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The Transcriptional Integrator CREB-Binding Protein Mediates Positive Cross Talk between Nuclear Hormone Receptors and the Hematopoietic bZip Protein p45/NF-E2

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Thyroid hormone (T3) and retinoic acid (RA) play important roles in erythropoiesis. We found that the hematopoietic cell-specific bZip protein p45/NF-E2 interacts with T3 receptor (TR) and RA receptor (RAR) but not retinoid X receptor. The interaction is between the DNA-binding domain of the nuclear receptor and the leucine zipper region of p45/NF-E2 but is markedly enhanced by cognate ligand. Remarkably, ligand-dependent transactivation by TR and RAR is markedly potentiated by p45/NF-E2. This effect of p45/NF-E2 is prevented by maf-like protein p18, which functions positively as a heterodimer with p45/NF-E2 on DNA. Potentiation of hormone action by p45/NF-E2 requires its activation domain, which interacts strongly with the multifaceted coactivator cyclic AMP response element protein-binding protein (CBP). The region of CBP which interacts with p45/NF-E2 is the same interaction domain that mediates inhibition of hormone-stimulated transcription by AP1 transcription factors. Overexpression of the bZip interaction domain of CBP specifically abolishes the positive cross talk between TR and p45/NF-E2. Thus, positive cross talk between p45/NF-E2 and nuclear hormone receptors requires direct protein-protein interactions between these factors and with CBP, whose integration of positive signals from two transactivation domains provides a novel mechanism for potentiation of hormone action in hematopoietic cells.

Nuclear hormone receptors (NHRs) are transcription factors which directly mediate intracellular signals in the form of lipophilic ligands such as thyroid hormone (T3) and retinoic acid (RA). A number of factors regulating the specificity of hormone action have been elucidated in recent years. Recognition of target gene binding is regulated by the orientation and spacing of NHR-binding sites, as well as by the homo- or heterodimerization status of the specific receptor (reviewed in reference 17). Ligand binding is itself highly specific, and the liganded receptor communicates with the basal transcription machinery both directly, e.g., by direct receptor interaction with an intrinsic component such as TFIIB (5, 15, 18, 22, 43) and indirectly, e.g., by an intermediary coactivator protein (7, 19, 33, 44).

One important class of coactivators includes CBP (the cyclic AMP response element protein-binding protein) and the highly related p300 protein (10, 14). These proteins are important not only for their role in positive transcriptional regulation from DNA-binding sites but for their likely role as mediators of cross talk between NHRs and other classes of transcription factors, most notably, the bZip AP1 proteins (9, 20, 25). Proteins which contribute to cellular AP1 activity, including jun and fos, have long been known to interact with NHRs (13, 24, 40, 45), including T3 receptor (TR) and RA receptor (RAR) (31, 41, 48), and this interaction has an inhibitory function in vivo (reviewed in references 34 and 37). Thus, on genes containing NHR-binding sites but lacking AP1-binding sites, positive regulation by liganded NHR is inhibited by activation of AP1. Conversely, liganded NHR can inhibit AP1-mediated transcription. The physical interactions between AP1 components and NHRs are not ligand dependent and therefore are likely to be insufficient for negative cross talk (24, 31, 40, 45, 48). CBP has recently been implicated as playing a central role in this negative cross talk (25). Both NHRs and AP1 proteins bind directly to CBP (9, 25), and the presence of NHR ligand leads to functional competition for CBP/p300 (25). NHRs bind to a region of the N terminus of CBP which does not bind AP1, and AP1 proteins bind to an interior region of CBP which, conversely, does not bind to NHRs (4, 25). Thus, it is possible that, rather than displace one another, liganded NHRs and AP1 proteins send separate transcriptional regulatory signals to CBP, whose role is to present an integration of these signals to the basal transcriptional machinery. In principle, such integration could be positive as well as negative, but thus far the well-described examples of cross talk between NHRs and other transcription factors have been inhibitory.

In the course of studying protein-protein interactions involving TRs, we discovered that multiple TR and RAR isoforms specifically interact with the hematopoietic factor p45/NF-E2. p45/NF-E2 is a bZip transcription factor which was initially discovered as an important regulator of β-globin transcription in erythroid cells and is expressed in erythroleukemia cells, as well as other hematopoietic cells, including progenitor-like cells and cells of the erythroid, megakaryocyte, and mast cell lineages and their committed precursors (1). This was of interest because thyroid hormones play an important role in erythropoiesis (6). Moreover, an oncogenic variant of chicken TRα, v-ErbA, contributes to erythroleukemia in chickens by a mechanism which involves inhibition of the function of TR, RAR, or both (reviewed in reference 16 and 36). Remarkably, we found that p45/NF-E2 potentiated the ligand-dependent transcription functions of both TRs and RARs on target genes which do not contain binding sites for p45/NF-E2. This positive

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TABLE 1. Oligonucleotides used in this study

Primer	Sequence
p10	tagactcgaggaattcatggaacagaagccaagc
p11	tgctcgagggatccttactgctcgtctttgtccagg
p12	tactcgaggaattcatgactcctaacagtatgac
p13	tgctcgagagatctttagagctcatccttgtctagatagc
p25b	tgctcgagagatctttattacatgccaacatagatgcat
p28	tgctcgagggatccttattacatgcccacagcgatgcac
p45	ctcggatccatggcaacagacctggtgctg
p46	ctcggatcctcagtcctcaaagacttccaag
p55	ctcggatccatgactcctaacagtatgac
p70	ggatcctcaatctgtagcctccattttgg
p78	ctcagatctatgcccccgtgtcctcctc
p97	carggatcctcaaagacctccagga
p101	gatccaggtcaccaggaggtcag
p102	gatcctgacctcctggtgacctg
p108	actggatccctggaaaccattgtgca
p111	acaggatccatgatccgtcgacggggc
p112	acaggatcctcaatctgtagcctcct
p113	acaagatctttagtcccgaactagagcc
p136	gtgggcatggccatggacctggt
p137	accaggtccatggccatgccac
p138	cacagatctatgcctgtagtgattaag
p139	ctaggatccatggaacagaagccaagc
p140	ctaagatcttcagcattttgtggtttcat
p143	atactcagatcttactggttacccaggat
p144	atcagagatctatgggaattcaaaacaca
p145	ttaggatccatggccgagaacttgctggac
p146	ttaggatccactagctggagatcccaggat
p147	ttaggatccgaggtgcagacagatgatgct
p148	ttaggatcctagtcgttttggtttggggat

cross talk was abolished by the bZip protein p18, which serves as a cofactor for positive transcriptional regulation by p45/NF-E2 on NF-E2-binding sites. The physical interaction between NHRs and p45/NF-E2 required the DNA-binding domains of both proteins but was stimulated by NHR ligand. The potentiation of NHR function required the activation domain of p45/NF-E2, which interacted with the AP1-binding site of CBP. This interaction was crucial for the potentiation of TR function by p45/NF-E2, because this region of CBP alone had no effect on TR function per se but abolished the ability of p45/NF-E2 to serve as a coactivator of liganded TR. Thus, the interaction of NHR and p45/NF-E2 on NHR-binding sites represents a novel example of positive cross talk. The likely mediation of this effect by simultaneous interaction with CBP provides a strong confirmation of the integration model of CBP function. Furthermore, our results extend the function of CBP to include integration of multiple positive signals with the potential for functional significance in hematopoiesis in the case of TR and p45/NF-E2.

MATERIALS AND METHODS

Oligonucleotides and vectors. The plasmid vectors used in this study were pGBT-9 (Clontech), pSG5 (Stratagene), pCMX (gift of R. Evans), pCMX-HA (47), pCMX-Gal4 (47), pGEX-KG (Pharmacia), and pCRII (Invitrogen). The oligonucleotides used in this study are listed in Table 1.

