positive charge) because replacing the acidic pair of amino acids (E404,D405) located immediately downstream of the conserved motif, with either Ala or Lys does not significantly affect transactivation. Although the glutamic acid residue at position 401 is essential for T_3R-dependent transactivation, a less drastic diminution of ligand-dependent transactivation was documented for the mouse ER and human GR after mutation of the equivalent residue (Danielian *et al.*, 1992). Also, in contrast to ER and GR, the importance of the conserved hydrophobic amino acids flanking E401 in T_3Rα in transactivation is less obvious. The F403/Y substitution and, even more pronouncedly, the F403/A substitution diminishes the ability to transactivate whereas F399/A has only minor effects on transactivation. The differences between the phenotypes produced by these substitutions in T_3Rα and those produced in ER and GR may reflect functional divergence of the transactivation surfaces presented by ER, GR and T_3R, related perhaps to different requirements for binding of their respective ligands. Conversely, these differences may reflect a component of cell-type specificity in the activity of this transactivation surface, since our experiments were performed in P19 EC (Figure 1B), RAC65 and COS-7 cells (data not shown) and Parker and collaborators (Danielian *et al.*, 1992) used different cell lines.

Transcriptional interference

Transcriptional interference or squelching [for reviews see Lewin (1990) and Ptashne and Gann (1990)] can be obtained between transcription factors possessing related types of transactivation domains (such as acidic, or proline-, glutamine- or serine/threonine-rich) and probably relates to sequestration of co-factors. The region in T_3Rα required for ligand-dependent transactivation is conserved between various members of the receptor superfamily (Danielian *et al.*, 1992; see also Figure 6). We therefore assessed the ability of wild-type and mutant T_3Rα to interfere with transactivation elicited by other members of the receptor superfamily in response to their cognate ligand.

We show that T_3-dependent inhibition of RA-induced transactivation is due to transcriptional interference based on the following observations. Firstly, the T_3 dose-dependence curves for transcriptional activation and suppression coincide. Secondly, the regions of the receptor required for ligand-dependent transactivation and suppression are overlapping or even identical. The ability of mutant T_3Rs to transactivate in a T_3-dependent manner correlates very well with their ability to suppress transactivation by the closely related RAR. Even more strikingly, ligand-activated type II receptors are also able to interfere with transactivation elicited by the functionally distinct type I

receptors and vice versa. Again, the ability to interfere maps to the C-terminal ligand-dependent transactivation domain AF-2 and mutations that abolish ligand-dependent transactivation also abolish ligand-dependent transcriptional interference. Finally, the interference is a specific effect related to AF-2 because ligand-activated $cT_3R\alpha$ does not interfere with transactivation elicited by the ligand-independent AF-1 of type I receptors and vice versa. The specificity of the squelching phenomenon is further supported by the fact that it appears to occur only between closely related transactivation functions, since co-expression of an acidic (VP16), glutamine-rich (SP1) or proline-rich (CTF1) transactivator does not result in suppression of ligand-dependent transactivation. Transcriptional interference has been previously described to occur between type I receptors; however, the domain was not delineated (Bocquel *et al.*, 1989; Meyer *et al.*, 1989; Tora *et al.*, 1989; Tasset *et al.*, 1990). Our results corroborate and extend the finding that AF-1 and AF-2 are functionally distinct.

The suppression phenomenon documented here differs from the T_3-independent inhibition of RAR-mediated transcription by sequestration of RXR described previously (Barettino *et al.*, 1993). Mutations that diminish the ability of T_3R to heterodimerize with RXR, and hence to sequester RXR, do not affect their ability to suppress RA-dependent transactivation, as is the case with C5 and V1 chimeras (Figure 2A; Barettino *et al.*, 1993). Furthermore, transcriptional interference is not solely a property of type II receptors, but can also be obtained with type I receptors which do not interact with RXR.

