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Characterization of the hormone-binding domain of the chicken c-*erbA*/thyroid hormone receptor protein

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To identify and characterize the hormone-binding domain of the thyroid hormone receptor, we analyzed the ligand-binding capacities of proteins representing chimeras between the normal receptor and P75$^{gag\text{-}v\text{-}erbA}$, the retrovirus-encoded form deficient in binding ligand. Our results show that several mutations present in the carboxy-terminal half of P75$^{gag\text{-}v\text{-}erbA}$ co-operate in abolishing hormone binding, and that the ligand-binding domain resides in a position analogous to that of steroid receptors. Furthermore, a point mutation that is located between the putative DNA and ligand-binding domains of P75$^{gag\text{-}v\text{-}erbA}$ and that renders it biologically inactive fails to affect hormone binding by the c-*erbA* protein. These results suggest that the mutation changed the ability of P75$^{gag\text{-}v\text{-}erbA}$ to affect transcription since it also had no effect on DNA binding. Our data also suggest that hormone-independent activity of P75$^{gag\text{-}v\text{-}erbA}$ provided a selective advantage to the avian erythroblastosis virus during the original selection for a highly oncogenic strain of the virus.

Key words: erbA oncogene/thyroid hormone receptor

Introduction

It was recently demonstrated (Sap *et al.*, 1986; Weinberger *et al.*, 1986) that the c-*erbA* proto-oncogene encodes a high-affinity receptor for the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4), and that its virally transduced homologue v-*erbA* is defective in binding its ligand (Sap *et al.*, 1986). A comparison between the cellular and viral genes revealed a number of mutations in v-*erbA* (see also Figure 1); part of the retroviral *gag* gene is fused to the *erbA*-specific sequence resulting in the synthesis of the P75$^{gag\text{-}v\text{-}erbA}$ hybrid protein lacking the first 12 amino-terminal amino acids found in the c-*erbA* protein. The *erbA*-specific part of P75$^{gag\text{-}v\text{-}erbA}$ also contains a 9-amino-acid-long deletion very close to the carboxy terminus and 13 amino acid substitutions distributed along the sequence. Two of these are located close to the amino-terminus, two in the putative DNA-binding domain and the remaining nine in the region corresponding to the ligand binding domain in steroid receptors.

v-*erbA* itself is non-oncogenic, but it enhances the transforming capacities of v-*erbB*, the second gene present in the genome of avian erythroblastosis virus (AEV) (Vennström and Bishop, 1982). Two effects of v-*erbA* in erythroblasts have been characterized: first, it arrests the residual differentiation exhibited by erythroblasts transformed by v-*erbB* alone, and second, it abolishes the strict culture conditions necessary for the *in vitro* propagation of these cells, allowing their growth in media with wide ranges in pH and salt concentration (Frykberg *et al.*, 1983; Kahn *et al.*, 1986; Damm *et al.*, 1987). Moreover, v-*erbA* promotes growth of fibroblasts by lowering serum requirements (Gandrillon *et al.*, 1987), and can complement v-*erbB* genes in fibroblast transformation both *in vitro* (Jansson *et al.*, 1987) and *in vivo* (Gandrillon *et al.*, 1987). Finally, the co-operativity between the two *erb* genes is not specific: v-*erbA* has been shown to co-operate in erythroblast transformation with various types of oncogenes that normally transform fibroblasts (Kahn *et al.*, 1986).

Recent results (Zenke *et al.*, 1988) indicate an inhibitory effect of v-*erbA* on the expression of the gene for band 3, the major anion transporter of the erythrocyte, as well as on the erythroid δ-aminolevulinic acid synthetase (ALA-S), a key enzyme in hemin biosynthesis. The normal thyroid hormone receptor has not been reported to affect the expression of these genes, although T_3 is known to increase the