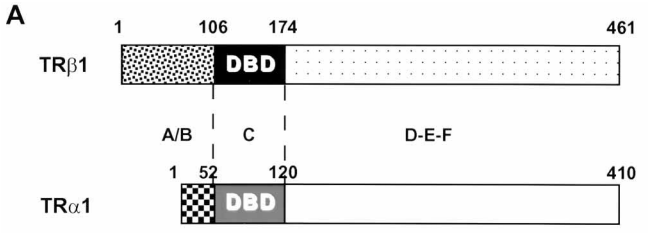
NHR constructs. pCMX-HA-v-ErbADBD-TRα1 was made by overlapping PCR, first with primers p138 and p137 with pBS-v-ErbA as the template to amplify v-ErbA-ABC and with primers p136 and p97 with pCMX-TRα1 as the template to amplify the ligand-binding domain (LBD) of TRα1 and then with primers p138 and p97 with purified products from the first PCRs as templates. pCMX-HA-TRα1DBD-v-ErbA was made by overlapping PCR, first with primers p139 and p137 with pCMX-TRα1 as the template and primers p136 and p140 with pBS-v-ErbA as the template and then with primers p139 and p140 with purified products from the first PCRs. Some expression vectors were made by PCR with the primers listed in Table 1 to amplify relevant cDNA as follows: pSG5.TRβ1, primers p12 and p46; pCMX-HA-TRβ1, primers p55 and p46; pCMX-HA-TRβ1LBD, primers p45 and p46. Plasmid pCMX-TRα1 was made by inserting an *Eco*RI fragment containing the entire rat TRα1 coding region into pCMX. The following expression vectors were gifts: pBS-v-ErbA (R. Koenig), pRS-gag-v-ErbA (M. Privalsky), pRS-gag-v-ErbA82mt (M. Privalsky), pSG5-RARα (P. Chambon), pSG5-RXRα (P. Chambon), and pCMX-RARαΔ403 (R. Evans).

NF-E2 constructs. Expression vectors p45/NF-E2, p45/NF-E2ΔAD (previously called pMT.TRN4), and p18 were all derived from pMT-2 as previously described (1, 2). Some expression vectors were made by PCR with the primers listed in Table 1 to amplify relevant cDNA as follows: pCMX-HA-NF-E2, primers p78 and p70; pCMX-HA-p18, primers p146 and p145; pCMX-HA-NF-AD, primers p78 and p113; pCMX-gal4-NF-E2, primers p78 and p70; pCMX-gal4-NF-AD, primers p109 and p110; pCMX-gal4-NF-DBD, primers p111 and p112; pCMX-gal4-NF-Zipper, primers p108 and p112.

Other constructs. Yeast two-hybrid constructs were made by PCR as follows: pGBT9-TRβ1ABC, primers p12 and p25b; pGBT9-TRα1ABC, primers p10 and p28; pGBT9-TRβ1CDEF, primers p45 and p46; pGBT9-TRβ1AB, primers p12 and p13; pGBT9-TRα1AB, primers p10 and p11. Glutathione *S*-transferase (GST) fusion constructs were made by PCR as follows: pGEX-KG-TRβ1, primers p55 and p46; pGEX-KG-NF-E2, primers p78 and p70. CBP constructs were made by PCR with mCBP as the template (gift of R. Goodman) for pCMX-HA-mCBP(1-450) (primers p145 and p146), pCMX-HA-mCBP(451-682) (primers p143 and p144), and pCMX-HA-mCBP(1000-1500) (primers p147 and p148).

Two-hybrid screen. The N-terminal 174-amino-acid sequence (corresponding to the A, B, and C domains) of rat TRβ1 was PCR amplified with primers p12 and p25b and inserted into pGBT9 (Clontech) as an *Eco*RI-*Bam*HI fragment. Yeast strain HF7c [*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::*(*GAL* 17-mers × 3)-*CYCI-lacZ*] containing pGBT9-TRβ1ABC(1-174) expresses a fusion protein containing the Gal4 DBD(1-147) and TRβ1ABC(1-174). To identify TRβ1ABC-interacting proteins, yeast strain HF7c/pGBT-9-TRβ1ABC(1-174) was transformed with DNA from a 17-day mouse embryo library in pGAD10 (Clontech) and plated on synthetic dropout (SD) medium lacking tryptophan, leucine, and histidine. Colonies that grew on the selective plates were restreaked and assayed for β-galactosidase β-gal activity by the filter lift assay. Library plasmids from positive clones were then recovered by electroporation and further analyzed by DNA sequencing as described in the manual for the Clontech two-hybrid system. The other bait vectors used are shown in Fig. 1B.

In vitro interaction assays. GST fusion proteins were isolated from *Escherichia coli* DH5α cells containing the appropriate expression vectors. Typically, bacteria were grown at 37°C to an optical density at 600 nm of 1.0 and induced for 5 h by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Cells were then pelleted and lysed by sonication in TEN buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl). GST fusion proteins were then purified with glutathione-Sepharose beads (Pharmacia) in accordance with the manufacturer's instructions. To perform a GST pulldown, GST fusion protein bound to GST beads (typically, 20 μl of a 50% slurry) in GST binding buffer (50 mM KCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 0.5% nonfat dry milk, 5 mM dithiothreitol) was mixed with 5 μl of the in vitro-translated proteins of interest and incubated at room temperature for



B

Bait(pGBT-9-)	pGAD10-p45/NF-E2(112-373) Growth on SD-Trp-Leu-His	β-gal
TRβ1A/B/C(1-174)	+	+
TRβ1A/B(1-106)	-	-
TRβ1C/D/E/F(107-461)	+	+
TRα1A/B(1-52)	-	-
TRα1A/B/C(1-120)	+	+

FIG. 1. Interactions between TR and p45/NF-E2 in yeast. (A) Domain structures of rat TRβ and TRα. (B) Interaction between different TRα and TRβ polypeptides with p45/NF-E2 in yeast implicates the DBD of TR. Interaction was scored both by growth and β-gal activity as shown.

30 min with gentle rocking. In vitro-translated proteins were precleared twice (for 15 min each time) by mixing with GST bound to GST beads in the same buffer. The beads were then washed five times with 1 ml of washing buffer (GST binding buffer or binding buffer plus 150 mM NaCl plus 0.1% sodium dodecyl sulfate [SDS], as indicated in the figure legends). Ligand was added to the binding and washing buffer where indicated. The bound proteins were eluted by boiling in 30 μl of 2× SDS loading buffer and analyzed by SDS-polyacrylamide gel electrophoresis. Unless otherwise indicated, the input lane in each experiment represents 10% of the total amount used. The GST fusion proteins were stained with Coomassie blue to ensure equal loading, and the bound proteins were visualized by autoradiography.

Cell culture and transfection. COS-7 cells were maintained in Dulbecco modified Eagle medium (DMEM; high glucose) with 10% fetal calf serum and transfected at 40% confluency in 60-mm-diameter dishes by the calcium phosphate precipitation method. Two hours prior to transfection, cells were placed in 5 ml of DMEM–10% stripped fetal calf serum. For transfection, 0.5 to 1 μg of a reporter and 100 ng of a cytomegalovirus β-gal expression plasmid were typically used for each dish. We used 250 ng of the receptor expression vector and 1 μg of the p45/NF-E2 expression vector unless otherwise indicated. Equivalent amounts of empty expression vectors (pSG5, pCMX, pCMX-HA, pMT-2, pRS-gag-v-ErbA82mt, pCDM, or pCMXgal4) were included in cells transfected with submaximal amounts of receptor or effector expression vectors. Reporter constructs were 8DR4-thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT), kindly supplied by Ron Koenig (26), $(DR5)_2$-TK-CAT (made with primers p101 and p102), and $(CRBPI)_2$-TK-luciferase (12). The calcium phosphate precipitates were left on cells overnight and then washed away with phosphate-buffered saline (PBS), and the medium was changed to 5 ml of DMEM with 10% stripped fetal calf serum with or without cognate ligands, as indicated in Results. Cells were further incubated at 37°C in 5% CO_2 for another 36 to 48 h before harvesting and processing for β-gal and luciferase or CAT assay by standard protocols. The measured number relative light units or acetylated chloramphenicol was normalized to β-gal activity, which served as an internal control for transfection efficiency. The figures show the results of representative experiments; each experiment was repeated two to five times.