It is assumed that the target for transcriptional interference is either a co-activator or a component of the basal transcription machinery. We have previously shown that the adenoviral E1A and its cellular counterpart, the EC cell-specific E1A-like activity, act as co-factors in RA-dependent transactivation of the $RAR\beta_2$ promoter (Berkenstam *et al.*, 1992; Keaveney *et al.*, 1993). This activation pathway may be specific for RAR/RXR heterodimers and EC cells (Berkenstam *et al.*, 1992). The suppression documented in the present study appears to be a general phenomenon not restricted to a particular receptor and/or cell type (data not shown). Co-transfection of adenovirus E1A and the TBP does not relieve suppression of RA-induced transactivation of the $RAR\beta_2$ promoter elicited by ligand-activated $cT_3R\alpha$ (D.Barettino and H.G.Stunnenberg, unpublished results). Similarly, the general transcription factor TFIIB (Ha *et al.*, 1991; Malik *et al.*, 1991) has been postulated as a target for transactivators such as VP16 (Lin and Green, 1991; Lin *et al.*, 1991; Choy and Green, 1993) and the orphan nuclear receptor COUP-TF (Sagami *et al.*, 1986; Tsai *et al.*, 1987). Furthermore, protein–protein interactions between the nuclear receptors COUP-TF, PR, ER and $T_3R\beta$ and the general factor TFIIB have been described (Ing *et al.*, 1992; Baniahmad *et al.*, 1993; Fondell *et al.*, 1993). However, co-transfection of hTFIIB, although having a moderate stimulatory effect on the level of transcription from the $RAR\beta_2$ promoter, does not counteract the repressive effect of $cT_3R\alpha$ after simultaneous addition of RA and T_3 ligands (D.Barettino and H.G.Stunnenberg, unpublished results). Although this experiment cannot formally exclude the possibility that TFIIB is a target for squelching, it seems unlikely that sequestration of only this component of the basic transcription machinery can account for the observed suppression.

Autonomous transactivation domain

The most C-terminal 35 residues of $cT_3R\alpha$ (amino acids 374–408), when fused to the Gal4 DBD act as an autonomous activation domain that is not governed by ligand. A chimera containing the 13 C-terminal amino acids, Gal4-T_3R(396–408), encompassing merely the highly conserved amino acid motif, is unable to activate transcription. A number of observations suggest that the ligand-independent transactivation elicited by the Gal4-T_3R(374–408) fusion protein has amino acid requirements and squelching properties similar to those of the ligand-dependent AF-2 in the context of the full-length receptor. Deletions or substitution mutations of the C-terminal fragment diminish the ability of the full-length receptor and the Gal4-T_3R(374–408) chimeric protein to transactivate with the exception of the mutations of the Phe403 residue. As mutations of these amino acid residues affect significantly the ability of $T_3R\alpha$ to bind ligand, this exception may indicate a ligand-based contribution to transactivation. The C-terminal region (amino acids 374–408) also conveys the ability to Gal4 DBD to interfere with ligand-activated transactivation elicited by a heterologous nuclear receptor and vice versa. Full-length nuclear receptors are able to interfere efficiently with transactivation mediated by the Gal4-T_3R(374–408) chimera; however, this chimeric activator is less efficient in interference with transactivation mediated by ligand-activated receptors. This may reflect a suboptimal configuration of the activation surface in the context of this chimeric protein. Moreover, the contribution of interactions between the dimerization partner RXR and Gal4-RAR(DE) chimeras to transactivation has been documented (Nagpal *et al.*, 1993), and the lack of these heteromeric interactions may also contribute to the lower efficiency of transactivation and suppression by the Gal4-T_3R(374–408) chimera. In addition, we cannot exclude the possibility that the activation surface requires contribution from elsewhere in the receptor molecule for full activity. In conclusion, the C-terminal region of $T_3R\alpha$ displays all the features anticipated for an autonomous transactivation domain. Our data show that the conserved motif is important, but not sufficient for transactivation when assayed outside of the context of the full-length receptor.

The transcriptional interference experiments suggest that the AF-2 of type I and II receptors may represent part of a surface for protein–protein interaction with either a common coactivator or a component of the basal transcription machinery, or both. Although other mechanisms can be envisaged, our interpretation is in line with the striking structural and functional conservation of the AF-2 domain of the different receptors and with the strong correlation between the effects of mutations in this domain in transcriptional activation and interference. Neither can we exclude the possibility that other regions of the ligand binding domain participate in cell- and promoter-specific transactivation pathways that may exist in addition to the proposed general mechanism that involves the AF-2.

The AF-2 domain

A computer-assisted sequence comparison of the C-terminal part of nuclear receptors covering the 'extended' AF-

