A Domain Containing Leucine-Zipper-Like Motifs Mediate Novel *in Vivo* Interactions between the Thyroid Hormone and Retinoic Acid Receptors

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The thyroid hormones and retinoic acid are potent modulators of differentiation, development, and gene expression. The transcriptional activities of these ligands are mediated by closely related nuclear receptors which bind and activate identical hormone responsive DNA elements. We noticed that a region within the ligand binding or E domain is well conserved between receptors for these hormones. This region contains hydrophobic heptad repeats that are structurally similar to the leucine-zipper dimerization domain. To study the function of this conserved domain, we examined the transcriptional responses of thyroid hormone receptor/c-erbA deletion mutants which lacked the heptad repeats. We previously reported that the chick c-erbA-α possesses hormone-independent (constitutive) activity in cells which express endogenous rat thyroid hormone receptor. We now demonstrate that this activity is abolished upon deletion of the conserved heptad repeats. This suggests that the heptad repeats mediate *in vivo* interactions between chick c-erbA and rat thyroid hormone receptors. To further test this hypothesis deletion mutants of chick c-erbA were constructed which contained all eight heptad repeats but which lacked the zinc-finger DNA binding domain. Although these mutants are transcriptionally inactive, they act in a dominant-negative fashion to block *trans*-activation by both the chick c-erbA-α and the endogenous thyroid hormone and retinoic acid receptors. We suggest that the heptad repeats mediate the formation of inactive mutant/wild-type hetero-dimers. Dimer formation suggests a mechanism to account for the dominant-negative phenotypes displayed by nonhormone binding variants of c-erbA, the proto-oncoprotein v-erbA and patients with the generalized thyroid hormone resistance syndrome. (Molecular Endocrinology 3: 1610–1626, 1989)

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INTRODUCTION

The thyroid hormones (T_3 and T_4) modulate a vast array of biological responses. In mammals, T_3 regulates the expression of hormones, metabolic enzymes, structural proteins, and cellular protooncogenes (1–3). In amphibians, T_3 initiates an intricate series of events that results in tadpole metamorphosis (1). Retinoic acid (RA) and other retinoids have striking effects on cell growth and differentiation (4) and are thought to establish morphogenetic gradients which dictate pattern formation in the developing chick limb bud (5–8). These diverse cellular responses appear to be initiated by the binding of these hormones to their nuclear receptors (9).

Characterization of thyroid hormone receptors (T_3R) has been facilitated by the observation that cellular homologues of the v-erbA oncoprotein bound T_3 and T_4 with affinities similar to the previously defined receptor (10, 11). Two divergent classes of c-erbA genes, designated as α and β, are encoded by distinct chromosomal loci (12, 13). Both the α- and β-genes encode multiple receptor subtypes that arise from alternative splicing (14–17). The amino acid sequences of the c-erbA proteins are conserved among the superfamily of nuclear receptors that mediate the transcriptional activities of steroids, retinoids, vitamin D_3, and possibly other

as yet unidentified ligands (9). Sequence similarity between the thyroid hormone and RA receptors (RAR) is most striking within the zinc-finger DNA binding domain, which is thought to target the receptor to specific hormone-responsive DNA elements. Indeed, the T_3R and RAR can act as ligand-dependent transcription factors which bind and activate identical DNA response elements (10).

We previously found that in certain cells c-erbAs can activate transcription in the absence of thyroid hormone (18). Specifically, in T_3-responsive GH_4C_1 and GH_1 rat pituitary cells, expression of chick c-erbA-α-1 (subsequently referred to as chick c-erbA) or human c-erbA-β-1 resulted in hormone-independent (constitutive) activation of rat GH (rGH) or rat PRL (rPRL) reporter genes containing T_3 response elements (T_3RE). High levels of c-erbA expression resulted in maximal constitutive activity that could not be further increased by T_3. At lower levels of expression the cells' endogenous rat receptors still exhibited hormone responsiveness, but basal activity was constitutively increased. Thus, the heterologous chicken or human receptors can act in a dominant-positive fashion to relieve the cells' hormonal requirement for *trans*-activation.

Inspection of the carboxy terminal ligand binding or E domain revealed the presence of eight potential α-helical heptads containing hydrophobic residues at positions 1, 5, and 8 of each helix. These amino acids would form a hydrophobic surface along one face of a coiled-coil helix. A similar motif has been shown to mediate dimerization of transcription factors such as GCN-4, c-*jun*, C/EBP, c-*myc*, and the κ-light chain enhancer binding protein E-47 (19–24). These motifs also mediate hetero-dimer formation between c-*jun* and c-*fos* (25–32). In this study we show that deletion mutants lacking these hydrophobic helices lose the constitutively active phenotype, suggesting that this dominant-positive phenotype requires a potential dimerization domain.

To further test the dimerization hypothesis, we constructed c-erbA mutants that retain the potential dimerization domain but lack sequences necessary for *trans*-activation. Similar mutants, termed dominant-negatives, have been described for the multimeric *trp*, *lac*, and λ repressors (33). Our results demonstrate that deletion mutants of c-erbA which lack the zinc-finger DNA-binding motif are transcriptionally inactive. However, when coexpressed with either endogenous receptor or exogenous c-erbA, these mutants inhibit the ability of the wild-type receptors to activate transcription. Further mutational analysis indicates that like the dominant-positive phenotype, this dominant-negative phenotype also maps to the putative hydrophobic dimerization domain.

The amino acids on the surface of the putative helix (positions 1, 5, and 8) were found to be conserved not only among different subtypes of the T_3R but also among the α, β, and γ-subtypes of the RAR (34–38). This observation raised the possibility that these two receptors might act as hetero-dimers *in vivo*. Indeed, we find that the dominant-negative mutants of c-erbA also act to inhibit retinoic acid stimulation from a retinoic acid responsive reporter construct. These findings suggest that dimerization may provide a mechanism to establish novel interactions among networks of related receptors.

RESULTS

The Intact Chick c-erbA Possesses a *Trans*-Dominant Constitutive Activity

Since large deletions of the carboxy terminal ligand binding or E domain result in constitutive activation of steroid receptors (39–42), the constitutive activity of c-erbA might have resulted from proteolytic cleavage of this domain in GH_4C_1 and GH_1 cells. To rule out this possibility, chick c-erbA was expressed in GH_4C_1 cells and the nuclear and extranuclear fractions were immune precipitated with four different antisera that recognize a wide range of epitopes of $p^{47\,c\text{-}erbA}$ (Fig. 1A) (43). Antibody 28 (Ab 28), antibody 21 (Ab 21), antibody 23 (Ab 23), and antibody 49 (Ab 49) specifically recognized a 47 kilodalton (kDa) protein in the nuclei of transfected GH_4C_1 cells (Fig. 1B, erb:+) but not in mock transfected controls (Fig. 1B, erb:−). This 47 kDa protein was not seen with nonimmune serum or when ab 28 was preabsorbed with its cognate antigen (Fig. 1B, Ab28A). No other specific bands were seen even after overexposure of the autoradiogram. The extranuclear fraction also revealed a single 47 kDa protein with no evidence for proteolytic cleavage (unpublished observations and see Fig. 4). Similar results were found in transfected 235-1 cells where the chick c-erbA behaves in a hormone-dependent fashion (18). Like the endogenous rat receptor, the 47 kDa chick c-erbA can be released from the nuclear fraction by treatment with sodium dodecyl sulfate (SDS) (Fig. 1B), 0.4 M KCl (18), or micrococcal nuclease (data not shown). Thus, chick c-erbA is expressed as a protein of the predicted size and can tightly associate with the nuclei of transfected cells. Most importantly, since we find no evidence for proteolytic cleavage, the constitutive activity appears to be a property of the intact chick c-erbA.

The Constitutive Activity Maps to Heptad Repeats Within the E Domain of Chick c-erbA

GH_4C_1 cells expressing endogenous rat T_3R were cotransfected with various chick c-erbA deletion mutants along with a T_3 responsive rPRL-chloramphenicol acetyltransferase (CAT) construct (Fig. 2A). The cells were cultured in T_3/T_4-depleted medium to assay the constitutive activity of c-erbA. Cells transfected with rPRL-CAT alone showed low levels of basal CAT activity (Fig. 2A, no c-erbA). Expression of the wild-type chick c-erbA (Fig. 2A, 1–408) resulted in a constitutive, 17-fold activation of rPRL-CAT (Fig. 2A, 1–408). Carboxy terminal mutants exhibit complete (Fig. 2A, 1–392) or