Immunoblotting. Western blotting was performed with crude nuclear extracts (10 μl) from transfectants and from mouse erythroleukemia cells. Electrophoretic transfer of proteins to nitrocellulose after SDS-polyacrylamide gel electrophoresis and detection with anti-p45/NF-E2 serum were carried out as previously described (1).

Immunofluorescence. COS-7 cells were transfected with 15 μg (total) of pCMX, pCMX-HA-CBP(451-682), and/or pMT-2-p45/NF-E2 as described above, except that the cells were grown on glass coverslips. Cells were washed 48 h later with 0.1 mM calcium chloride in PBS and then fixed for 30 min in 4% paraformaldehyde at room temperature. After washing with PBS, cells were incubated with 0.1% Triton X-100–PBS for 15 min. Mouse monoclonal anti-hemagglutinin (HA) antibody 12CA5 was used at a 1:75 dilution in 1% milk–PBS. After several washes, cells were incubated with the secondary antibody, fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G (Sigma) at a dilution of 1:200 in 1% milk–PBS. Cells were washed with 1% milk–PBS, and the coverslips were then mounted on microscope slides by using Vectashield (Vector) for fluorescence analysis.

RESULTS

The N terminus of thyroid hormone receptors interacts with the basic leucine zipper transcription factor p45/NF-E2 in yeast. The N-terminal 174-amino-acid sequence of rat TRβ1 was fused to the Gal4 DNA-binding domain and used as bait to screen a day 17 mouse embryo expression library by using a yeast two-hybrid strategy designed to uncover novel protein-protein interactions involving the N terminus of thyroid hormone receptors. This region of TRβ1 contains the N-terminal A/B domain (28), which has a cryptic function but may encode a transcriptional activation domain (18), as well as the Zn finger-containing DNA-binding domain (DBD; Fig. 1A). One positive clone which was independently isolated four times encoded the C terminus of the hematopoietic basic leucine zipper transcription factor p45/NF-E2, beginning with amino acid 112 and extending to the end of the protein (amino acid 373). Additional studies were performed with yeast to assess the specificity and sublocalization of the interaction by using the criteria of growth and color selection as indicated in Fig. 1B. This region of NF-E2 did not interact with the A/B domain (amino acids 1 to 106) of TRβ1 alone but did interact with the C-terminal region of TRβ1, beginning with the DBD. This suggested that the DBD of TRβ1 (amino acids 107 to 174) was responsible for the interaction, since it was common to the interacting TRβ polypeptides. In addition, the A/B domain of TRα1 (amino acids 1 to 52) did not interact with p45/NF-E2, but addition of the DBD of TRα1 (amino acids 53 to 120), which is 96% (65 of 68 amino acids) identical to that of TRβ1, was sufficient for interaction. These results strongly suggested that p45/NF-E2 interacts with TRα and TRβ through their highly homologous DBDs.

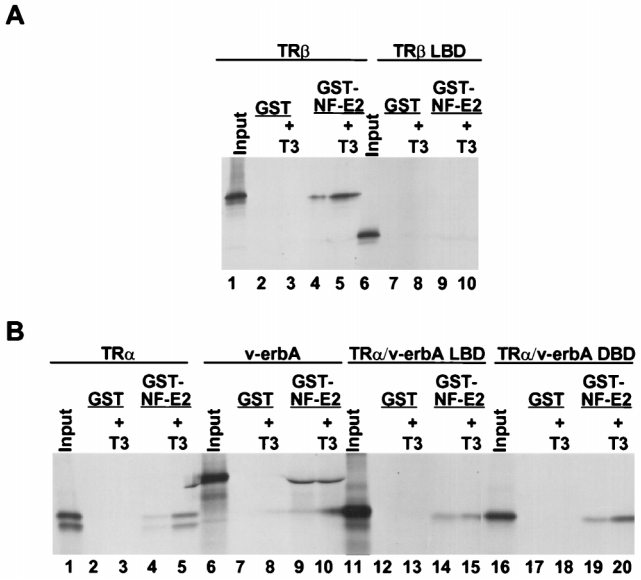


FIG. 2. Interactions between TR and p45/NF-E2 in vitro. (A) p45/NF-E2 interacts with TRβ but not with the TRβ LBD. Interaction of ^{35}S-labeled TR proteins with GST or GST–NF-E2(p45) is shown. T3 stimulates the interaction between TRβ and NF-E2(p45). Pulldowns were performed in the presence and absence of 1 μM T3. (B) p45/NF-E2 interaction with TRα is stimulated by T3, but interaction with v-erbA is not. Interaction of ^{35}S-labeled TRα (lanes 1 to 5), v-ErbA (lanes 6 to 10), and chimeric proteins TRα (with the v-ErbA C terminus) (lanes 11 to 15) and TRα (with the v-ErbA DBD) (lanes 16 to 20) with GST or GST–NF-E2. Pulldowns were performed in the presence and absence of 1 μM T3.

Ligand stimulates interaction of the TR DBD and p45/NF-E2. The genetic evidence for interaction between the TR DBD and p45/NF-E2 in yeast was confirmed in vitro by using p45/NF-E2 synthesized in bacteria as a fusion protein with GST. Figure 2A shows that GST–p45/NF-E2, but not GST alone, interacted with in vitro-translated TRβ1 (lanes 1 to 5). The C-terminal LBD of TRβ1 did not interact with p45/NF-E2 (lanes 6 to 10). Interestingly, the interaction between TRβ and p45/NF-E2 was increased three- to fivefold in the presence of 1 μM T3 (compare lanes 4 and 5). In contrast, no stimulation of nonspecific binding of TRβ to GST was observed (lanes 2 and 3). Furthermore, the TRβ LBD, which binds T3, was nevertheless unable to bind to p45/NF-E2 in the presence of T3. Thus, ligand binding by the TRβ C terminus stimulated binding to p45/NF-E2; however, the N-terminal DBD is absolutely required for the interaction. Figure 2B shows similar results for TRα. Thus, TRα interacted with p45/NF-E2 in a manner which was stimulated by T3 (lanes 4 and 5). Although it is clear that p45/NF-E2 is necessary for thrombopoietic differentiation (42), NF-E2 was first described as a factor which is involved in erythropoietic differentiation and, indeed, lack of NF-E2 expression correlates with failure of globin gene expression in an erythroleukemia cell line (32). Intriguingly, v-ErbA is an oncogenic form of TRα1 whose ability to interfere with normal erythropoietic differentiation is related to its ability to

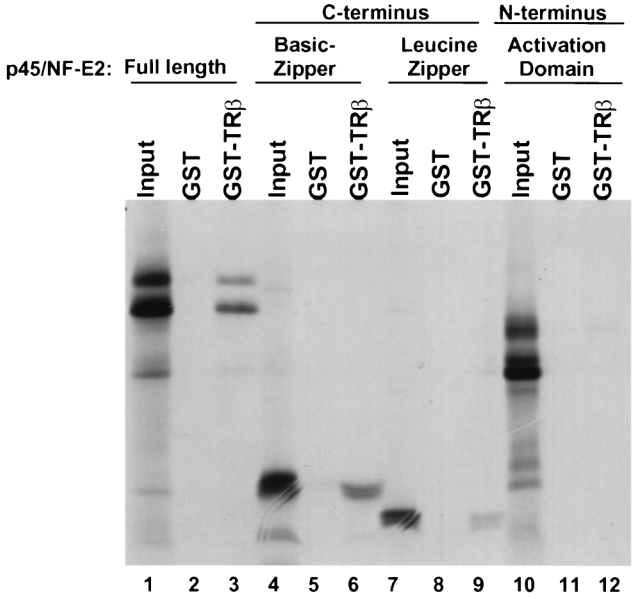


FIG. 3. The leucine zipper of p45/NF-E2 interacts with TR. p45/NF-E2 polypeptides (amino acids 1 to 373, 270 to 373, 288 to 373, and 1 to 269) were in vitro translated as Gal4 fusion proteins and incubated with GST or GST-TRβ in the presence of 1 μM T3. Lanes 1, 4, 7, and 10 represent 20% of the input protein.