2, as functionally defined by the Gal-fusion experiment, is shown in Figure 6. It reveals a region of homology present in most nuclear receptors in a location equivalent to that in $cT_3R\alpha$, i.e. immediately following the dimerization domain. The homology is high over a very short region from which the consensus motif -ΦΦXEΦΦ- can be derived, Φ being a hydrophobic amino acid. This motif, previously pointed out by Parker and collaborators (Danielian *et al.*, 1992), is present in most of the members of the nuclear receptors superfamily. However, in receptors from the COUP-TF group, the motif contains a conservative substitution of aspartic for glutamic acid in the central acidic residue. NGFI-B and its homologs Nur77/N10 and NAK-1 (Hazel *et al.*, 1988; Milbrandt, 1988; Ryseck *et al.*, 1989; Nakai *et al.*, 1990) contain a degenerate version of the motif, in which a positive charged amino acid substitutes for the highly conserved central glutamic acid residue. The significance of this change is unknown, but it should be pointed out that a transactivation domain could not be detected in the C-terminal region of Nur77 (Davis *et al.*, 1993). The conserved motif is absent in some mammalian orphan receptors, like ELP (Tsukiyama *et al.*, 1992), EAR-1/rev-erbA (Lazar *et al.*, 1989, 1990; Miyajima *et al.*, 1989), TR-2 (Chang and Kokontis, 1988) and the *Drosophila* proteins Ftz-F1α (Lavorgna *et al.*, 1991), E75 (Seagraves and Hogness, 1990), egon (Rothe *et al.*, 1989), knirps (Nauber *et al.*, 1988) and knirps-related (Oro *et al.*, 1988). However, knirps, egon and knirps-related proteins, although having the typical steroid finger DBD, lack overall homology with other receptors in the C-terminal part of the protein.

The conserved transactivation motif ΦΦXEΦΦ could represent a new class of activation domain, with a net negative charge. Nevertheless, this new acidic activation domain does not seem to be related to the acidic activation domain of VP16, because VP16 cannot interfere with transactivation by ligand-activated receptors and vice versa. Protein folding predictions indicate that the region comprising the conserved motif (referred to as 'helix 2' in Figure 6) can adopt an amphipathic α-helical conformation as previously postulated (Zenke *et al.*, 1990). This potential helix is preceded by a short stretch of amino acids that is variable both in sequence composition and length, varying from eight to 12 amino acids, and could form either a loop or a turn. In between this 'loop' and the end of the dimerization interface (the so-called ninth heptad; Forman and Samuels, 1990) is a stretch of 15 amino acids that shows only limited homology within the receptor superfamily and is predicted to form an α-helical structure. Interestingly, several human $T_3R\beta$ mutations linked to the familial generalized resistance to thyroid hormone syndrome (GRTH; Weiss and Refetoff, 1992, for review) map to the region of the receptor discussed here. These mutations are mainly characterized by decreased T_3 binding. In light of our data it seems possible that this region may play a role in folding and/or positioning of the conserved transactivation 'helix', for example by providing a surface for its packing. We cannot exclude, however, the possibility that some of the residues in the first 'helix' and 'loop' regions may play a role in transcriptional activation. Further analysis of this region and its role in transactivation will be required to elucidate this point.

The question that arises is how a transactivation domain that in principle can function autonomously, as documented by our Gal fusion experiments, is governed by the ligand in the context of the full-length receptor. Several reports indicate the occurrence of ligand-induced conformational changes in the C-terminal half of PR (Allan *et al.*, 1992a,b; Vegeto *et al.*, 1992; Weigel *et al.*, 1992), ER (Fritsch *et al.*, 1992; Beekman *et al.*, 1993), T_3R (Bhat *et al.*, 1993; Toney *et al.*, 1993) and RAR/RXR (Allan *et al.*, 1992a; Keidel *et al.*, 1994). Different conformational changes in PR, ER and RAR have been detected upon administration of agonists (which activate AF-2) or antagonists (which block AF-2) (Vegeto *et al.*, 1992; Weigel *et al.*, 1992; Beekman *et al.*, 1993; Keidel *et al.*, 1994). The binding of the ligand may indirectly contribute to transactivation by inducing a structural rearrangement of the ligand binding domain of the receptor that leads to exposure of the transactivation domain previously hidden in the unliganded state, or facilitating cooperation between multiple activation domains spread in the receptor molecule. Alternatively, ligand-induced allosteric changes may lead to the release of a putative repressor protein that blocks the activity of the AF-2 domain in the absence of ligand. It appears also possible that the ligand itself may contribute directly to the transactivation surface. Further studies are required to address these possibilities.