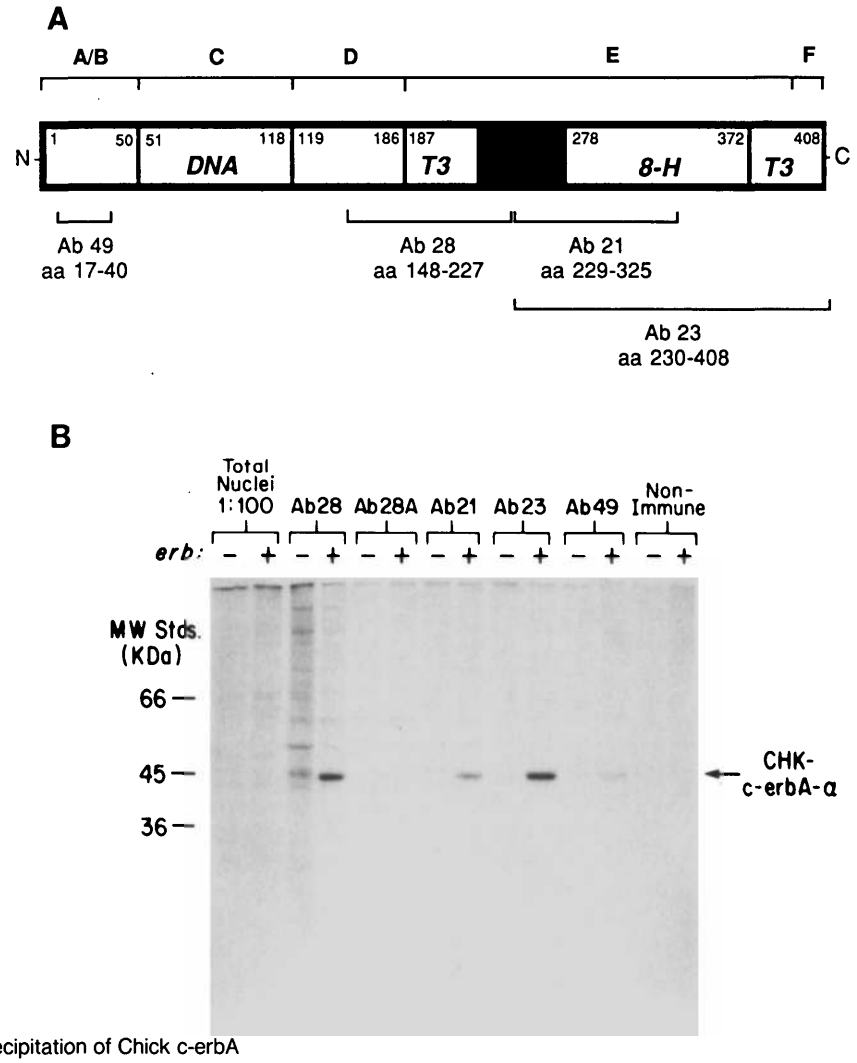


Fig. 1. Immune Precipitation of Chick c-erbA

A, Chick c-erbA is a 408 amino acid protein (47 kDa) consisting of six subdomains (A–F) defined by sequence conservation with other nuclear hormone receptors (85). The domains are: A/B (amino acid 1–50), C (amino acid 51–118), D (amino acid 119–186), E (amino acid 187–395), and F (amino acid 396–408). The C or DNA binding domain (DNA) contains two putative zinc-finger motifs and are thought to mediate sequence-specific binding to target DNA sequences. Amino acids at the ends of the E domain that are required for hormone binding are labeled (T_3). A region extending from amino acids 278–372 contains eight heptad repeats (8-H) of hydrophobic amino acids that have the potential to form an α-helical structure. Ab 49, 28, 21, and 23 (43) and their chick c-erbA epitopes are illustrated. Ab 23 and 28 were raised against v-erbA fragments, Ab 49 against a c-erbA peptide and Ab 21 was raised against a human c-erbA-α fragment. The amino acids listed below each antibody refer to the corresponding epitopes on chick c-erbA. B, GH_4C_1 cells were transfected with (erb:+) or without (erb:−) 10 μg of the chick erb-A expression vector. Cells were labeled with L-[^{35}S]methionine and the nuclear fraction immune precipitated with Ab 28, 21, 23, and 49 or nonimmune serum, as indicated. Ab28A refers to Ab28 that had been preabsorbed to its cognate antigen. The immune precipitates were electrophoresed on 10% SDS-polyacrylamide gels and fluorographed at −70 C for 42 h. For comparison, a 1:100 dilution of the nuclear fraction before immune precipitation is shown in the first two lanes (total nuclei, 1:100). Molecular weight standards are shown on the left and the migration position of chick c-erbA is indicated by the labeled *arrow*.

partial (Fig. 2A, 1–373) constitutive activity even though they cannot bind T_3 (44), indicating that the constitutive activity does not result from the binding of trace amounts of ligand. In contrast, deletions extending from the carboxy terminus through residues 342^{thr}, 284^{gln}, or 260^{arg} were all inactive (Fig. 2A). Since the different c-erbA mutants are expressed with varying efficiencies, the amounts of expression vectors used in this study (Fig. 2A) were chosen so that all the mutants would be expressed at similar levels. Figure 2B shows that the carboxy terminal deletion mutants are expressed as proteins of the predicted size and are found in approximately equal amounts in both the nuclear and extranuclear fractions. These results demonstrate that mutants lacking amino acids 342^{thr}–373^{arg} are expressed in cells but have lost the ability to mediate constitutive activation.

Inspection of the carboxy terminal region required for constitutive activity of chick c-erbA revealed the presence of 8 heptad repeats extending from amino acids

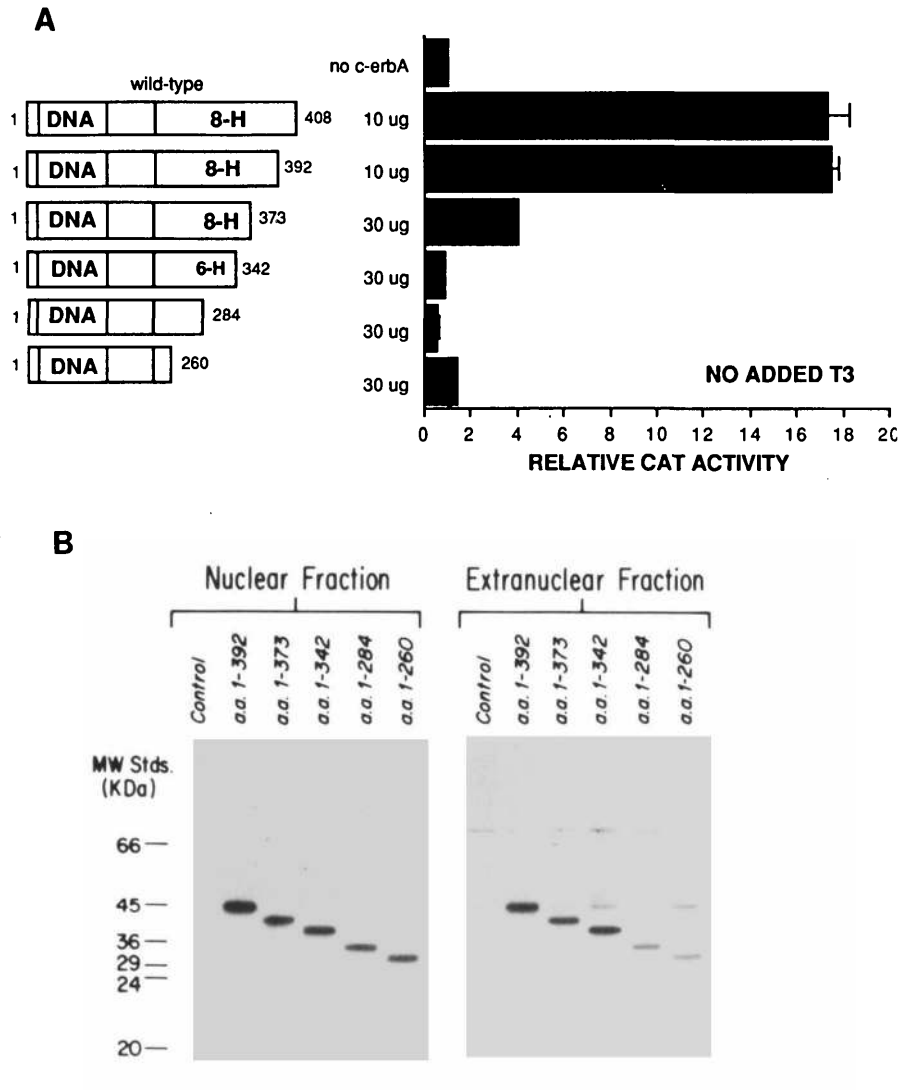


Fig. 2. Deletion Mapping of the Constitutive Activity of Chick c-erbA

A, GH_4C_1 cells were transfected with 10 μg −338/+75 rPRL-CAT alone (no c-erbA) or with the indicated amounts of each expression vector. The total amount of RSV promoter sequences were maintained constant by addition of the expression vector lacking all cDNA sequences (pRSVi⁻). After 48 h in hormone-depleted medium, CAT activity was determined and the percent of chloramphenicol acetylation in the absence of c-erbA was defined to have a relative CAT activity of 1. The remaining CAT activities are normalized to this value. DNA refers to the DNA binding or C domain. 8-H indicates the presence of all eight heptad repeats (Fig. 1A and Fig. 3); 6-H indicates the presence of heptad repeats 1–6 (Fig. 3). B, The cells in Fig. 2A were labeled with L-[^{35}S] methionine and the nuclear and extranuclear fractions immune precipitated with Ab 28. Immune precipitates were electrophoresed in 10% SDS-polyacrylamide gels and fluorographed for 8 h at −70 C. Molecular weight standards (MW Std.) are shown on the *left*. The carboxy terminal deletion mutants appear as single bands of the predicted molecular weight: 1^{met}–392^{thr} (45 kDa, 15 methionines), 1^{met}–373^{arg} (43 kDa, 13 methionines), 1^{met}–342^{thr} (39 kDa, 12 methionines), 1^{met}–284^{gln} (33 kDa, 11 methionines), and 1^{met}–260^{arg} (30 kDa, 10 methionines). After accounting for differences in methionine content all mutants appeared to accumulate to similar levels.