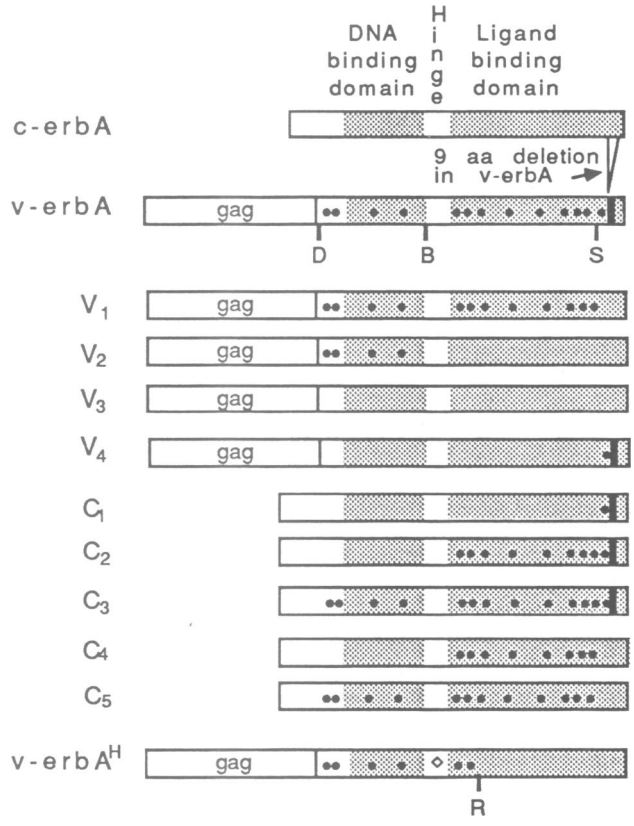

Fig. 1. Schematic representation of the structure of chicken c-*erbA*, v-*erbA* and their chimeras. The constructions were made using restriction endonuclease sites common to both genes. The deletion (I) and the point mutations (•) in v-*erbA* and the mutation (◇) at position 144 in v-*erbA*^{H} are indicated. B, *Bam*HI; D, *Dra*III; R, *Eco*RV; S, *Sac*I.

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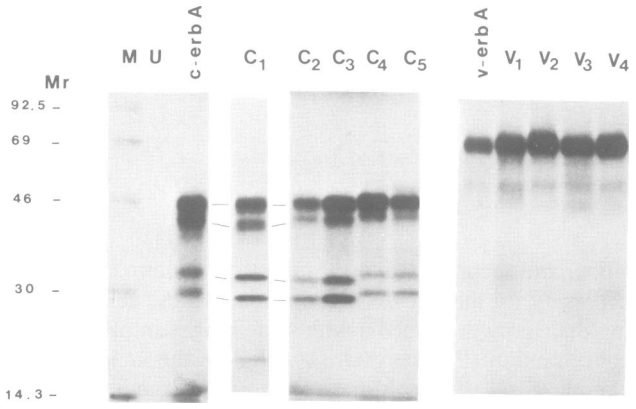

Fig. 2. *In vitro* synthesis of the chimeric *erbA* proteins. Plasmids encoding the genes were transcribed *in vitro* and the RNA translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Aliquots of each translation mixture were analyzed in 10% SDS–polyacrylamide gels. Marker proteins (M) and unprogrammed translation mixtures (U) were included as controls.

expression of another ion transporter, Na^+/K^+ATPase, and of the liver specific ALA-S (Lo and Edelman, 1976; Lo and Lo, 1980; Sassa *et al.*, 1979). The altered properties of the v-*erbA* protein consequently appear to suppress the expression of two genes not previously known to be regulated by the normal thyroid hormone receptor.

To identify the mutations in P75$^{gag\text{-}v\text{-}erbA}$ responsible for the defectiveness in hormone binding and, as a long-term goal, to understand better the differences in transcription regulation exerted by the cell- and virus-encoded *erbA* proteins, we tested the ligand binding properties of several hybrid v-/c-*erbA* proteins. The results show first that the ligand-binding domain of the thyroid hormone receptor is located in an analogous position as compared to steroid receptors. Second, the data demonstrate that several mutations in the domain homologous to the ligand-binding region of steroid receptors contribute to the defective ligand binding by P75$^{gag\text{-}v\text{-}erbA}$, suggesting that the hormone-independent action by the v-*erbA* protein provided a selective advantage to AEV during its evolution towards an acutely oncogenic retrovirus. Finally, analysis of the effects on the c-*erbA* protein of a point mutation previously found to affect the biological activity of P75$^{gag\text{-}v\text{-}erbA}$ (Damm *et al.*, 1987) showed that the lesion did not affect either hormone binding or binding of the protein to DNA, suggesting that the mutation affects transcriptional regulation by other, as yet undefined, mechanisms.