Materials and methods

Plasmids

Reporter plasmids R140-Luc, T_3RE_{MoMLV}-TK-Luc, $(T_3RE_{pal})_3$-TK-Luc and 5 × GalRE have been described previously (Vivanco Ruiz *et al.*, 1991; Smith and Bohmann, 1992). Reporter construct $(GRE)_2$-TK-CAT (J.Schmitt and H.G.Stunnenberg, unpublished) contains two copies of the GREs of the rat tyrosine amino-transferase promoter (Jantzen *et al.*, 1987) in front of the TK_{109} promoter driving the expression of the CAT gene. The cDNAs encoding chicken $T_3R\alpha$ (Sap *et al.*, 1986), avian erythroblastosis virus v-erbA (Sap *et al.*, 1989), human RXRα (Mangelsdorf *et al.*, 1990), human $RAR\beta_2$ (De Thé *et al.*, 1987), V- and C-series of $cT_3R\alpha$/v-erbA chimeras (Muñoz *et al.*, 1988; Zenke *et al.*, 1990), human VD_3R (Baker *et al.*, 1988), rat GR (Miesfeld *et al.*, 1986), and rat GR mutant M770,L771/A (Schmitt and Stunnenberg, 1993) were cloned into the mammalian expression vector pSG-5 (Green *et al.*, 1988). Mutations in the $cT_3R\alpha$ E-domain were introduced in pSG-$cT_3R\alpha$ by replacing the wild-type sequences of the *Xho*I–*Bam*HI (E404,D405/A, E404,D405/K, F403/A, F403/Y) or the *Sac*I–*Xho*I (F399/A) fragments with mutated double-stranded oligonucleotides. Mutations E401/K and E401/Q were introduced by site-directed mutagenesis using PCR (Landt *et al.*, 1990; Barettino *et al.*, 1994). Gal4-T_3R(374–408) was constructed by cloning an *Fsp*I–*Xba*I fragment from pSG-$cT_3R\alpha$ into the *Xma*I (Klenow-filled) and *Xba*I sites in the polylinker of expression vector pSGS424 (Sadowski and Ptashne, 1989). The resultant in-frame fusion has the following junction: $Gal4/S_{147}$-P-E-F-P-G-$M_{374}/cT_3R\alpha$. The complete cassette Gal4-T_3R(374–408) was subcloned as a *Bgl*II fragment into the *Bgl*II site of expression vector pSG-5. pSG-Gal4-T_3R(396–408) was constructed by cloning a *Sac*I (made blunt with S1 nuclease)–*Xba*I fragment of pSG-$cT_3R\alpha$ into *Sma*I- and *Xba*I-digested pSG-Gal4-T_3R(374–408). The resulting in-frame fusion has at its junction the sequence $Gal4/S_{147}$-P-E-F-P-$P_{396}/cT_3R\alpha$. Transactivation domain mutants F403/A, F403/Y, E401/Q and C1 were cloned from the above described pSG-$cT_3R\alpha$-based vectors as *Sac*I–*Xba*I fragments into *Sac*I- and *Xba*I-digested pSG-Gal4-T_3R(374–408). Expression vector pSG-Gal4(1–147) was constructed by cloning a *Bgl*II–*Sma*I fragment from pSG424 into *Bam*HI- and *Bgl*II- (Klenow filled) digested pSG-5. Gal4-GR(EF) expression vector, including amino acids 486–777 of human GR fused to Gal4(1–147), was a gift of C.Logie. Expression vectors for Gal4 fusions with the DE domains of rat $T_3R\alpha$ (amino acids 120–410) and with the transactivation domains of VP16, CTF/NF1 and Sp1 have been previously described (Baniahmad *et al.*, 1992 and Seipel *et al.*, 1992, respectively).

Cell culture and transient transfection

P19 and RAC65 EC cells were cultured and transfected using the calcium phosphate procedure as previously described (Berkenstam *et al.*, 1992; Barettino *et al.*, 1993). Experiments were performed with medium containing charcoal-stripped serum. In experiments involving GR and ER constructs, phenol red-free medium was used. Semi-confluent cells (6 cm dish) received up to 10 μg of DNA, including, if not otherwise stated, 5 μg of the corresponding luciferase reporter, 0.5 μg of RSV-CAT internal control plasmid (or 5 μg of the corresponding CAT reporter and 0.1 μg of CMV-luciferase internal control plasmid), and the indicated amounts of expression plasmids or pSG-5 expression vector. After 12–14 h of exposure to the co-precipitate, cells were incubated in fresh medium with ligands (10^{-6} M *all-trans*-RA, 10^{-7} M T_3, 10^{-6} M 1,25-dihydroxy-vitamin D_3, 10^{-7} M dexamethasone) or solvent. Cells were harvested after 24 h for determination of luciferase and CAT activities. All reporter activities were corrected for the internal control reporter values. Equivalent expression of the different chimeric or mutant proteins was tested by Western blot and/or bandshift assay.