278–372 (Fig. 3), which contain one of the following hydrophobic amino acids at positions 1 and 8 of the heptad: leucine (L), methionine (M), isoleucine (I), valine (V), or phenylalanine (F). The fifth position contains either hydrophobic residues or the charged amino acids arginine (R) or glutamic acid (E). The amino acids comprising positions 1, 5, and 8 of each heptad are particularly well conserved between T_3R and RAR (Fig. 3). If these heptads folded into coiled-coil helices, then the conserved residues at position 1, 5, and 8 would align along the same surface of the helix (22). Similar hydrophobic helices mediate the dimerization of transcription factors (GCN–4, c-*jun*/c-*fos*, C/EBP, c-*myc*, and E-47) (19–32) as well as structural proteins (nuclear lamins, keratins, and paramyosin) (45–49). The data in Fig. 2A suggest that the constitutive activity correlates with the

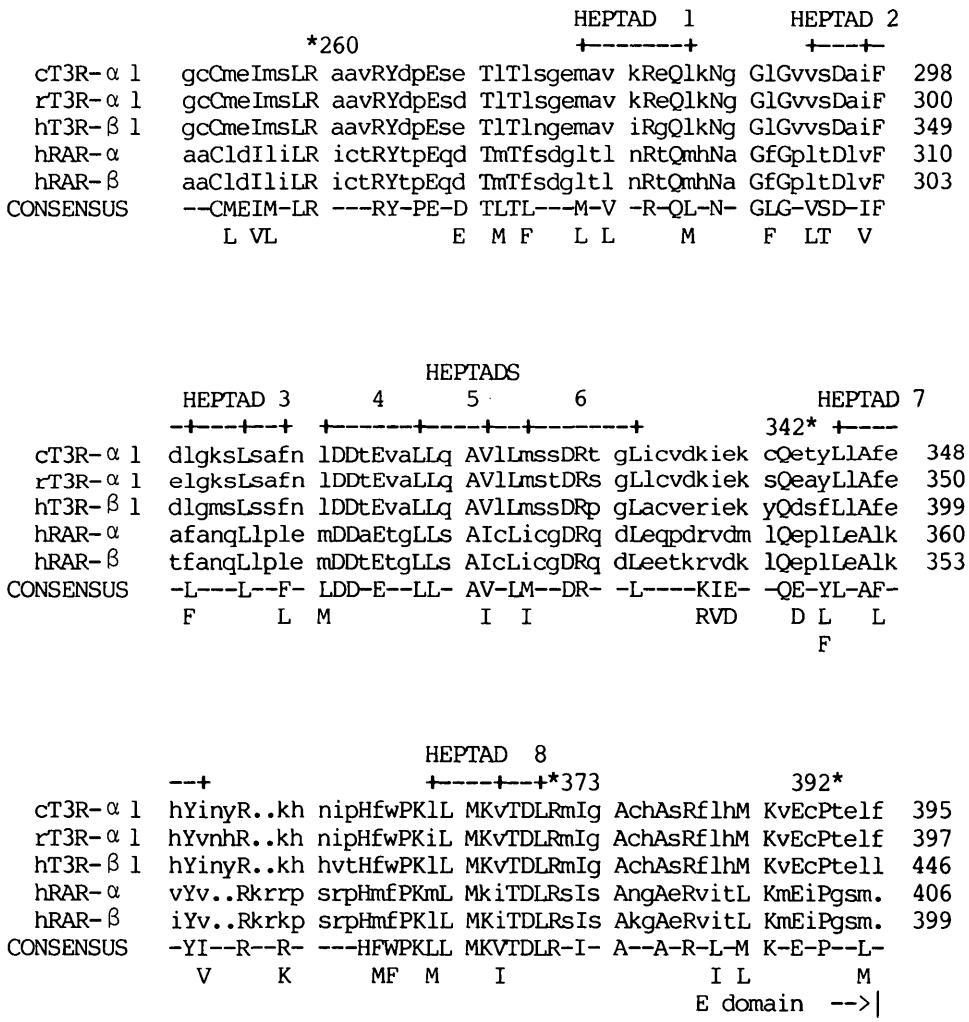


Fig. 3. The Heptad Repeats of Chick c-erbA are Conserved between the T_3R and RAR

The amino acid sequence of the eight heptad repeats are shown for the chicken c-erbA α-1 (cT3R-α1), rat c-erbA-α1 (rT3R-α1), human c-erbA-β-1 (hT3R-β1), and the α- and β-subtypes of the human RAR (hRAR). All eight heptad repeats are labeled. Conserved hydrophobic residues at positions 1, 5, and 8 are indicated by a +. *Capital letters* indicate residues that are identical in all receptors. The consensus sequence is shown in capital letters and includes positions containing identical or conserved residues. The carboxy terminal amino acid of mutants extending to residues 260, 342, 373, and 392 (Fig. 2) are indicated with an *. Note that heptads 2–3 and 4–6 represent contiguous repeats. Proline residues are absent from all heptads with the exception of heptad 3 of the hRARs and heptad 5 of the hT3R-β1. The Chou-Fasman algorithm (Genetics Computer Group, University of Wisconsin) predicts α-helices in heptads 1, 2–3, and 8 of chick c-erbA. The Garnier-Ogusthorpe-Robson algorithm (Genetics Computer Group, University of Wisconsin) is consistent with α-helices in heptads 1, 4–6, 7, and 8.

presence of the heptad repeats since all constitutive activity is lost when the seventh and eighth heptads (Fig. 3) are deleted (Fig. 2A, 1–342). The 1^{met}–373^{arg} mutant contains all eight heptads but exhibits partial constitutive activity (Fig. 2A, 1–373). This mutant is truncated at residue 373—just one amino acid from the carboxy terminal end of the eighth heptad (amino acid 372), suggesting that flanking sequences are necessary for full stability of the eighth heptad.

c-erbA Mutants Lacking the DNA Binding Domain Inhibit *Trans*-Activation by Endogenous Thyroid Hormone Receptors

Since the heptad repeats are structurally related to known dimerization domains, we considered the possibility that the T_3R may act as a dimer (or multimer) *in vivo*. To functionally test this possibility, we studied chick c-erbA mutants which contained the putative dimerization domain, but which lacked domains required for transcriptional activation. If expressed in excess of the wild-type receptor, mutant/wild-type hetero-dimers would accumulate which might be transcriptionally inactive. Analgous dominant-negative mutants have been shown to inhibit multimeric *lac*, *trp*, and λ repressors (33). Similarly, networks of keratin filaments are known to collapse upon expression of a keratin mutant that contains similar heptad repeats (49). We describe two chick c-erbA amino terminal deletion mutants which have dominant negative activity. The first

mutant (amino acids 120^{met}–408^{val}) lacks the A/B domain as well as the DNA-binding or C domain (Fig. 1A). The second mutant (amino acids 199^{met}-408^{val}) lacks all of the A/B, C, and D domains as well as 11 amino acids of the E domain which are required for T_3 binding (44). In both mutants, all eight hydrophobic heptads (8-H) are intact. We refer to these mutants as DBD^- since they lack the DNA Binding Domain.

GH_4C_1 cells were transfected with DBD^- expression vectors and the nuclear and extranuclear fractions immune precipitated (Fig. 4) with Ab 28. A predominant 45 kDa band was seen in the nuclear and extranuclear fraction of all samples including mock-transfected controls (Fig. 4). The intensity of this band varies from preparation to preparation (compare with Fig. 1B). This band is nonspecific and does not represent endogenous rat receptor. Unlike chick c-erbA, it was still found when immune precipitation was performed with antiserum that has been preabsorbed with its cognate antigen (data not shown).

Cells transfected with the wild-type (Fig. 4, WT) chick c-erbA expressed the predicted 47 kDa protein which was equally abundant in the nuclear and extranuclear fractions. The 120^{met}–408^{val} mutant was expressed as the predicted 33 kDa protein. This mutant was found in the nuclear fraction but accumulated to much higher levels in the extranuclear fraction (Fig. 4, 120–408), suggesting that the first 119 amino acids may contain sequences necessary for efficient nuclear localization. Similar results were obtained with Ab 21 and 23 (data not shown). When the last wash of the nuclear fraction was immune precipitated the 120^{met}–408^{val} mutant was not detected (data not shown), indicating that the nuclear 33 kDa protein does not represent contamination from the extranuclear fraction. The 120^{met}–408^{val} mutant can be released from nuclei by micrococcal nuclease (data not shown), suggesting that the small fraction of this DBD^- mutant that is associated with the nucleus may be bound indirectly to DNA. The 199^{met}–408^{val} mutant was less efficiently expressed in these cells. Although it cannot be detected in nuclei, mutant 199^{met}–408^{val} was seen as a 24 kDa protein of low abundance in the extranuclear fraction (Fig. 4, 199–408). Similar results were found in 235–1 cells (data not shown).

These results indicate that both DBD^- mutants are synthesized in cells but have lost sequences necessary for tight nuclear binding. Since the mutants are small proteins with lowered affinity for nuclei, they may diffuse out of the nucleus during cell-lysis. Therefore, the *in vivo* distribution of these mutants may vary from the results presented here. Nonetheless, it appears that

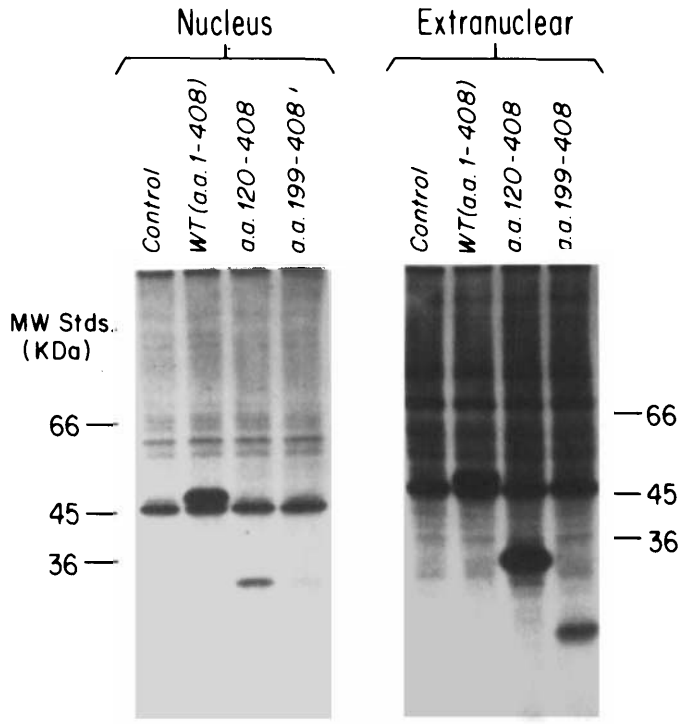


Fig. 4. Immune Precipitation of Chick c-erbA DBD^- Mutants

GH_4C_1 cells were transfected without (control) or with 15 μg expression vectors for the wild-type chick c-erbA (WT, 1^{met}–408^{val}), DBD^- mutant 120^{met}–408^{val} or DBD^- mutant 199^{met}–408^{val}. Nuclear and extranuclear fractions were immune precipitated with Ab 28 (Fig. 1A) and electrophoresed in 10% and 11% SDS-polyacrylamide gels, respectively. The gels were fluorographed for 36 h at −70 C. All three proteins appear as single bands of the predicted size: wild-type 1^{met}–408^{val} (47 kDa, 15 methionines), mutant 120^{met}–408^{val} (33 kDa, 12 methionines), and mutant 199^{met}–408^{val} (24 kDa, 10 methionines). Note that mutant 199–408 cannot be detected in the nuclear fraction. The distribution of the wild-type or mutant proteins between the nuclear and extranuclear fractions was not effected by T_3 (data not shown).

sequences within or around the first 119 amino acids confer tight nuclear localization to chick c-erbA. Insertional mutagenesis of v-erbA has shown that sequences corresponding to position 135 of chick c-erbA are required for tight nuclear binding (50). In the rat glucocorticoid receptor a nuclear localization signal has been mapped (51) to a domain corresponding to residues 119–135 of chick c-erbA, suggesting that this region of chick c-erbA may be necessary for efficient nuclear localization.