dominantly inhibit the effects of T3 and RA by interfering with TR- and RAR-mediated transcription (11, 38, 39). One of the biggest differences between v-erbA and cellular TRα is that v-erbA is unable to bind T3 due to a nine-amino-acid deletion in its extreme C terminus. Figure 2B shows that this property of v-erbA was reflected in its interaction with p45/NF-E2. Like TRα, v-ErbA also interacted with p45/NF-E2, but unlike TRα, this interaction was ligand independent (compare lanes 9 and 10). These properties of TRα and v-ErbA were due to their C termini, because replacement of the C terminus of TRα with that of v-ErbA abolished the stimulatory effect of ligand upon interaction with p45/NF-E2 (compare lanes 14 and 15). Conversely, a chimeric protein containing the v-ErbA DBD in the context of the TRα1 C terminus interacted with p45/NF-E2 in a manner which was greatly enhanced by T3 (compare lanes 19 and 20).

p45/NF-E2 interacts with TR via its basic leucine zipper region. We next determined the region of p45/NF-E2 which interacts with TR. TRβ1 was synthesized as a GST fusion protein and used to study interaction with p45/NF-E2 by using p45/NF-E2 polypeptides fused to Gal4(1-147). Figure 3 confirms the specific interaction between p45/NF-E2 and TRβ (lanes 1 to 3). Consistent with the results obtained with yeast, where the original TR-interacting clone corresponded to the C terminus of p45/NF-E2, GST-TRβ1 interacted in vitro with the C-terminal basic leucine zipper region of p45/NF-E2 (amino acids 270 to 373, lanes 4 to 6) but not with its N-terminal activation domain (amino acids 1 to 269, lanes 10 to 12). Furthermore, lanes 7 to 9 show that the most C-terminal leucine zipper region (amino acids 288 to 373) of p45/NF-E2 was sufficient for interaction with GST-TRβ1, although this interaction was somewhat weaker than that of the combined basic-zipper region which may thus be required for maximal interaction. The experiments shown were done in the presence of T3; however, for unclear reasons, the effects of T3 on the interaction of p45 with GST-TR were less dramatic than those shown earlier between GST-p45 and TR (data not shown).

p45/NF-E2 potentiates ligand-stimulated transactivation by TR. TR has been shown to interact with other leucine zipper transcription factors, including c-Jun (31, 48); in those cases, the functional interactions between the TR and leucine zipper proteins have almost invariably been found to be negative, i.e., one transcription factor signaling pathway interferes with the other. Thus, it was of interest to determine the functional consequence of the interaction between TR and p45/NF-E2 for T3-dependent transcription. We utilized a transient transfection assay in which transcriptional activation of the CAT reporter gene driven by the thymidine kinase basal promoter was activated by T3 plus cotransfected TRβ1 due to a T3 response element (TRE) 5′ to the promoter (Fig. 4A, lanes 1 to 4). Cotransfection of p45/NF-E2 markedly increased T3-mediated transcription (inset; compare lanes 4 and 6). The effect of p45/NF-E2 was dose dependent (lanes 5 to 8). In the absence of TR, p45/NF-E2 had little effect upon transcription (lanes 9 and 10). Furthermore, the effect of p45/NF-E2 was observed only in the presence of T3 ligand.

Figure 4B shows that, similar to its effects on T3 activation of

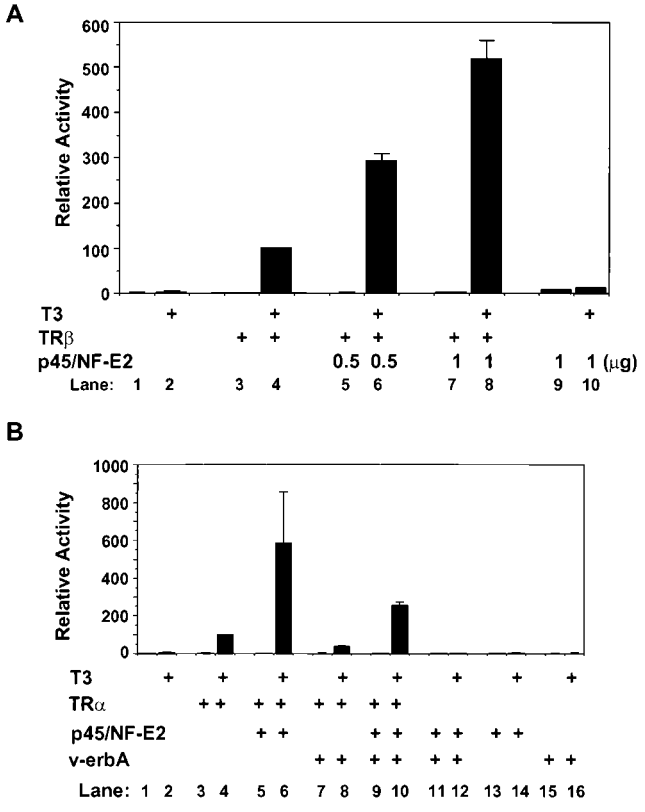


FIG. 4. p45/NF-E2 potentiates T3-dependent transcriptional activation by TR. (A) p45/NF-E2 potentiates T3-dependent transcriptional activation by TRβ. COS-7 cells were transfected with an 8DR4-TK-CAT reporter (26) in the presence or absence of expression vectors for TRβ (pCMX-TRβ1, 150 ng) and/or p45/NF-E2, as indicated, and in the presence or absence of 100 nM T3, as indicated. Results shown are the mean and range of duplicate samples, normalized to the β-gal activity from a cotransfected plasmid. The activity of TRβ-transfected cells in the presence of T3 was assigned a value of 100 U (lane 4). (B) p45/NF-E2 potentiates T3-dependent transcriptional activation by TRα, with proportional inhibition by v-ErbA. COS-7 cells were transfected with the 8DR4-TK-CAT reporter, in the presence or absence of expression vectors for TRα, v-ErbA, and/or p45/NF-E2, as indicated, and in the presence or absence of 100 nM T3, as indicated. Results shown are the mean and range of duplicate samples, normalized to the β-gal activity from a cotransfected plasmid. The activity of TRα-transfected cells in the presence of T3 was assigned a value of 100 U (lane 4).

TRβ1, p45/NF-E2 increased the T3 stimulation of transcription by TRα1 about five- to sixfold (compare lanes 4 and 6). It was also of interest to determine the effects of p45/NF-E2 on the dominant negative properties of v-ErbA. As with unliganded TRα, there was little or no effect of p45/NF-E2 on CAT activity in the presence of v-ErbA (lanes 5 and 11). Unlike TR, ligand had no additional effect in the case of v-ErbA (lane 12), a result which is not surprising since v-ErbA does not bind T3. We next investigated the ability of v-ErbA to dominantly inhibit T3 action. In Fig. 4B, the effect of v-ErbA was ~60% inhibition of the effect of T3 in the presence of TRα (compare lane 8 with lane 4). The effects of p45/NF-E2 are shown in lane 10. Compared with the T3 stimulation of TRα in the presence of p45/NF-E2 shown in lane 6, the effect of v-ErbA was to reduce this activity by ~60%; this percent change is similar to the effect of v-ErbA on T3 stimulation of TRα in the absence of p45/NF-E2, suggesting that v-ErbA and p45/NF-E2 have independent effects on T3 action. This similarity is consistent with the likely mechanism of the dominant negative effects of v-ErbA action, i.e., competition for the TRE, the retinoid X receptor (RXR) heterodimer partner, or both, which would be unaffected by the interaction with p45/NF-E2. It is worth noting that although the fold decline in T3 stimulation is similar in the presence and absence of p45/NF-E2, the absolute amount of T3 stimulation is still about five- to sixfold greater in the presence of p45/NF-E2 (compare lanes 8 and 10). Thus, v-ErbA and p45/NF-E2 simultaneously and independently exert opposing effects whose integration determines the overall magnitude of T3 stimulation of TR transcriptional activity.

p45/NF-E2 interacts with and potentiates the actions of RAR but not those of RXR. Since p45/NF-E2 interacts with the DBD of TR, a region which is conserved among the nuclear receptor superfamily, we evaluated the specificity of the interaction by determining whether retinoid receptors can interact with p45/NF-E2. Figure 5A shows that RARα specifically interacts with p45/NF-E2 and that this interaction is increased in the presence of all-*trans* RA (lanes 4 and 5). RARγ was also tested, and it interacted with p45/NF-E2 as well (data not shown). RARαΔ403 is a dominant negative mutant of RARα, which is functionally similar to v-ErbA because it lacks the C-terminal activation domain which is important for conformation-dependent effects of ligand on transactivation and transrepression. Figure 5A shows that RARαΔ403 interacts with p45/NF-E2 but, similar to v-ErbA, this interaction is not enhanced by ligand. Figure 5A also shows that, in contrast to TR and RAR, RXR does not interact with p45/NF-E2 in either the absence or the presence of the RXR-specific ligand SR11237 (lanes 11 to 15).