Ligand binding experiments

COS-7 cells were transfected using the calcium phosphate procedure according to Vivanco Ruiz *et al.* (1991). Semi-confluent cells (9 cm dish) received 12 μg of pSG-5 vector expressing wild-type or mutant $cT_3R\alpha$, along with 1 μg of CMV luciferase as internal control and 6 μg pUC18 carrier DNA. After 12–14 h of exposure to the co-precipitate, cells were incubated in fresh medium for 10–12 h, and then in serum-free medium for a further 12–14 h. For nuclear extract preparation, cells (9 cm dish) were harvested, washed in ice-cold PBS, resuspended in 200 μl of ice-cold 10 mM Tris–HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF and immediately pelleted by centrifugation. Cells were lysed in 200 μl of the above buffer containing 0.4% Nonidet P-40 for 10 min at 4°C. Nuclei were pelleted by centrifugation, and resuspended in 60 μl of 20 mM Tris–HCl (pH 7.5), 20% glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1 mM PMSF. After swelling, 120 μl of the same buffer containing 1.2 M KCl was added (final KCl concentration 0.48 M) and incubated for 30 min at 4°C. The extract was cleared by centrifugation and stored at –80°C.

Binding reactions (10 μl) contained 2.5 μl of nuclear extract in 25 mM Tris–HCl (pH 7.5), 10% glycerol, 120 mM KCl, 2 mM EDTA, 1 mM DTT, 0.025 mM PMSF and different concentrations (between 0.01 and 5 nM) of [^{125}I]triiodothyronine (Amersham, sp. act. > 1200 μCi/μg). Parallel reactions including a 1000-fold excess of cold T_3 were set to determine the non-specific binding. After a 6 h incubation on ice in the dark, binding was assayed using hydroxylapatite essentially as described (Bodine *et al.*, 1984). Scatchard plots were constructed with the specific binding values obtained and used to calculate dissociation constants (K_d).

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References

Allan,G.F., Leng,X., Tsai,S.Y., Weigel,N.L., Edwards,D.P., Tsai,M.J. and O'Malley,B.W. (1992a) *J. Biol. Chem.*, **267**, 19513–19520.

Allan,G.F., Tsai,S.Y., Tsai,M.J. and O'Malley,B.W. (1992b) *Proc. Natl Acad. Sci. USA*, **89**, 11750–11754.

Arriza,J.L., Weinberger,C., Cerelli,G., Glaser,T.M. and Handelin,B.L. (1987) *Science*, **237**, 268–275.

Baes,M., Gulick,T., Choi,H.-S., Martinoli,M.G., Sihma,D. and Moore,D.D. (1994) *Mol. Cell. Biol.*, **14**, 1544–1552.

Baker,A.R., McDonnell,D.P., Hughes,M., Crisp,T.M., Mangelsdorf,D.J., Haussler,M.R., Pike,J.W., Shine,J. and O'Malley,B.W. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 3294–3298.

Baniahmad,A., Steiner,C., Kohne,A.C. and Renkawitz,R. (1990) *Cell*, **61**, 505–514.

Baniahmad,A., Köhne,A.C. and Renkawitz,R. (1992) *EMBO J.*, **11**, 1015–1023.

Baniahmad,A., Ha,I., Reinberg,D., Tsai,S., Tsai,M.-J. and O'Malley,B.W. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8832–8836.

Barettino,D., Bugge,T.H., Bartunek,P., Vivanco Ruiz,M.d.M., Sonntag-Buck,V., Beug,H., Zenke,M. and Stunnenberg,H.G. (1993) *EMBO J.*, **12**, 1343–1354.

Barettino,D., Feigenbutz,M., Valcárcel,R. and Stunnenberg,H.G. (1994) *Nucleic Acids Res.*, **22**, 541–542.

Becker-André,M., André,E. and DeLamarter,J.F. (1993) *Biochem. Biophys. Res. Commun.*, **194**, 1371–1379.

Beekman,J.M., Allan,G.F., Tsai,S.Y., Tsai,M.-J. and O'Malley,B.W. (1993) *Mol. Endocrinol.*, **7**, 1266–1274.

Berkenstam,A., Vivanco Ruiz,M.d.M., Barettino,D., Horikoshi,M. and Stunnenberg,H.G. (1992) *Cell*, **69**, 401–412.

Bhat,M.K., Parkison,C., McPhie,P., Liang,C.M. and Cheng,S.Y. (1993) *Biochem. Biophys. Res. Commun.*, **195**, 385–392.

Bocquel,M.T., Kumar,V., Stricker,C., Chambon,P. and Gronemeyer,H. (1989) *Nucleic Acids Res.*, **17**, 2581–2595.

Bodine,P.V., Schmidt,T.J. and Litwack,G. (1984) *J. Steroid Biochem.*, **20**, 683–689.

Chang,C. and Kokontis,J. (1988) *Biochem. Biophys. Res. Commun.*, **155**, 971–977.