The functional activities of the DBD$^-$ mutants were tested in GH_4C_1 cells using a −338/+75 rPRL-CAT reporter plasmid (Fig. 5A). Cells transfected with rPRL-CAT alone (Fig. 5A, no c-erbA) express low levels of CAT in the absence of T_3. CAT activity increased 5-fold in response to 0.75 nM T_3, reflecting *trans*-activation by endogenous receptor. Cotransfection with 10 μg wild-type chick c-erbA (1^{met}–408^{val}) resulted in a constitutive 20-fold activation (Fig. 5A, 1–408, no T_3) which is lowered approximately 50% with T_3 (Fig. 5A, 1–408, 0.75 nM T_3). Cotransfection with 1–10 μg 120^{met}–408^{val} DBD$^-$ mutant had no significant effect on basal CAT activity (Fig. 5A, 120–408, no T_3). Thus, unlike the wild-type protein, this mutant does not possess constitutive transcriptional activity, presumably because it lacks the zinc-finger motif and/or other regions essential for *trans*-activation. More interestingly, this mutant acts in a dominant-negative fashion to block T_3 stimulation by the endogenous receptors (Fig. 5A, 120–408, 0.75 nM T_3).

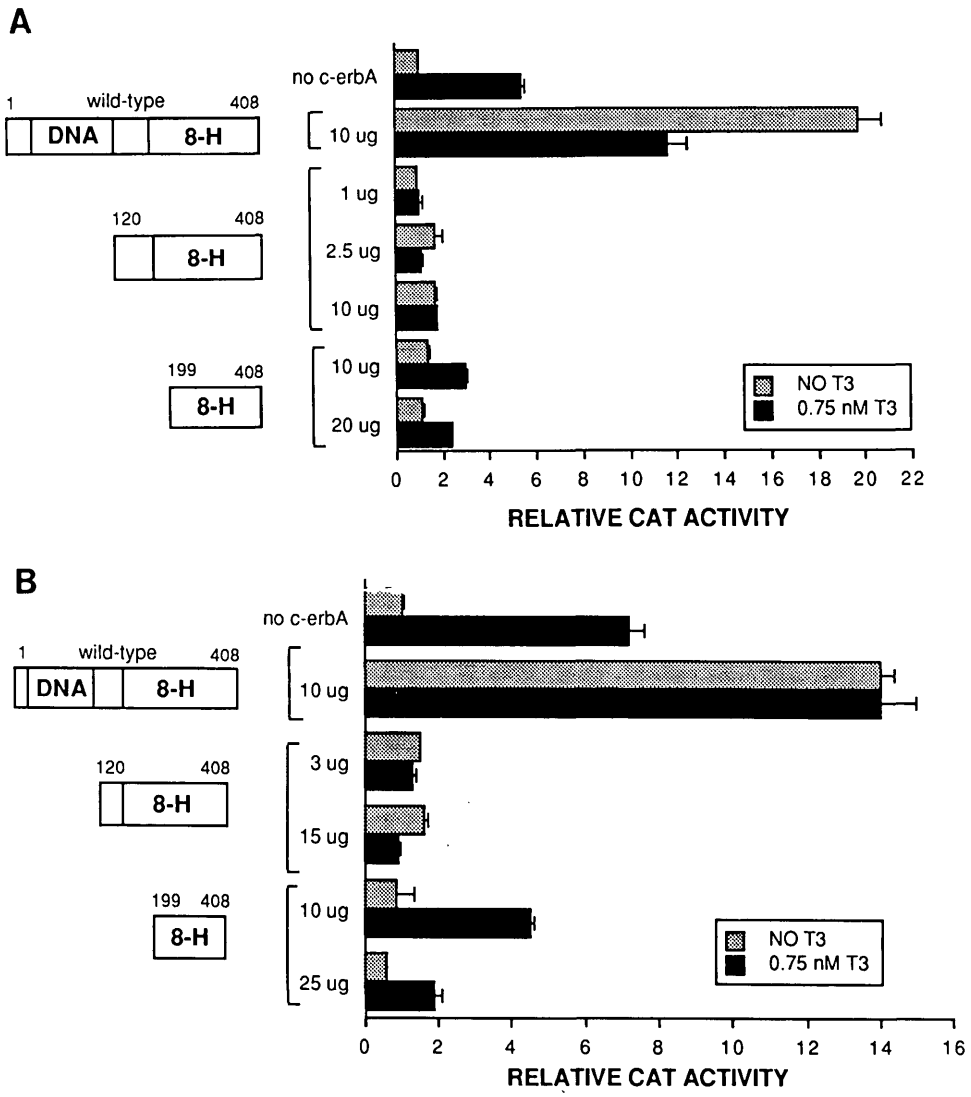


Fig. 5. DBD$^-$ Mutants of Chick c-erbA Inhibit *Trans*-activation by Rat GH_4C_1 Cell Receptors

A, GH_4C_1 cells containing endogenous rat T_3Rs were transfected with 15 μg −338/+75 rPRL-CAT alone (no c-erbA) or with the indicated amounts of the wild-type and mutant chick c-erbA expression vectors. RSV promoter sequences were maintained at constant levels by addition of the expression vector lacking any cDNA sequences. The cells were incubated for 48 h with (*black bars*) or without (*gray bars*) 0.75 nM T_3. CAT activity was determined and the percent acetylation seen with rPRL-CAT alone (no c-erbA, no T_3) was defined as 1. All other values were normalized to this level of CAT activity. DNA indicates the presence of the DNA binding or C domain; 8-H represents the eight heptad repeats (Fig. 3). B, This experiment is similar to A except that 20 μg −236:−178/−104:+11 rGH-CAT was used as the reporter construct.

Since the 120^{met}–408^{val} mutant can still bind hormone, it can be argued that this mutant is simply sequestering T_3, thereby making the ligand unavailable to the wild-type receptor. This is unlikely because with 0.75 nM T_3 in the media, intracellular levels of T_3 are in excess over the mutant. Furthermore, at 37 C, the intracellular pools of T_3 are in rapid equilibrium with the vast pool of extracellular T_3 (1, 52, 53). However, to rule out this possibility, the nonhormone binding DBD^- mutant, 199^{met}–408^{val}, was examined. Cotransfection of 10–20 μg of this mutant results in a dose-dependent decrease in T_3 responsiveness (Fig. 5A, 199–408), indicating that inhibition of T_3 responsiveness does not result from sequestration of T_3. Moreover, complete inhibition was observed with another DBD^- mutant that cannot bind hormone (see Fig. 8A, 120–392). Note that significant but incomplete inhibition is observed with 20 μg 199^{met}–408^{val} expression vector consistent with the lower level of expression of the 199^{met}–408^{val} mutant (Fig. 4).

Figure 5B illustrates similar results using a rGH-CAT construct containing a rGH T_3RE (−236/−178) just upstream of the rGH promoter (−104/+11) (54). Unlike rPRL-CAT, T_3 does not reduce the constitutive activity of the wild-type chick c-erbA (compare Fig. 5, A and B, 1–408). The reason for these differences are under investigation. No effect of the 120^{met}–408^{val} was seen in cells transfected with a control reporter that lacks a T_3RE (−104/+11 rGH-CAT) (55) (data not shown). This is consistent with the results in Fig. 5 indicating that the DBD^- mutants had no effect on basal (no T_3) CAT activity, supporting the notion that the dominant-negative mutants specifically inhibit receptor activation at T_3REs.

Dominant-Negative Mutants of Chick c-erbA Inhibit *Trans*-Activation by the Wild-Type Chick c-erbA

We next examined whether the dominant-negative chick mutants could block the activity of wild-type chick c-erbA. GH_4C_1 cells were transfected with a rPRL-CAT reporter, wild-type c-erbA and increasing doses of the dominant-negative mutant 120^{met}–408^{val}. The effect of the dominant-negative mutant on the constitutive activity of chick c-erbA can be examined in cells treated without T_3 (Fig. 6A, no T_3, *gray bars*). CAT activity is low in cells transfected with only rPRL-CAT (Fig. 6A, no c-erbA, no T_3), while eight μg wild-type c-erbA vector resulted in constitutive activation (Fig. 6A, 1–408, no T_3). Cotransfection of increasing amounts of the 120^{met}–408^{val} mutant, with a constant amount (8 μg) of the wild-type vector resulted in a dose-dependent inhibition of constitutive activity (Fig. 6A, WT + 120–408, no T_3). Inhibition with a similar dose-response was observed when the same cells were treated with T_3 (Fig. 6A, 0.75 nM T_3, *black bars*).