The effect of the interaction with p45/NF-E2 on RAR function was tested next by using a transient transfection assay with COS-7 cells transfected with an RA response element $(DR5)_2$-TK-CAT construct along with various expression plasmids as shown in Fig. 5B. The cells were found to have low levels of endogenous RARs whose activity could be inhibited by RARα403 (compare lanes 2 and 4). p45/NF-E2 increased the activity of the endogenous RAR (lanes 2 and 6). Similar to what was observed with v-ErbA, RARαΔ403 was also able to inhibit the p45/NF-E2 stimulation of the RA response (lanes 6 and 8). Ectopic expression of RARα greatly increased transcription from the RAR-responsive reporter in the presence of RA (lanes 2 and 10). Cotransfection of p45/NF-E2 also increased the RA responsiveness of the transfected RARα (lanes 10 and 12). Figure 5C shows that, consistent with the in vitro interaction studies, p45/NF-E2 did not significantly increase SR11237 and RXR-dependent activation of an RXR response element-containing reporter gene (lanes 4 and 6).

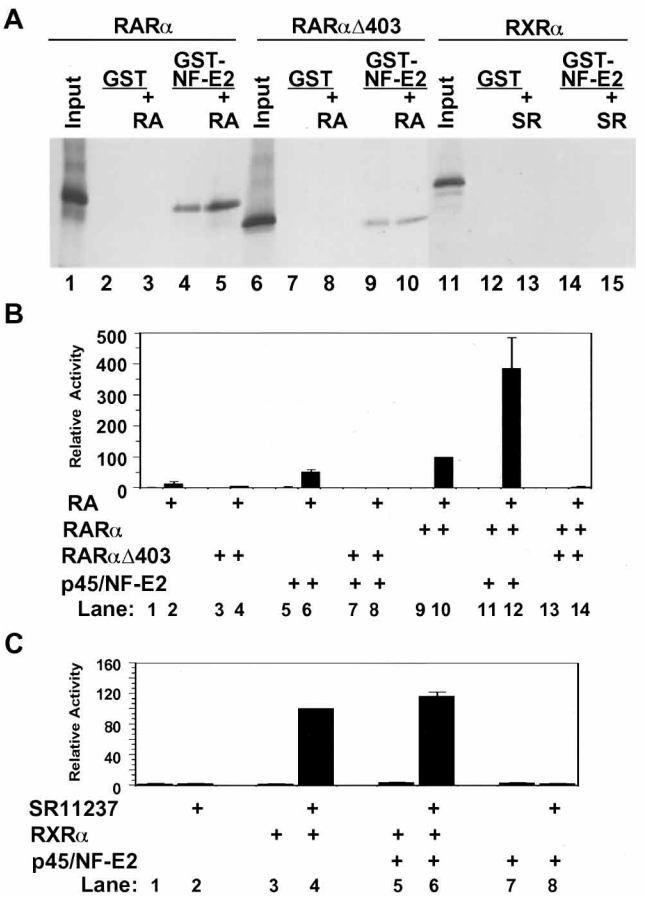


FIG. 5. p45/NF-E2 interacts with and potentiates transcription by RAR but not that by RXR. (A) RAR, but not RXR, interacts with p45/NF-E2 in vitro. Radiolabeled, in vitro-translated RARα, RARαΔ403, or RXRα was incubated with GST or GST–NF-E2(p45), as indicated, in the presence or absence of 1 μM all-*trans* RA or 1 μM SR11237 (lanes SR). GST beads were washed in GST binding buffer plus 150 mM NaCl and 0.1% SDS five times before elution. (B) p45/NF-E2 potentiates RA-dependent transcription. COS-7 cells were transfected with a $(DR5)_2$-TK-CAT reporter in the presence or absence of expression vectors for RARα, RARαΔ403, and/or p45/NF-E2, as indicated, and in the presence or absence of 1 μM RA, as indicated. Results shown are the mean and range of duplicate samples, normalized to the β-gal activity from a cotransfected plasmid. Activity of RARα-transfected cells in the presence of RA was assigned a value of 100 U (lane 10). (C) p45/NF-E2 does not potentiate RXR-stimulated transcription. COS-7 cells were transfected with a $(CRBPI)_2$-TK-luciferase reporter in the presence or absence of expression vectors for RXRα and/or p45/NF-E2, as indicated, and in the presence or absence of 1 μM SR11237, as indicated. Results shown are the mean and range of duplicate samples, normalized to the β-gal activity from a cotransfected plasmid. Activity of RXRα-transfected cells in the presence of SR11237 was assigned a value of 100 U (lane 4).

The maf-like p45/NF-E2 heterodimerization partner p18 abolishes positive cross talk between TR and p45/NF-E2. In hematopoietic cells, p45/NF-E2 exists as a heterodimer with another bZip protein, p18, which is related to the oncogene v-maf (2). p18 increases the DNA-binding specificity of the heterodimer for the bases outside the AP-1 core of the NF-E2 consensus binding site (2, 21). Heterodimerization with p18 is sufficient for NF-E2 activity (27). Figure 6 shows that p18, on its own, had little, if any, effect upon T3-dependent transcription mediated by TRβ1 (compare lanes 2 and 4). In contrast, as shown earlier, p45/NF-E2 potentiated TRβ1 function about fivefold (lane 6). Cotransfection of p18 inhibited this effect in a dose-dependent manner (lanes 8 to 12), but not below the level of activation seen with liganded TRβ1 alone. Thus, the

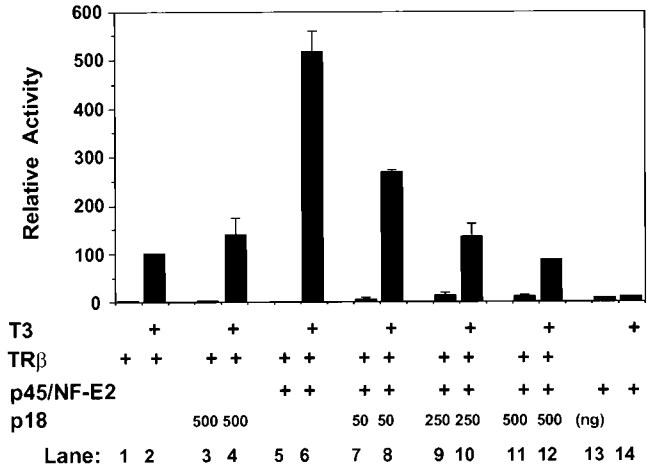


FIG. 6. p18 inhibits potentiation of T3 action by p45/NF-E2. COS-7 cells were transfected with a DR4-TK-CAT reporter in the presence or absence of expression vectors for TRβ, p45/NF-E2, and/or p18/NF-E2, as indicated, and in the presence or absence of 100 nM T3, as indicated. Results shown are the mean and range of duplicate samples, normalized to the β-gal activity from a cotransfected plasmid. Activity of TRβ-transfected cells in the presence of T3 was assigned a value of 100 U (lane 2).

heterodimer partner of p45/NF-E2, that enhances NF-E2 DNA-binding specificity and is thought to play a positive role in gene regulation by NF-E2 from NF-E2-binding sites, actually plays an inhibitory role in the ability of p45/NF-E2 to potentiate transcription from hormone-responsive elements.