Choy,B. and Green,M.R. (1993) *Nature*, **366**, 531–536.

Danielian,P.S., White,R., Lees,J.A. and Parker,M.G. (1992) *EMBO J.*, **11**, 1025–1033.

Davis,I.J., Hazel,T.G., Chen,R.-H., Blenis,J. and Lau,L.F. (1993) *Mol. Endocrinol.*, **7**, 953–964.

De Thé,H., Marchio,A., Tiollais,P. and Dejean,A. (1987) *Nature*, **330**, 667–670.

Dreyer,C., Krey,G., Keller,H., Givel,F., Helftenbein,G. and Wahli,W. (1992) *Cell*, **68**, 879–887.

Folkers,G.E., van der Leede,B.J. and van der Saag,P.T. (1993) *Mol. Endocrinol.*, **7**, 616–627.

Fondell,J.D., Roy,A.L. and Roeder,R.G. (1993) *Genes Dev.*, **7**, 1400–1410.

Forman,B.M. and Samuels,H.H. (1990) *Mol. Endocrinol.*, **4**, 1293–1301.

Fritsch,M., Leary,C.M., Furlow,J.D., Ahrens,H., Schuh,T.J., Mueller,G.C. and Gorski,J. (1992) *Biochemistry*, **31**, 5303–5311.

Giguere,V., Ong,E.S., Segui,P. and Evans,R.M. (1987) *Nature*, **330**, 624–629.

Giguere,V., Yang,N., Segui,P. and Evans,R.M. (1988) *Nature*, **331**, 91–94.

Goodrich,J.A., Hoey,T., Thut,C.J., Admon,A. and Tjian,R. (1993) *Cell*, **75**, 519–530.

Green,S., Walter,P., Kumar,V., Krust,A., Bonert,J.M. and Chambon,P. (1986) *Nature*, **320**, 134–139.

Green,S., Issemann,I. and Sheer,E. (1988) *Nucleic Acids Res.*, **16**, 369.

Gronemeyer,H. (1992) *FASEB J.*, **6**, 2524–2529.

Ha,I., Lane,W.S. and Reinberg,D. (1991) *Nature*, **352**, 689–695.

Hazel,T.G., Nathans,D. and Lau,L.F. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 8444.

Hoey,T., Weinzierl,R.O., Gill,G., Chen,J.L., Dynlacht,B.D. and Tjian,R. (1993) *Cell*, **72**, 247–260.

Hollenberg,S.M. and Evans,R.M. (1988) *Cell*, **55**, 899–906.

Honda,S., Morohashi,K., Nomura,M., Takeya,H., Kitajima,M. and Omura,T. (1993) *J. Biol. Chem.*, **268**, 7494–7502.

Ikeda,Y., Lala,D.S., Luo,X., Kim,E., Moisan,M.-P. and Parker,K.L. (1993) *Mol. Endocrinol.*, **7**, 852–860.

Ing,N.H., Beekman,J.M., Tsai,S.Y., Tsai,M.J. and O'Malley,B.W. (1992) *J Biol. Chem.*, **267**, 17617–17623.

Ingles,C.J., Shales,M., Cress,W.D., Triezenberg,S.J. and Greenblatt,J. (1991) *Nature*, **351**, 588–590.

Jantzen,H.M., Strähle,U., Gloss,B., Stewart,F., Schmid,W., Boshart,M., Miksicek,R. and Schütz,G. (1987) *Cell*, **49**, 29–38.

Kastner,P., Krust,A., Turcotte,B., Stropp,U., Tora,L., Gronemeyer,H. and Chambon,P. (1990) *EMBO J.*, **9**, 1603–1614.

Keaveney,M., Berkenstam,A., Feigenbutz,M., Vriend,G. and Stunnenberg,H.G. (1993) *Nature*, **365**, 562–566.

Keidel,S., LeMotte,P. and Apfel,C. (1994) *Mol. Cell. Biol.*, **14**, 287–298.

Koelle,M.R., Talbot,W.S., Seagraves,W.A., Bender,M.T., Cherbas,P. and Hogness,D.S. (1991) *Cell*, **67**, 59–77.

Krust,A., Kastner,P.H., Petkovich,M., Zelent,A. and Chambon,P. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 5310–5314.

Ladias,J.A.A. and Karathanasis,S.K. (1991) *Science*, **251**, 561–565.

Landt,O., Grunert,H.-P. and Hahn,U. (1990) *Gene*, **96**, 125–128.