Our data indicate a direct relationship between the total levels of functional receptor and the dose of the dominant-negative mutant required to elicit inhibition. In Fig. 5A, 1 μg 120^{met}–408^{val} expression vector was sufficient to inhibit the activity of the endogenous receptor. In Fig. 6A the total level of functional receptors was increased by expression of wild-type chick c-erbA. We have previously shown that transfection of cells with 8 μg wild-type c-erbA expression vector results in accumulation of approximately 90 fmol c-erbA/100 μg DNA (18). Assuming a 10% transfection efficiency, cells that were transfected with the wild-type c-erbA (Fig. 6, WT) express approximately 900 fmol c-erbA/100 μg DNA. This represents a 6-fold increase over the levels of the endogenous receptor (150 fmol/100 μg DNA). In the face of this c-erbA challenge, 50 μg mutant expression vector were required to inhibit CAT activity (Fig. 6A, WT + 120–408), whereas 1 μg was sufficient to elicit complete inhibition in the absence of added c-erbA (Fig. 5A, 120–408). This suggests that the dominant-negative mutants directly interfere with receptor activity.

To exclude the possibility that the dominant-negative mutants function by inhibiting the nuclear localization or synthesis of the wild-type receptor, the nuclear fraction from cells transfected as in Fig. 6A were examined by immune precipitation (Fig. 6B). The nonspecifically precipitated 45 kDa protein is seen in all lanes. Transfection with 10 μg wild-type vector (Fig. 6B, wild-type) or 50 μg 120^{met}–408^{val} mutant (Fig. 6B, 120–408) results in expression of the predicted 47 kDa and 33 kDa proteins. Cotransfection of 50 μg mutant vector had little effect on the nuclear accumulation of the wild-type protein (Fig. 6B, WT + 120–408). Therefore, under conditions where the 120^{met}–408^{val} acted in a dominant-negative fashion (Fig. 6A) this mutant did not affect the nuclear localization or synthesis of the wild-type protein.

We next tested whether the 120^{met}–408^{val} mutant acted as a dominant-negative in 235–1 cells (Fig. 7). This cell line is deficient in T_3R (18) and, therefore T_3 does not activate rPRL-CAT in these cells (Fig. 7, no c-erbA). Expression of chick c-erbA resulted in a T_3-dependent stimulation of CAT activity (Fig. 7, 1–408). This stimulation is brought about by a decrease in CAT activity in the absence of T_3, followed by a T_3-dependent increase from these suppressed levels. Brent *et al.* (55) reported similar results using rGH-CAT constructs in other cells. These findings suggest that ligand-free c-erbA functions in 235–1 cells as a constitutive repressor and that T_3 stimulates expression by relieving the repression. When the 120^{met}–408^{val} mutant was coexpressed with 10 μg wild-type c-erbA, inhibition of T_3 stimulation was seen (Fig. 7, WT + 120–408). This is consistent with our findings in GH_4C_1 cells which indicate that the dominant-negative mutants of c-erbA can inhibit the wild-type chick receptor (Fig. 6). As in GH_4C_1 cells, immune precipitation experiments in 235–1 cells indicated that the 120^{met}–408^{val} mutant had no effect on the nuclear localization of the wild-type protein (data not shown). Thus, dominant-negative activity requires the presence of a functional T_3R.

Dominant-Negative Activity Requires the Hydrophobic Heptads

Since the DBD^- constructs contain the heptad repeats (Fig. 3), additional mutants were studied to determine

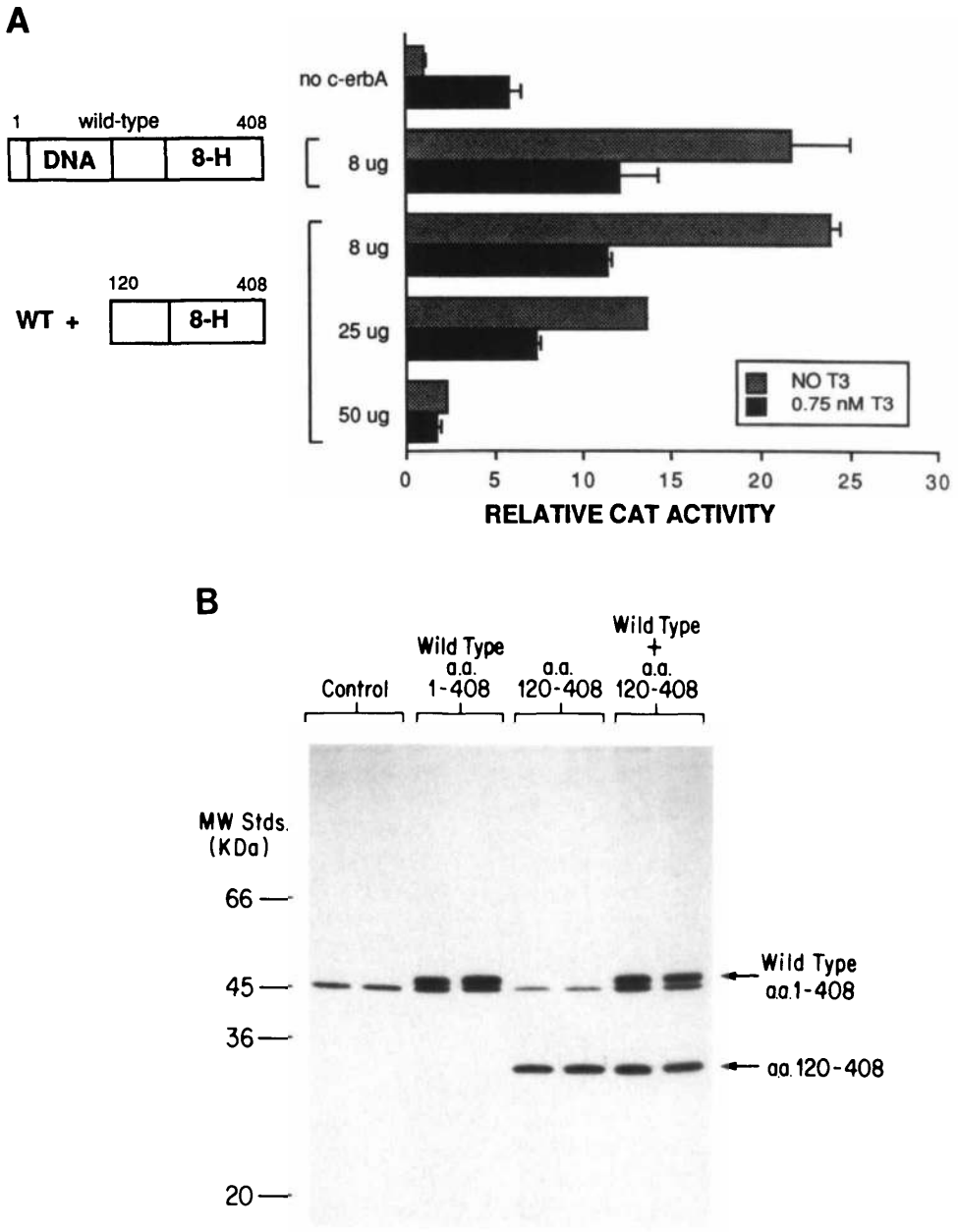


Fig. 6. DBD⁻ Mutants Inhibit *Trans*-activation by Wild-Type Chick c-erbA in GH_4C_1 Cells

A, GH_4C_1 cells containing endogenous rat thyroid hormone receptors were transfected without chick c-erbA (no c-erbA), with 8 μg wild-type chick c-erbA (1^{met}–408^{val}), or with 8 μg wild-type chick c-erbA (WT) in the presence of increasing doses (8–50 μg) the DBD⁻ mutant 120^{met}–408^{val}. −338/+75 rPRL-CAT (15 μg) was used as a reporter. All other conditions are the same as in Fig. 5. DNA indicates the presence of the DNA binding or C domain; 8-H represents the eight heptad repeats (Fig. 3). B, GH_4C_1 cells were transfected in duplicate without (control) or with 10 μg chick c-erbA (wild-type, 1–408), 50 μg DBD⁻ mutant 120^{met}–408^{val} (120–408) or with both 10 μg wild-type and 50 μg DBD⁻ mutant 120^{met}–408^{val} (wild-type + 120–408). RSV promoter sequences were maintained at constant levels by addition of the expression vector lacking any cDNA sequences. The nuclear fraction was immune precipitated with ab 28 and electrophoresed in 10% SDS-polyacrylamide gels. The gels were fluorographed for 6 h at −70 C. Molecular weight standards (MW Stds.) are shown on the *left* and the migration position of the wild-type and mutant proteins are indicated by the arrows on the *right*.

if this domain is required for the dominant-negative effect. Two carboxy terminal deletion mutants were derived from the 120^{met}–408^{val} mutant and their activities were assayed in GH_4C_1 cells (Fig. 8A). T_3 acts through the endogenous receptor to increase activity of the rPRL-CAT reporter (Fig. 8A, no c-erbA) and the 120^{met}–408^{val} suppresses this stimulation (Fig. 8A, 120–408). Mutant 120^{met}–392^{thr} contains all eight heptads and completely suppresses T_3 responsiveness (Fig. 8A, 120–392). As a result of the 16 amino acid carboxy terminal deletion, this mutant cannot bind T_3, further indicating that hormone binding is not required for the

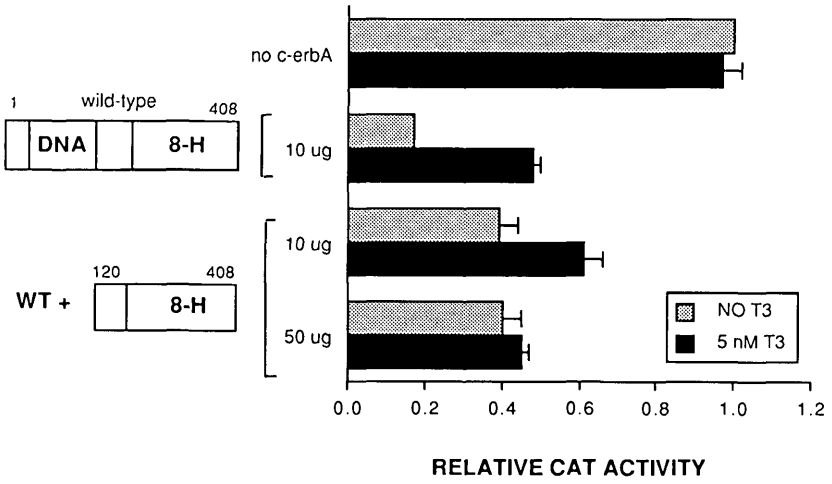


Fig. 7. DBD^- Mutants Inhibit *Trans*-activation by Wild-Type Chick c-erbA in Receptor Deficient 235–1 Cells