The activation domain of p45/NF-E2 is required for potentiation of TR action. We next determined the region of p45/NF-E2 required for potentiation of T3 action. Figure 7A shows that although the N-terminal transcriptional activation domain of p45/NF-E2 was not required for the interaction with TR, it was necessary for the potentiation of activity (compare lanes 2, 4, and 6). Figure 7B shows that both wild-type p45/NF-E2 and the mutant lacking the N-terminal transactivation domain, p45/NF-E2ΔAD, were expressed in the transfected cells (lane numbers correspond to those in Fig. 7A). It is of note that p45/NF-E2ΔAD (amino acids 270 to 373) was transcriptionally neutral despite the fact that it contains the TR interaction domain defined earlier and binds to TRβ (Fig. 7A, lane 6, and data not shown). This suggests that increased transcription due to TR interaction with p45/NF-E2 is due to recruitment of the p45/NF-E2 activation domain to the TR bound to DNA. The TR-p45/NF-E2 interaction is required because overexpression of the p45/NF-E2 activation domain alone (amino acids 1 to 269) had no significant effect on T3 action (data not shown). Figure 7C shows that this region of p45/NF-E2 is, indeed, a transferable activation domain which functions on a Gal4-responsive reporter gene when fused to the Gal4 DBD, consistent with previous observations of its sequence similarity to other bZip transactivation domains (21), as well as the observation that removal of the N-terminal 206 residues of p45 destroyed its positive transcriptional function (27).

The activation domain of p45/NF-E2 interacts with the nuclear receptor coactivator CBP at a site which is bound by leucine zipper proteins involved in negative cross talk. Thus far, we have shown that p45/NF-E2 interacts with TR and RAR in a manner that is stimulated by ligand and that p45/NF-E2 potentiates the ligand-dependent transactivation function of these NHRs in a manner which requires the activation domain of p45/NF-E2. This is distinct from cross talk between NHRs and c-Jun/AP-1 (34), as well as other bZip proteins, such as the viral BLZF1 protein (35), which interfere with ligand-dependent transactivation by nuclear receptors. In the case of c-Jun, the interference occurs via interaction with the transcriptional coactivator CBP at a site distinct from that bound by the nuclear hormone receptor (25). We hypothesized that the positive cross talk between p45/NF-E2 and TR could involve positive integration of different inputs into CBP, in contrast to the prior examples of negative regulation. We therefore tested p45/NF-E2 for the ability to interact with CBP. Figure 8 shows that p45/NF-E2, as well as the isolated p45/NF-E2 activation domain, interacted strongly with GST–CBP(451-682) (lanes 4 and 9), which is the region previously shown to interact with c-Jun (4). Full-length p45/NF-E2 interacted weakly with GST–CBP(1-450) (lane 3), containing the region that interacts with NHRs, but the p45/NF-E2 activation domain (amino acids 1 to 269) did not interact with this region of CBP at all (lanes 3 and 8). In contrast, lanes 14 and 15 show that TR interacted strongly with CBP(1-450), in a manner

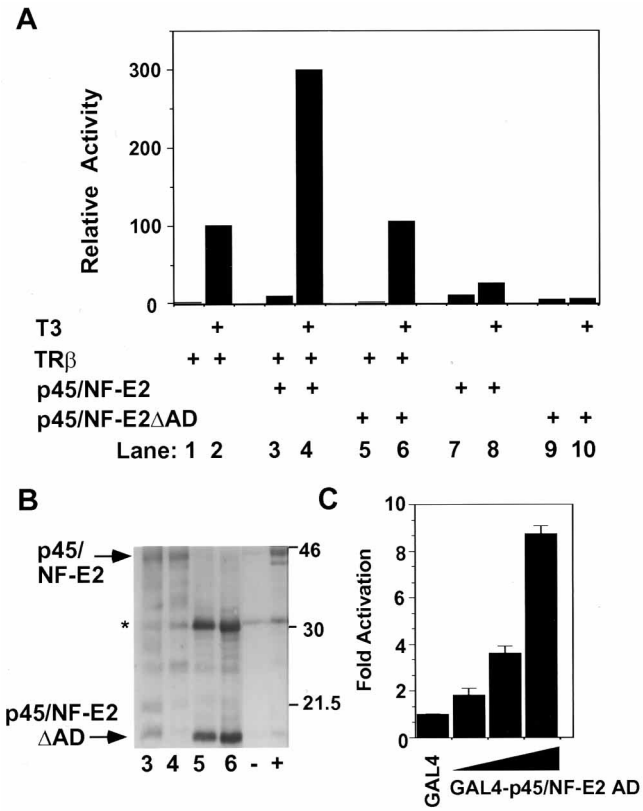


FIG. 7. The activation domain of p45/NF-E2 is required for its ability to potentiate T3 action. (A) Transfection. COS-7 cells were transfected with a DR4-TK-CAT reporter in the presence of absence of expression vectors for TRβ, p45/NF-E2, and/or p45/NF-E2ΔAD (AD, N-terminal activation domain), as indicated, and in the presence or absence of 100 nM T3, as indicated. Results shown are normalized to the β-gal activity from a cotransfected plasmid. Activity of TRβ-transfected cells in the presence of T3 was assigned a value of 100 U (lane 2). (B) Western analysis of expression of p45/NF-E2 and p45/NF-E2ΔAD proteins in transfected cells. Lane numbers correspond to those in panel A. −, untransfected cells; +, murine erythroleukemia cell extract control. Arrows indicate the migration of NF-E2 proteins. The asterisk indicates an additional band seen with the p45/NF-E2ΔAD expression plasmid. (C) The N terminus of p45/NF-E2 is a bona fide activation domain. The Gal4 DBD(1-147) or the Gal4 DBD fused to the activation domain (amino acids 1 to 269) of p45/NF-E2 in increasing amounts was transfected into COS-7 cells with a (Gal4 ×5)-simian virus 40-luciferase reporter. Results shown are the mean and range of duplicate samples, normalized to β-gal activity, and expressed as fold activation compared with the Gal4 DBD alone.

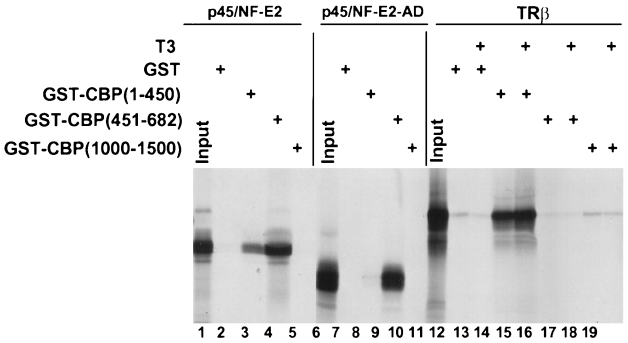


FIG. 8. The activation domain of p45/NF-E2 interacts with the bZip interaction domain of CBP. In vitro-translated, radiolabeled p45/NF-E2, the p45/NF-E2 activation domain (AD) (amino acids 1 to 269), and TRβ were incubated with GST and GST fused to the indicated CBP polypeptides as shown. For TRβ, pulldowns were performed in the presence or absence of 100 nM T3 as indicated. Lanes 1, 6, and 11 represent 20% of the total input.

which was modestly stimulated by T3. TR also did not interact with GST–CBP(451-682), as previously described (25). As a further specificity control, neither p45/NF-E2 nor TR interacted with another region of CBP (amino acids 1000 to 1500).