Lavorgna,G., Ueda,H., Clos,J. and Wu,C. (1991) *Science*, **252**, 848–851.
Lazar,M.M., Hodin,R.A., Darling,D.S. and Chin,W.W. (1989) *Mol. Cell. Biol.*, **9**, 1128–1136.
Lazar,M.A., Jones,K.E. and Chin,W.W. (1990) *DNA Cell Biol.*, **9**, 77–83.
Lee,W.S., Kao,C.C., Bryant,G.O., Liu,X. and Berk,A.J. (1991) *Cell*, **67**, 365–376.
Leid,M., Kastner,P. and Chambon,P. (1992a) *Trends Biochem. Sci.*, **17**, 427–433.
Leid,M. *et al.* (1992b) *Cell*, **68**, 377–395.
Lewin,B. (1990) *Cell*, **61**, 1161–1164.
Lin,Y.S. and Green,M.R. (1991) *Cell*, **64**, 971–981.
Lin,Y.S., Ha,I., Maldonado,E., Reinberg,D. and Green,M.R. (1991) *Nature*, **353**, 569–571.
Liu,F.L. and Green,M.R. (1990) *Cell*, **61**, 1217–1224.
Lubahn,D.B., Joseph,D.R., Sar,M., Tan,J., Higgs,H.N., Larson,R.E., French,F.S. and Wilson,E.M. (1988) *Mol. Endocrinol.*, **2**, 1265–1275.
Malik,S., Hisatake,K., Sumimoto,H., Horikoshi,M. and Roeder,R.G. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9553–9557.
Mangelsdorf,D.J., Ong,E.S., Dyck,J.A. and Evans,R.M. (1990) *Nature*, **345**, 224–229.
Meyer,M.E., Gronemeyer,H., Turcotte,B., Bocquel,M.T., Tasset,D. and Chambon,P. (1989) *Cell*, **57**, 433–442.
Miesfeld,R., Rusconi,S., Godowski,P.J., Maler,B.M., Okret,S., Wilkström,A.-C., Gustafsson,J.-A. and Yamamoto,K.R. (1986) *Cell*, **46**, 389–399.
Milbrandt,J. (1988) *Neuron*, **1**, 183–188.
Miyajima,N., Kadowaki,Y., Fukushige,S.I., Shimizu,S., Semba,K., Yamanashi,Y. and Yamamoto,T. (1988) *Nucleic Acids Res.*, **16**, 11057–11074.
Miyajima,N., Horiuchi,R., Shibuya,Y., Fukushige,S.-I., Matsubara,K.-I., Toyoshima,K. and Yamamoto,T. (1989) *Cell*, **57**, 31–39.
Mlodzik,M., Hiromi,Y., Weber,U., Goodman,C.S. and Rubin,G.M. (1990) *Cell*, **60**, 211–224.
Muñoz,A., Zenke,M., Gehring,U., Sap,J., Beug,H. and Vennström,B. (1988) *EMBO J.*, **7**, 155–159.
Nagpal,S., Saunders,M., Kastner,P., Durand,B., Nakshatri,H. and Chambon,P. (1992) *Cell*, **70**, 1007–1019.
Nagpal,S., Friant,S., Nakshatri,H. and Chambon,P. (1993) *EMBO J.*, **12**, 2349–2360.
Nakai,A., Kartha,S., Sakurai,A., Toback,F.G. and DeGroot,L.J. (1990) *Mol. Endocrinol.*, **4**, 1438–1443.
Nauber,U., Pankratz,M.J., Kielin,A., Seifert,E., Klemm,U. and Jäckle,H. (1988) *Nature*, **336**, 489–492.
Ohno,C.K. and Petkovich,M.P. (1992) *Mech. Dev.*, **40**, 13–24.
Oro,A.E., Ong,E.S., Margolis,J.S., Posakony,J.W., McKeown,M. and Evans,R.M. (1988) *Nature*, **336**, 493–496.
Oro,A.E., McKeown,M. and Evans,R.M. (1990) *Nature*, **347**, 298–301.
Pignoni,F., Baldarelli,R.M., Steingrímsson,E., Diaz,R.J., Patapoutian,A., Merriam,J.R. and Lengyel,J.A. (1990) *Cell*, **62**, 151–163.
Ptashne,M. and Gann,A.A.F. (1990) *Nature*, **346**, 329–331.
Pugh,B.F. and Tjian,R. (1990) *Cell*, **61**, 1187–1197.
Roeder,R.G. (1991) *Trends Biochem. Sci.*, **16**, 402–408.
Rothe,M., Nauber,U. and Jäckle,H. (1989) *EMBO J.*, **8**, 3087–3094.