This experiment is similar to the one in Fig. 6A except 235–1 cells were transfected with −3000/+75 rPRL-CAT (20 μg) and cultured with or without 5 nM T_3. WT indicates 10 μg wild-type chick c-erbA vector; DNA indicates the presence of the DNA binding or C domain; 8-H represents the eight heptad repeats (Fig. 3).

dominant-negative activity. Deletion of all eight heptads in mutant 120^{met}–260^{arg} results in loss of the dominant-negative phenotype (Fig. 8A, 120–260). Immune precipitation of transfections from Fig. 8A demonstrate that both the 120^{met}–392^{thr} and the 120^{met}–260^{arg} mutants are synthesized but do not efficiently localize to the nucleus. The 120^{met}–260^{arg} protein does not appear to accumulate to as high levels as the 120^{met}–392^{thr} protein. Since these proteins are labeled with L-[^{35}S]methionine, the apparent low accumulation of 120^{met}–260^{arg} can be accounted for in part by the loss of five of 12 methionines upon deletion of residues 261–392. Since suppression by the 199^{met}–408^{val} mutant (Fig. 5, A and B, 199–408) occurred at even lower levels of expression, sufficient quantities of 120^{met}–260^{arg} were synthesized to elicit possible dominant-negative activity.

Dominant-Negative Mutants of c-erbA Inhibit Retinoic Acid Receptor Activity

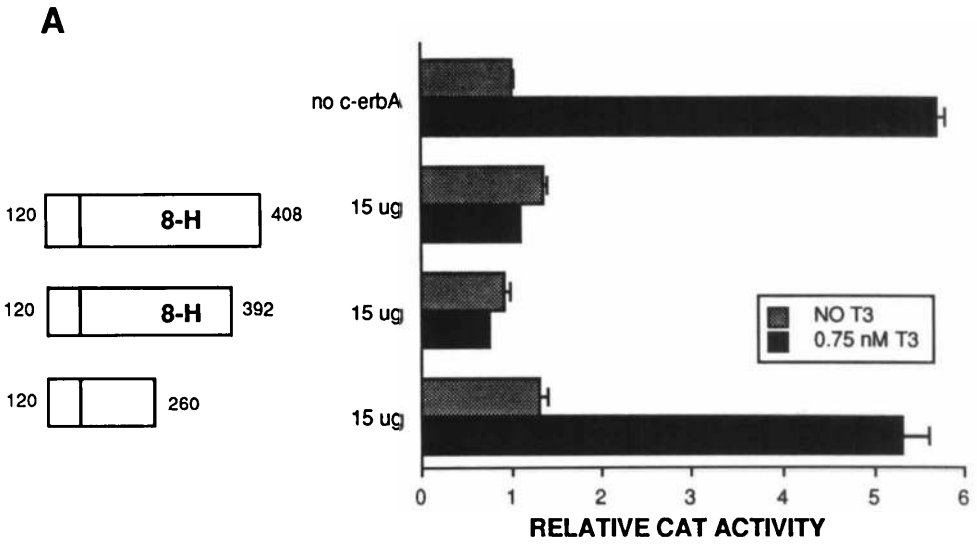
Figure 3 indicates that positions 1, 5, and 8 of the heptad repeats are conserved among subtypes of the T_3R and RAR (34–38). This encouraged us to examine the possibility that dominant-negative c-erbA mutants might also inhibit transcriptional activities of the RAR. Consistent with the findings of Umesono *et al.* (56), we found that T_3REs located between −208/−178 and −178/−163 of the rGH gene can also function as RA response elements (RRE) (Fig. 9 and unpublished observations). T_3 (0.75 nM) or RA (1 μM) (57, 58) resulted in a 4-fold stimulation of CAT when GH_1 cells were transfected with a −208/+11 rGH-CAT (Fig. 9, −208 rGH-CAT). Like T_3, RA stimulation required the T_3RE/RRE (data not shown). Thus, in agreement with Bedo *et al.* (59) and Morita *et al.* (60), we find that 1 μM RA activates RAR in GH cells. Cotransfection with 5 μg dominant-negative mutant 120^{met}–408^{val} had no effect on RA stimulation (data not shown), but as in Fig. 5, completely blocked stimulation by T_3. However, cotransfection of 50 μg of this mutant completely blocked stimulation by both T_3 and RA (Fig. 9, −208 rGH-CAT + 120–408). To assess the specificity of this inhibition we examined the effect of the dominant-negative mutant on T_3/RA-independent stimulation of the rGH promoter. Forskolin, an activator of adenylate cyclase, has been shown to stimulate rGH promoter sequences (61) that lack a T_3RE/RRE (−104/+11) (62). Although the dominant-negative mutant completely inhibits T_3 and RA activation it minimally affected forskolin stimulation (Fig. 9), indicating that the dominant-negative mutant selectively blocks activation by the T_3R and RAR.

DISCUSSION

What Mechanism Accounts for the Dominant-Negative and Dominant-Positive Phenotypes?

We have demonstrated that the wild-type chick c-erbA possesses a positive constitutive activity that overrides the hormone-dependent activity of the T_3R in rat GH_4C_1 cells. Conversely, DBD^- mutants that are deficient in *trans*-activation, inhibit the transcriptional activities of both the endogenous rat receptor and chick c-erbA. Both the dominant-positive and the dominant-negative activities map to the heptad repeat region within the E domain of chick c-erbA. We suggest two models which can account for these findings: a dimerization model and a transcription complex model.

In the dimerization model, the heptad repeats would mediate the association of two (or more) receptor molecules *in vivo*. DBD^- mutants containing intact heptad repeats could form complexes with the wild-type receptor. When expressed in excess, the mutants would drive the wild-type receptor into complexes with the



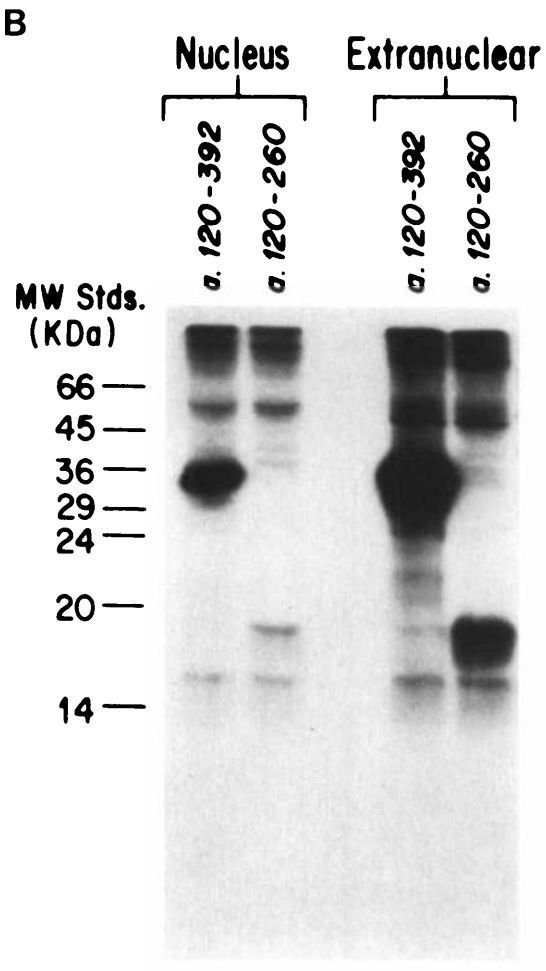


Fig. 8. Dominant-Negative Activity Requires the Heptad Repeats

A, GH_4C_1 cells were transfected without c-erbA (no c-erbA) or with 15 μg indicated DBD^- mutants. −3000/+75 rPRL-CAT (15 μg) was used as the reporter. The cells were cultured and assayed as in Fig. 5. 8-H represents the eight heptad repeats that are present in these mutants (Fig. 3). This region is absent in mutant 120^{met}–260^{arg}. B, The cells in A were labeled with L-[^{35}S]methionine and the nuclear and extranuclear fractions immune precipitated with Ab 28. The immune precipitates were analyzed in 15% SDS-polyacrylamide gels and fluorographed for 40 h. The mutants appear as single bands of the predicted size: 120^{met}–392^{thr} (31 kDa, 12 methionines) and 120^{met}–260^{arg} (16 kDa, 7 methionines). Molecular weight markers (MW Stds.) are shown on the *left*.

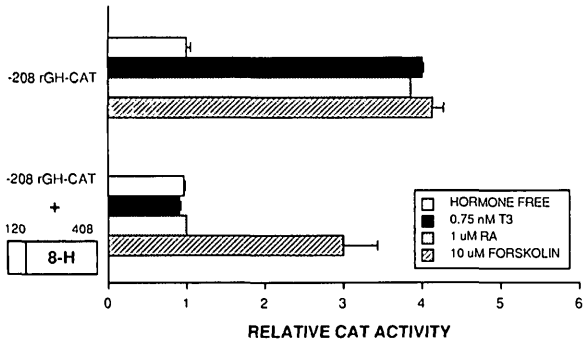


Fig. 9. Dominant-Negative Mutants Inhibit Responses to Thyroid Hormone and RA

GH_1 cells were transfected with 20 μg −208/+11 rGH-CAT alone or with 50 μg 120^{met}–408^{val} expression vector. The total amount of RSV promoter sequences were maintained constant by addition of the expression vector lacking all cDNA sequences (pRSVi⁻). The cells were placed into hormone-depleted medium with or without the indicated concentrations of hormones. After 48 h CAT activity was assayed and the activity of −208/+11 rGH-CAT in hormone-free medium was defined as a relative CAT activity of 1. The remaining CAT activities were normalized to this value. The 120^{met}–408^{val} mutant contains all eight heptad repeats (8-H, Fig. 1A and Fig. 3), but lacks the DNA binding domain.

inactive mutant. This model predicts that the dominant-negative mutants are by themselves inactive. Their inhibitory activity arises when they interact with wild-type receptor to form inactive receptor-mutant complexes. The dominant-positive activity of chick c-erbA has only been observed in cells which express rat receptors [GH_4C_1 and GH_1 (18)]; this activity is not seen in receptor deficient 235-1 [Fig. 7 (18)], HeLa or COS cells (unpublished observations). According to the dimerization model, chick-rat hetero-dimers would assume a conformation that does not require hormone for activity. Consistent with this possibility, Brent *et al.* (55) have shown that the rat c-erbA-β-1 requires hormone for activity in cells expressing endogenous rat receptor (GH_4C_1).