The p45/NF-E2 interaction region of CBP functions as a dominant inhibitor of p45/NF-E2 potentiation of TR function. To determine whether the interaction with CBP was responsible for the positive cross talk between p45/NF-E2 and liganded TR, we tested the effects of expression of the p45/NF-E2-interacting domain of CBP (i.e., amino acids 451 to 682) on the ability of p45/NF-E2 to potentiate transactivation by liganded TR. Since TR does not interact with this domain, we reasoned that this domain would have little effect on TR function in the absence of p45/NF-E2. However, if the interaction between p45/NF-E2 and CBP were responsible for the ability of p45/NF-E2 to potentiate TR action, then CBP(451-682) would be predicted to dominantly inhibit this effect by competing with endogenous CBP for binding to p45/NF-E2. Figure 9A shows the results of a transient transfection experiment in which HA epitope-tagged CBP(451-682) was, indeed, found to have little or no effect on the ability of T3 to activate TRβ-mediated transcription from a TRE. In contrast, CBP(451-682) blocked the ability of p45/NF-E2 to potentiate TRβ-mediated transcription in a dose-responsive manner. These effects required high plasmid concentrations, as has been observed with other effects of transfected CBP (10); this is presumably due to inefficient expression of the transfected protein or high endogenous CBP (and p300) concentrations or both. In any case, these results are consistent with an in vivo interaction between p45/NF-E2 and CBP(451-682) that prevents p45/NF-E2 from interacting with endogenous CBP and thereby prevents positive cross talk between p45/NF-E2 and liganded TR. The interaction of the transfected HA-CBP with p45/NF-E2 most likely occurred in the cell nucleus, because the cellular localization of the transfected HA-CBP was nuclear both in the absence and in the presence of cotransfected p45/NF-E2 (Fig. 9B).

DISCUSSION

We have described physical and functional interactions between a subset of NHRs, including TR and RAR, and the bZip transcription factor p45/NF-E2. The physical interaction involves the DBDs of both proteins. This is consistent with other interactions between NHRs, including RAR, and bZip proteins, including jun and BZLF1 (34, 35). T3 and RA markedly enhanced the interaction between their cognate receptor and p45/NF-E2, suggesting a role for the LBD of the NHR in promoting the interaction. Ligand binding could alter the conformation of the NHR, resulting in increased availability of the DBD for interaction with p45/NF-E2. Alternatively, ligand binding might induce interaction between an additional domain of the NHR and p45/NF-E2.

p45/NF-E2 physically interacted with TR and RAR in the absence of its bZip heterodimerization partner p18. One role of p18 is to serve as a heterodimer partner to increase the affinity of p45 for NF-E2-binding sites, where the complex serves a positive function (21). However, p18 actually antagonized the ability of p45/NF-E2 to potentiate T3 action. Thus, the positive role of NF-E2 in TR function is mechanistically distinct from its regulation of transcription from NF-E2 sites. The mechanism by which p18 inhibits the positive cross talk between TR and p45/NF-E2 is uncertain but could involve steric hindrance or displacement of liganded TR from p45/NF-E2 since p18 interacts with the leucine zipper of p45/NF-E2 (2, 21). p18 also interacts with TR (data not shown), but unlike p45, the p18 protein neither contains a transcriptional activation domain (21) nor interacts with CBP (data not shown). Thus, p18 would not be expected to potentiate T3 action, consistent with the results herein. It would be of interest to determine the effects of liganded TR on transcriptional activation by the NF-E2 heterodimer. Forced expression of TR with the NF-E2 subunits did not alter NF-E2 DNA-binding activity quantitatively or qualitatively (data not shown). However, it was not feasible to directly examine the potential effects of TR on NF-E2 function because NF-E2 is thought to activate erythroid transcription through cooperation with other tissue-specific proteins (3), and no biologically relevant assay for NF-E2 function has been described in heterologous cells. We have not studied the forced expression of TR in erythroid cells, because endogenous NF-E2 activity is present and we cannot readily distinguish TR effects on p45/NF-E2 from other potential effects of TR. However, the positive cross talk between TR and NF-E2 is consistent with the positive roles of both T3 and NF-E2 in erythroid differentiation.

A number of proteins have recently been described as interacting with NHRs in a ligand-dependent manner. Some or all of these proteins, which include SRC-1 (33), RIP-140 (8), Trip1 (30), TIF-1 (29), and ERAPs (19), may function as coactivators for NHRs. The most compelling evidence for a functional coactivator pertains to SRC-1 because it has (i) the ability to interact with NHRs in a ligand-dependent manner, (ii) a transferable activation domain, and (iii) the ability to potentiate transcriptional activation by a number of NHRs.

Interestingly, although p45/NF-E2 was originally isolated as a sequence-specific DNA-binding protein, its properties related to NHR function are characteristic of coactivator proteins. Thus we have shown that p45/NF-E2 (i) interacts with NHRs in a ligand-dependent manner, (ii) contains an intrinsic transcriptional activation domain, and (iii) potentiates ligand-dependent transactivation by NHRs. The major difference between the properties of p45/NF-E2 and SRC-1 as coactivators is that NHR interaction with p45/NF-E2 is not completely dependent upon ligand, although in both cases the function is clearly dependent upon AF2 (as indicated by the lack of function of v-erbA and RARαΔ403 with p45/NF-E2), which is the hallmark of the ligand-dependent coactivators (7, 19). Thus, p45/NF-E2, a protein that functions as a sequence-specific transcription factor in one context, can also function as a coactivator of NHRs. In this regard, it is noteworthy that at least one isoform of SRC-1 contains a basic helix-loop-helix motif characteristic of another class of sequence-specific DNA-bind-

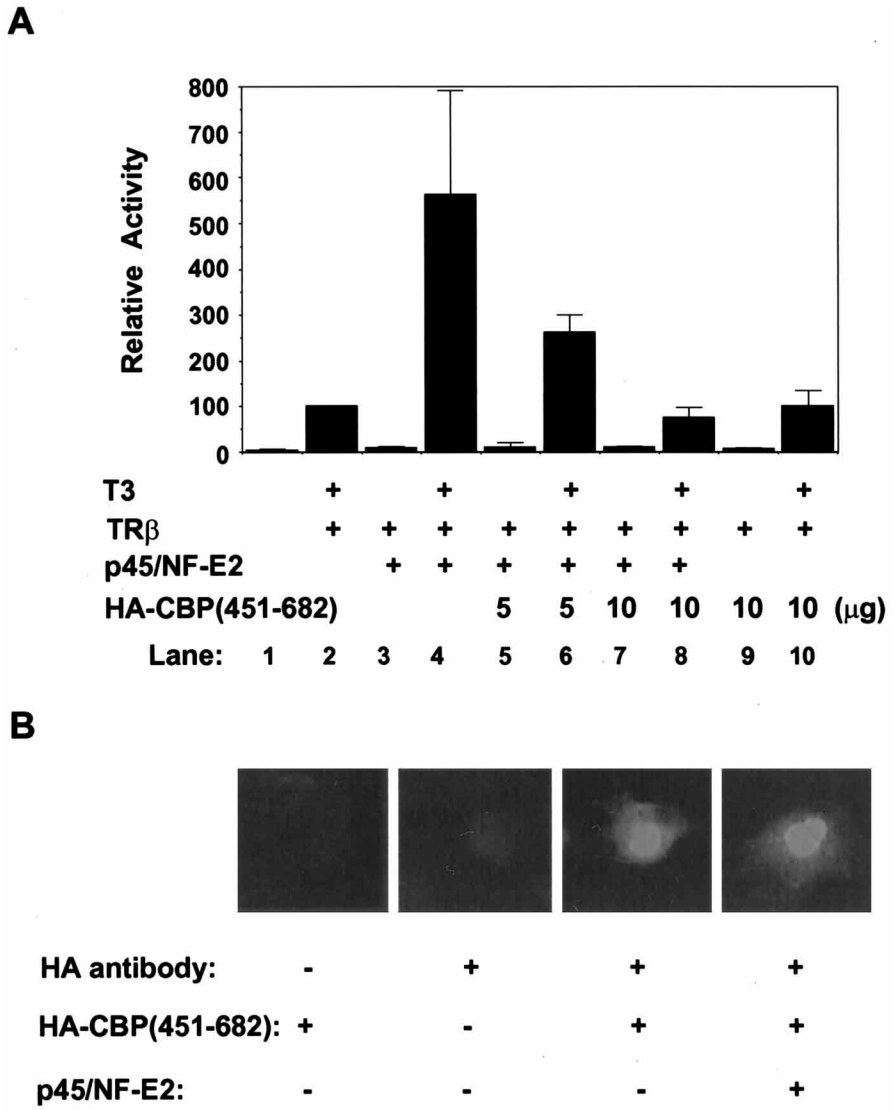


FIG. 9. The bZip interaction domain of CBP inhibits potentiation of T3 action by p45/NF-E2. (A) COS-7 cells were transfected with an 8DR4-TK-CAT reporter in the presence of absence of expression vectors for TRβ, p45/NF-E2, and/or HA–CBP(451-682), as indicated, and in the presence or absence of 100 nM T3, as indicated. Results shown are normalized to the β-gal activity from a cotransfected plasmid. Activity of TRβ-transfected cells in the presence of T3 was assigned a value of 100 U (lane 2). (B) Nuclear localization of HA–CBP(451-682). Transfections were performed as described in the text, and cells were assayed for immunofluorescence with an HA antibody.