Ryseck,R.-P., Macdonald-Bravo,H., Mattéi,M.-G., Ruppert,S. and Bravo,R. (1989) *EMBO J.*, **8**, 3327–3335.
Saatcioglou,F., Bartunek,P., Deng,T., Zenke,M. and Karin,M. (1993a) *Mol. Cell. Biol.*, **13**, 3675–3685.
Saatcioglou,F., Deng,T. and Karin,M. (1993b) *Cell*, **75**, 1095–1105.
Sadowski,I. and Ptashne,M. (1989) *Nucleic Acids Res.*, **17**, 7539.
Sagami,I., Tsai,S.Y., Wang,H., Tsai,M.J. and O'Malley,B.W. (1986) *Mol. Cell. Biol.*, **6**, 4259–4267.
Sap,J., Muñoz,A., Damm,K., Goldberg,Y., Ghysdael,J. and Vennström,B. (1986) *Nature*, **324**, 635–640.
Sap,J., Muñoz,A., Schmitt,J., Stunnenberg,H. and Vennström,B. (1989) *Nature*, **340**, 242–244.
Schmitt,J. and Stunnenberg,H.G. (1993) *Nucleic Acids Res.*, **21**, 2673–2681.
Schöler,H.R., Ciesiolka,T. and Gruss,P. (1991) *Cell*, **66**, 291–304.
Seagraves,W.A. and Hogness,D.S. (1990) *Genes Dev.*, **4**, 204–209.
Seipel,K., Georgiev,O. and Schaffner,W. (1992) *EMBO J.*, **11**, 1961–1968.
Sladek,F.M., Zhong,W., Lai,E. and Darnell,J.E.,Jr (1990) *Genes Dev.*, **4**, 2353–2365.
Smith,D.P., Mason,C.S., Jones,E.A. and Old,R.W. (1993) *Nucleic Acids Res.*, **22**, 66–71.
Smith,S.E. and Bohmann,D. (1992) *Cell Growth Differen.*, **3**, 523–529.
Stone,B.L. and Thummel,C.S. (1993) *Cell*, **75**, 307–320.
Stunnenberg,H.G. (1993) *BioEssays*, **15**, 309–315.
Tasset,D., Tora,L., Fromental,C., Scheer,E. and Chambon,P. (1990) *Cell*, **62**, 1177–1187.
Toney,J.H., Wu,L., Summerfield,A.E., Sanyal,G., Forman,B.M., Zhu,J. and Samuels,H.H. (1993) *Biochemistry*, **32**, 2–6.
Tora,L., White,J., Brou,C., Tasset,D., Webster,N., Scheer,E. and Chambon,P. (1989) *Cell*, **59**, 477–487.
Tsai,S.Y., Sagami,I., Wang,H., Tsai,M.-J. and O'Malley,B.W. (1987) *Cell*, **50**, 701–709.
Tsukiyama,T., Ueda,H., Hirose,S. and Niwa,O. (1992) *Mol. Cell. Biol.*, **12**, 1286–1291.
Vegeto,E., Allan,G.F., Schrader,W.T., Tsai,M.J., McDonnell,D.P. and O'Malley,B.W. (1992) *Cell*, **69**, 703–713.
Vivanco Ruiz,M.d.M., Bugge,T., Hirschmann,P. and Stunnenberg,H.G. (1991) *EMBO J.*, **10**, 3829–3838.
Wang,L.H., Tsai,S.Y., Cook,R.G., Beattie,W.G., Tsai,M.J. and O'Malley, B.W. (1989) *Nature*, **340**, 163–166.
Webster,N., Green,S., Jin,J.R. and Chambon,P. (1988) *Cell*, **54**, 199–207.
Weigel,N.L., Beck,C.A., Estes,P.A., Prendergast,P., Altmann,M., Christensen,K. and Edwards,D.P. (1992) *Mol. Endocrinol.*, **6**, 1584–1597.
Weinberger,C., Thompson,C.C., Ong,E.S., Lebo,R., Gruol,D. and Evans,R.M. (1986) *Nature*, **324**, 641–646.
Weinzierl,R.O., Dynlacht,B.D. and Tjian,R. (1993) *Nature*, **362**, 511–517.
Weiss,R.E. and Refetoff,S. (1992) *Annu. Rev. Med.*, **43**, 363–375.
Zawel,L. and Reinberg,D. (1992) *Curr. Opin. Cell Biol.*, **4**, 488–495.
Zenke,M., Muñoz,A., Sap,J., Vennström,B. and Beug,H. (1990) *Cell*, **61**, 1035–1049.
Zhou,Q., Lieberman,P.M., Boyer,T.G. and Berk,A.J. (1992) *Genes Dev.*, **6**, 1964–1974.

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