Our findings could also be accounted for by mechanisms in which the heptad repeats represent a Trans-Activation Domain (TAD). We refer to this as the transcription complex model. According to this model, hormone binding results in a conformation change that exposes the TAD which can then interact with and stimulate the transcription machinery of the basal promoter. The DBD^- mutants contain the TAD, associate with the transcription machinery, but fail to alter promoter activity because they lack the DNA binding domain. Dominant inhibition occurs when these mutants compete with the wild-type receptor for binding and subsequent activation of transcription complexes. It is unclear how this model could account for the constitutive activity of the wild-type chick c-erbA. Both models predict that the DBD^- mutants are by themselves inactive. Inhibitory activity can arise by blocking formation of wild-type dimers (dimerization model) or by interference with *trans*-activation (transcriptional complex model). Thus, the two models imply that the DBD^- mutants contain a domain that interacts with one of two distinct classes of proteins: the receptors themselves or promoter-specific transcription complexes. Our data cannot functionally distinguish between the above models.

The region necessary for the dominant-positive and dominant-negative effect (extending from amino acids 278–372) contains eight heptad repeats which are structurally related to known dimerization domains (19). The amino acids at positions 1, 5, and 8 of these repeats are well conserved between the T_3R and RAR and positions 1 and 8 contain one of the following hydrophobic amino acids: leucine, methionine, isoleucine, valine, or phenylalanine. The conserved residues at the fifth position are hydrophobic in four of the eight heptads. The remaining four heptads contain charged residues at the fifth position. Should this structure form a helix, the residues at the 1, 5, and 8 positions would form a hydrophobic surface along one face of the helix. Secondary structure predictions (Fig. 3 legend) are consistent with the formation of helices in these regions.

The heptad regions extend over 95 amino acids (278–372) and have a net charge of −1. Thus, this domain is not strikingly reminiscent of the acidic TADs that have been described in yeast (63, 64) and the herpes simplex viral protein VP16 (65). The SP1 transcription factor contains another class of TADs which is characterized by long tracts of multiple glutamine residues (66). Similar tracts cannot be identified in the heptad repeats of chick c-erbA. Thus, the region necessary for both the dominant-positive and dominant-negative effects bears strong structural resemblance to known dimerization domains. This does not rule out the possibility, however, that this region functions *in vivo* as a transcriptional activation domain.

Previous studies suggest that the T_3R exists as a monomer in solution (67). Specifically, receptor prepared from 0.4 M KCl nuclear extracts sediments as a 3.8S particle, has a stokes radius of 3.3 nm, a density of 1.36 g/cm^3, and a calculated M_r of 54 kDa. When nuclei are treated with micrococcal nuclease, the receptor is extracted as a 6.8S particle with a stokes radius of 6.0 nm, a density of 1.42 g/cm^3, and a M_r of 149 kDa. The 3.8S particle appears to represent a receptor monomer while the 6.8S particle was calculated to contain a 35–40 base pair (bp) DNA fragment with a total protein mass of 127 kDa. This was proposed to represent the receptor bound to itself (dimer) or another protein (67). If the 6.8S particle does represent a receptor dimer (homo- or hetero-dimer), then the dimer is not stable in solution unless it is bound to DNA. In contrast, GCN-4, c-*jun*/c-*fos*, C/EBP and c-*myc* can form stable dimers in the absence of DNA (20, 22, 23, 25, 31). The leucine zipper motifs in these proteins are close to the ideal zipper in that they contain leucine repeats at 7 amino acid intervals (positions 1 and 8 of the heptad). Mutagenesis studies indicate that substitutions of leucines with other residues (*e.g.* isoleucine, valine, ala-

nine, methionine, phenylalanine, or arginine) results in less stable dimer complexes (22, 25, 28–30, 32).

Since the leucine-zipper-like domain of the thyroid hormone receptor contains some nonleucine residues, it is likely that a T_3R dimer would be less stable and may only be detected *in vitro* when DNA binding maintains the dimer pairs in close proximity. In principle, it should be possible to directly test whether the wild-type c-erbA forms complexes with DBD^- mutants. These experiments are complicated by the likelihood that dimerization may only be detectable *in vitro* when both members of the dimer pair are bound to DNA. This is not possible with the DBD^- mutants since they lack the zinc-finger motif. Therefore, to detect putative wild-type/DBD^- hetero-dimers in solution it may be necessary to use concentrated solutions of purified proteins. Thus, these experiments may prove to be technically difficult until large quantities of the mutant and wild-type protein can be obtained.

The DNA binding domains of the glucocorticoid and estrogen receptors are thought to contain a dimerization activity (68, 69). It is not known whether the DNA binding domains of the T_3R or RAR possess similar activities. However, the heptad repeats act independently of the DNA binding domain and thus represents a distinct activity. Other investigators have found that the A/B, D, and E domains of the estrogen receptor contain sequences that are important for transcriptional interferences (70, 71). A similar phenomenon (squelching) has been described for yeast GAL4 protein (72). The regions involved in these activities do not appear to be structurally related to the heptad repeats that we describe.

Interactions among erbA Proteins

Multiple c-erbA subtypes including α-1, α-2, β-1, and β-2 have been cloned from rat GH cells (17, 73, 74). These cells have been shown by photoaffinity labeling to contain several molecular weight forms of the thyroid hormone receptor (75). Based on their molecular weights, these forms most likely represent hormone-binding subtypes of rat c-erbA (α-1, β-1, and β-2). The ability of our dominant-negative mutants to inhibit all receptor activity in GH cells suggests that interactions occur between members of the α- and β-subtypes of c-erbA.

A nonhormone binding form of c-erbA, c-erbA-α-2 (14, 16, 76) has been shown to inhibit the T_3 responsiveness of coexpressed rat receptors (74) even though it contains a functional DNA binding domain (73). Therefore, c-erbA-α-2 has the properties of our dominant-negative mutants. Preliminary studies indicate that DBD^- mutants of rat c-erbA-α-2 can act as dominant-negatives (Fitzpatrick, T., B. M. Forman, H. H. Samuels, unpublished observations), suggesting that inhibition by the wild-type α-2 protein may occur via hetero-dimer formation. The α-2 form arises from a splicing event within the eighth heptad. As a result, the α-2 protein contains a glutamic acid and valine at positions 5 and 8 of the last heptad, whereas the α-1 contains a valine (V) and leucine (L) at the fifth and eighth positions, respectively. Thus, the eighth heptad of the α-2 subtype may remain functional though the substitutions at the fifth and eighth positions are likely to result in a less stable dimer.

Interestingly, the 1, 5, and 8 positions of all eight heptads are conserved between v-erbA and its cellular homologue, chick c-erbA. Damm *et al.* (77) have recently shown that v-erbA possesses a dominant-negative phenotype. A mutant with a disrupted DNA binding domain lost the dominant-negative activity, thus the authors concluded that the dominant-negative activity of v-erbA resulted from its ability to compete with c-erbA for binding to a shared response element. However, the *in vivo* accumulation and cellular localization of this mutant was not examined and it remains possible that inactivity reflects poor accumulation of this mutant. Sap *et al.* (78) have also shown that v-erbA exhibits dominant-negative activity. However, these authors demonstrate that v-erbA does not bind T_3REs with high affinity, suggesting that DNA binding is not crucial for dominant-negative activity. Since the activity of v-erbA mutants which lacked the heptad repeats were not examined, it remains possible that this region contributes to the dominant-negative activity of v-erbA.

Implications for the Generalized Thyroid Hormone Resistance Syndrome

The generalized thyroid hormone resistance syndrome is a dominantly inherited disease that is characterized by a partial resistance to circulating levels of thyroid hormone. This phenotype is closely linked to a restriction fragment length polymorphism near a single c-erbA-β allele (79). Although this implicates a receptor defect in this disease it does not explain why this mutation should act in a dominant fashion to override the activities of the wild-type c-erbA-α and the unaffected c-erbA-β locus. The possibility that thyroid hormone receptors act as dimers suggests a mechanism whereby dominant-negative activity could arise. In principle, a mutation which maintains the activity of the heptad repeats but which disrupts DNA binding, ligand binding, or *trans*-activation could result in the formation of inactive mutant/wild-type hetero-dimers. Sequence and functional analysis of mutant cDNAs from affected individuals will facilitate an understanding of the mechanisms underlying this disease.

The Biological Significance of the Heptad Repeats

The leucine zippers in GCN-4, c-*jun*/c-*fos*, and C/EBP lie immediately adjacent to the DNA-binding motif and are thought to juxtapose the DNA binding domains from each member of the dimer pair to create the active DNA binding entity (22). The heptad repeats of c-erbA/RAR do not lie adjacent to the DNA binding domain (Fig. 1A), suggesting that the heptad domain of c-erbA does not serve to juxtapose DNA binding domains and

may not be essential for DNA binding or *trans*-activation. Since our data raise the possibility that T_3Rs interact with themselves and with the RAR it is possible that the heptad repeats function to create hetero-dimers between distinct but related receptors. Such receptor hetero-dimers could greatly increase the spectrum of cellular responses to extracellular signals and thus dimerization could serve a modulatory function. In GH_1 cells, RA and thyroid hormones have recently been shown to synergistically activate transcription of rGH-CAT constructs (59). Although many interpretations are possible, this synergistic effect could result from formation of highly active T_3R-RAR hetero-dimers.