ing proteins (25). Furthermore, SRC-1, like p45/NF-E2, also interacts directly with CBP (9, 20, 25, 46). Given that the NHRs could interact with p45/NF-E2 in the absence of ligand in vitro, it is somewhat surprising that ligand was required for p45/NF-E2 to serve as a coactivator in vivo. This could be related to the binding of corepressor N-CoR or SMRT to the unliganded NHRs (15, 47), which could conceivably prevent the functional interaction with p45 in vivo. Furthermore, it is possible that coactivation in vivo requires the TR-CBP-p45 complex and that T3 stabilizes this complex by enhancing both the TR-CBP and TR-p45 interactions.

Recently, it has become clear that CBP plays a central role in the integration of transcriptional signals involving a variety of pathways. CBP has multiple functional domains, which allow it to interact with a variety of transcriptional regulators, including sequence-specific transcription factors, basal factors, and protein kinases (summarized in reference 23). We have provided evidence that p45/NF-E2 interacts with CBP at the site of interaction of other bZip proteins, such as AP1. Furthermore, this interaction is likely to be of functional significance because overexpression of the p45/NF-E2 interaction domain prevents the positive cross talk with NHRs without otherwise affecting the function of the NHR.

Most of the evidence for functional interactions of different classes of proteins converging on CBP derive from experiments showing cross-inhibition, or negative cross talk between different pathways (23). At least three models could possibly explain the negative cross talk between NHRs and AP1. One model involves direct competition for CBP between NHR and AP1. This model seems unlikely given that the proteins interact with separable regions of CBP. Another possibility is that binding of one transcription factor in solution recruits CBP away from the other transcription factor, i.e., "squelching." Neither of these models is likely to explain positive cross talk between NHRs and p45/NF-E2, since the effect requires hormone, intact NHR, and intact p45/NF-E2. Rather, we prefer a third model

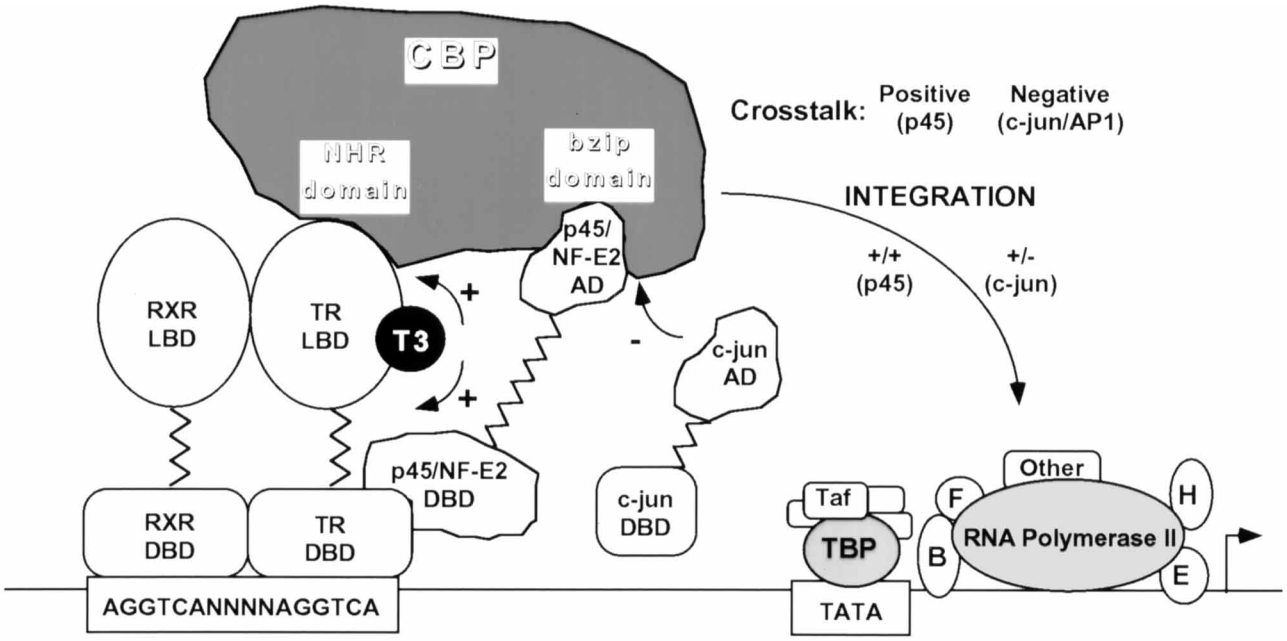


FIG. 10. Model of positive and negative cross talk mediated by the transcriptional integrator CBP. A gene with a TRE but not an AP1 or NF-E2 site is indicated. T3 activates the gene by facilitating interaction between TR and CBP(1-450), which sends a positive signal to RNA polymerase II, mediated by interaction with basal factors, acetylation of histones, or both. T3 binding to TR also causes recruitment of other activator proteins, including SRC-1 and TFIIB (data not shown). Interaction with the TR DBD via its leucine zipper region recruits p45/NF-E2 to this gene, and this interaction is further stabilized by interaction of the p45/NF-E2 activation domain with CBP(451-682). This interaction sends a second positive signal to RNA polymerase II. This same region of CBP is involved in the negative signal transmitted by Jun in the setting of T3-stimulated transcription. AD, activation domain.

in which the positive cross talk is likely to involve summation of two positive signals by CBP.

We envision this integration to occur as shown in Fig. 10 for T3-stimulated transcription. It should be noted that the model presented here, while consistent with our results and those of others, requires the ability of CBP, TR, and p45/NF-E2 to form a complex on DNA, which we have not directly shown. In this model, ligand binding by TR recruits CBP, which functions as a transcriptional coactivator. Other coactivators, such as SRC-1, may also be recruited and function together with CBP or independently (not shown in Fig. 10, for simplicity). On genes which do not contain binding sites for NF-E2, p45/NF-E2 is recruited to the TR-CBP complex on DNA because of its direct interaction with the TR DBD, in a manner which is greatly enhanced by T3. We propose that the TR–CBP–p45/NF-E2 complex is stabilized by the fact that p45/NF-E2 and TR interact with each other, as well as with CBP. In addition, the ability of T3 to enhance the TR-CBP and TR-p45 interactions would further stabilize the trimolecular complex. The transcriptional activation domain of p45/NF-E2, which directly interacts with CBP, provides a second, positive transcriptional signal to CBP. CBP thus truly integrates the signals from the independent interactions with the activation domains of both liganded TR and p45/NF-E2. A similar stabilizing role for CBP in mediating cooperativity between Myb and the bZip protein NF-M has also been suggested, although in that case both transcription factors bind directly to the target gene (32a). We suggest that the more commonly observed negative cross talk, such as that observed between TR and jun, as shown in Fig. 10, may reflect similar multiple protein-protein interactions stabilizing the association of CBP with jun on a target gene without an AP1 site. In this case, the jun interaction inputs a negative signal into CBP (by an unknown mechanism), which cancels or mitigates the effect of the positive input from liganded TR.

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