The residues in the 1, 5, and 8 positions of the c-erbA heptads are conserved between the T_3R and RAR, but not with receptors for the adrenal and sex steroids. Surprisingly, the 1, 5, and 8 positions are also conserved with the 1,25-dihydroxyvitamin D_3 receptor (VDR) (80) and reverse-erbA-α (15). Interestingly the T_3R, RAR, VDR, and reverse-erbA-α have similar DNA-binding domains. In particular, they share the same three amino acids that are responsible for recognition of specific hormone responsive DNA elements (81, 82). Our data indicate that a second functional domain is well conserved between this subfamily of nuclear hormone receptors, suggesting that these receptors may interact at both the DNA and protein levels to regulate an overlapping network of genes.

The thyroid hormones, RA, and vitamin D_3 are important modulators of growth, development, and differentiation. Regardless of their mechanisms of action, we have demonstrated that at appropriate concentrations, the dominant-negative c-erbA mutants can selectively block T_3 receptor function. In principle, analogous mutants could be designed to block vitamin D_3 and RA responsiveness. Therefore, targeted expression of dominant-negative mutants to selected tissues of transgenic animals may provide a useful tool to examine hormonal responses during development.

MATERIALS AND METHODS

Cell Culture and Electroporation

GH_4C_1 and 235–1 cells were maintained in Dulbecco's Modified Eagle's Medium (GIBCO, Grand Island, NY) containing 15 mM HEPES, 0.1 mg/ml pyruvate, 50 μg/ml streptomycin sulfate, 50 μg/ml penicillin sulfate (DHAP medium), and 5% fetal calf serum (GIBCO). GH_1 cells were cultured in Ham's F-10 with 0.12 mg/ml glucose, 40 μg/ml penicillin, 12.5% horse serum, and 2.5% fetal calf serum. The cells were refed 1 day before electroporation. For electroporation the cells were washed once with versene (0.7 mM mM EDTA, 2.7 mM KCl, 137 mM NaCl, 6.3 mM sodium phosphate, and 1.5 mM potassium phosphate, pH 7.4), harvested with versene (GH_4C_1, GH_1) or trypsin-versene (235–1 cells) and washed again with DHAP containing 10% resin-charcoal stripped calf serum (RC-CS). RC treatment effectively depletes serum of T_3, T_4, RA, and steroid hormones. The cells were maintained at 5–10 million cells/ml DHAP-10% RC-CS. Immediately before electroporation 50–100 million cells were centrifuged and the pellet placed into 0.4 ml electroporation buffer (PBS with 0.91 mg/ml glucose) containing the appropriate plasmids. The cells were placed on ice for 1 min in a 0.4 cm electroporation cuvette (Bio-Rad, Richmond, CA) and electroporated by discharging an Isco 494 power supply set at 1600–2000 V and 0.9 mA. The cells were allowed to recover on ice for 10 min before addition of 1.6 ml electroporation buffer containing 10% RC-CS. The cells were then added to DHAP-10% RC-CS containing the appropriate concentration of hormones. T_3 (0.75 nM) is sufficient for maximal stimulation of GH transcription but has little effect on the growth rate of GH_4C_1 cells and the GH_1 variant used in these studies. T_3 (5 nM) was used for experiments involving 235–1 cells and has no effect on their growth. Dim light was used for experiments involving RA.

The cells were refed 24 h after electroporation and maintained for another 24 h (48 h total) before preparation for whole cell labeling or CAT assays. For transfections with rPRL-CAT, cells were refed with phenol red free medium. Although different amounts of the Rous Sarcoma virus (pRSV) (18) expression vectors were used in these experiments, the total amount of RSV promoter sequences were kept constant by addition of a control RSV expression vector (pRSVi$^-$) which lacks c-erbA cDNA sequences. We find that transfection efficiencies vary by less than 10% when performed on the same day. Therefore, to avoid differences in transfection efficiencies we have performed all experiments in each figure on the same day and have repeated the experiments multiple times with similar results. CAT assays were performed as described (83). The data is shown as the range of duplicate samples from experiments performed on the same day.

Whole Cell Labeling and Immune Precipitation

Whole cell labeling was performed 48 h after electroporation. Cells that were incubated in hormone-depleted medium were used for labeling since T_3 treatment was found to have no effect on the nuclear localization of the wild-type or mutant c-erbA proteins. Cells were washed once with Ham's F-10/met$^-$leu$^-$ (methionine and leucine free) and then incubated for 10 min at 37 C in Ham's F-10/met$^-$leu$^-$. The cells were incubated for 2 h at 37 C with the same medium supplemented with 10% dialyzed RC-CS, 13 μg/ml L-leucine, and 0.19 μM (200 μCi/ml) L-[^{35}S]methionine (New England Nuclear, Boston, MA, NEG-009T). After 2 h the cells were placed on ice, washed 3 times with saline, and lysed in STM-Triton (250 mM sucrose, 25 mM Tris, pH 7.8, 1.1 mM $MgCl_2$, 0.2% Triton X-100) containing 1 μg/ml Aprotinin. The lysate was centrifuged at 1600 $\times$ *g* and detergent was added to the supernatant (extranuclear fraction) to a final concentration of 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100. The pellet was washed 3 times with STM-Triton, and the nuclear fraction was prepared by a 10-min extraction with RIPA buffer (25 mM Tris, pH 7.5, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100) containing 100 mM NaCl and 1 μg/ml Aprotinin.

Trichloroacetic acid precipitable counts were determined on the nuclear and extranuclear fraction. For immune precipitation, approximately half of the nuclear fraction (10^7 cpm) and half of the extranuclear fraction (5×10^7 cpm) were immune precipitated in 350 μl RIPA buffer containing 1–2 μl antiserum and 5 mg Protein A-Sepharose (8 μg Protein A/mg beads, Sigma P-3391). Preabsorbed serum was prepared by incubating 10 μg appropriate protein fragment with 50 μL antiserum for 1 h at 4 C. Immune precipitations were incubated at 4 C for 3 h. The Protein A-Sepharose beads were washed twice with RIPA buffer-400 mM NaCl and once with RIPA buffer-100 mM NaCl. The samples were then prepared for SDS polyacrylamide gel electrophoresis. The gels were fixed in 10% acetic acid/10% methanol and fluorographed with Autofluor (National Diagnostics). ^{14}C labeled molecular weight standards (Sigma M4399) were as follows: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde 3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; β-casein, 24 kDa; trypsin inhibitor, 20 kDa, and α-lactalbumin, 14 kDa.

Plasmid Constructions

The reporter plasmids −236/−178:−104/+11 rGH-CAT, −104/+11 rGH-CAT, −208/+11 rGH-CAT and −3000 (*Xba*I)/+75 (*Xho*I) rPRL-CAT were previously described (18, 54). The −338/+75 rPRL-CAT was constructed by ligating a *Hin*dIII(−338) −XhoI(+75) fragment of the rat (Sprague Dawley) PRL gene into the *Hin*dIII-*Xho*I site of pBLCAT2 (83). This plasmid lacks all viral thymidine kinase sequences and expresses CAT under control of the rPRL promoter. The chick c-erbA expression vector was derived as follows. pSP71 (Promega Biotec, Madison, WI) was cleaved with *Eco*RV and blunt-end ligated to a *Bam*HI linker (CGGATCCG, New England Biolabs, Beverly, MA, 1021). This plasmid, pSP71-Bam, was cleaved with *Bam*HI, and ligated to a 250 bp fragment containing a *Bgl*II restriction site, an Asp718 restriction site, translation termination codons in three reading frames, the SV40 polyadenylation signal and a *Bam*HI site (18). The resultant plasmid was designated pSP71-poly(A). A modified chick c-erbA cDNA (obtained from Bjorn Vennstrom, Karolinsk Institute, Stockholm) was cloned into the *Eco*RI site of pSP71-poly(A). This cDNA has an *Eco*RI site followed an ATG translation initiation codon contained within an *Nco*I site (GAATTCACC-ATG-G) (18). The resultant plasmid pSP71-chk-c-erbA-polyA lacks all 5′-untranslated sequences and encodes the wild-type chick c-erbA. The expression vector was derived by ligating the *Hin*dIII-*Bam*HI fragment of pSP71-chk-c-erbA-poly(A) into the *Hin*dIII-*Bam*HI site of the expression vector pRSVi$^-$ (18). This plasmid pRSVi$^-$/Asp718-chk-c-erbA contains the RSV promoter enhancer (3′-long terminal repeat) followed by the modified c-erbA cDNA, a *Bam*HI-*Bgl*II fusion, an Asp718 site, a three-frame translation termination codon and then the SV40 polyadenylation sequences. The sequence is as follows:

5′-GGATCT (*Bam*HI-*Bgl*II fusion) GGGTACCC (Asp718 linker) AGCTT (former *Hin*dIII site) GCTGATTGATTGACCG (termination codons) -SV40 poly(A) -GGATCC (*Bam*HI site) 3′.

Amino terminal deletion mutants extending to 120^{met} and 199^{met} were obtained by cleaving at *Nco*I sites in the chick c-erbA cDNA. The resulting fragments were cloned into the *Nco*I site surrounding the initiation codon of the pRSVi$^-$/Asp718 expression vector. Carboxy terminal deletion mutants were obtained by making 3′-deletions at natural restriction sites within the chick c-erbA: 392^{thr}-*Sac*I; 373^{arg}-*Fsp*I; 342^{thr}-*Rsa*I; 284^{gln}-*Pvu*II; 260^{arg}-*Bss*HII. Translation from these mutants terminates within the three-frame termination site described above. Thus, all the carboxy terminal mutants contain a small seven to nine amino acid tail that arises from codons that precede the three-frame stop codon. Carboxy terminal mutants are designated by the last amino acid that the mutant shares with the wild-type chick c-erbA.

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