Results

Construction and expression of v-/c-erbA chimeric genes

Chimeric *erbA* genes (Figure 1) were constructed using restriction endonuclease cleavage sites common to both the viral and cellular genes as described in detail in Materials and methods. In the following, chimeric genes containing a 5′ end from v-*erbA*, i.e. *gag*, will be designated V_{1-4}, and those with 5′ sequences from c-*erbA* C_{1-5}. The corresponding proteins were subsequently synthesized in reticulocyte lysates, and, as determined by SDS–gel electrophoresis, all constructs encoded the expected [^{35}S]methionine-labeled hybrid proteins (Figure 2). The constructs containing a 5′ end from c-*erbA* gave rise to two main polypeptides of M_r ~46 000 and ~40 000 as shown previously for the c-*erbA* protein (Sap *et al.*, 1986), whereas

Table I. Thyroid hormone-binding acitivity of *erbA* proteins

Protein	Specific binding[a]	Dissociation constant[b] (nm)
c-*erbA*	18	0.35
v-*erbA*	1.5	NM[c]
V_1	8.7	8.6
V_2	18	0.39
V_3	16	0.41
V_4	5.7	9.2
C_1	4.5	5.6
C_2	1.7	NM
C_3	1.9	NM
C_4	3.6	6.5
C_5	3.9	4.8

[a]Ratio between the amount of [^{125}I]T_3 bound in the absence or presence of a 1000-fold molar excess cold T_3 as competitor.
[b]K_d values were obtained from Scatchard analyses.
[c]NM, not measurable.

those containing an N terminus of viral origin produced a major band of M_r ~75 000 due to the presence of the *gag* domain. Similar but distinct patterns of minor bands were detected in all sets of expressed proteins, according to the composition of the chimeric protein. These bands are probably the result of translations from internal initiators rather than proteolytic cleavage, since they appeared even when protease inhibitors (leupeptin, aprotinin and PMSF) were present during translation (not shown).

Hormone-binding activities of the recombinant receptor proteins

The capacities of the recombinant proteins to bind thyroid hormones were first assessed by means of a filter binding assay (Inoue *et al.*, 1983; Sap *et al.*, 1986). The same amounts of *in vitro* produced protein, as determined by TCA precipitation, were tested for their capacity to bind hormone by incubation with 1 nM [^{125}I]T_3 in the presence or absence of a 1000-fold molar excess of unlabeled hormone. As shown in Table I, the chimeric proteins containing the carboxy-terminal half of P75$^{gag\text{-}v\text{-}erbA}$ were, like the viral protein itself, unable to bind hormone. Reconstitution of the ultimate carboxy terminus of P75$^{gag\text{-}v\text{-}erbA}$ with that of the cellular protein as in construct V_1 increased binding, but the carboxy-terminal half of the c-*erbA* protein was required to restore normal levels of binding activity, as shown with the constructs V_2 and V_3 (Table I).

To determine accurately the effect of the mutations on hormone binding we performed Scatchard analyses with all the chimeric proteins. This type of analysis has the additional advantage of being independent of the amount of protein present in the lysates. Table I shows the dissociation constants obtained for the complete series of chimeric proteins. In accord with our previous data, the K_d for the c-*erbA* protein was 0.35 nM, and the viral P75$^{gag\text{-}v\text{-}erbA}$ protein exhibited no detectable binding activity. A similar lack of activity was exhibited by the chimeric proteins C_2 and C_3, the carboxy-terminal halves of which were derived from P75$^{gag\text{-}v\text{-}erbA}$. However, replacement of the ultimate C-terminal region of P75$^{gag\text{-}v\text{-}erbA}$ containing the 9-amino-acid-long deletion with the corresponding region of the c-*erbA* protein yielded a hybrid protein (C_1) with a lower but appreciable dissociation constant (5.6 nM), as shown in Figure 3 and Table I. A similar K_d (6.5 nM) was determined for construct C_4

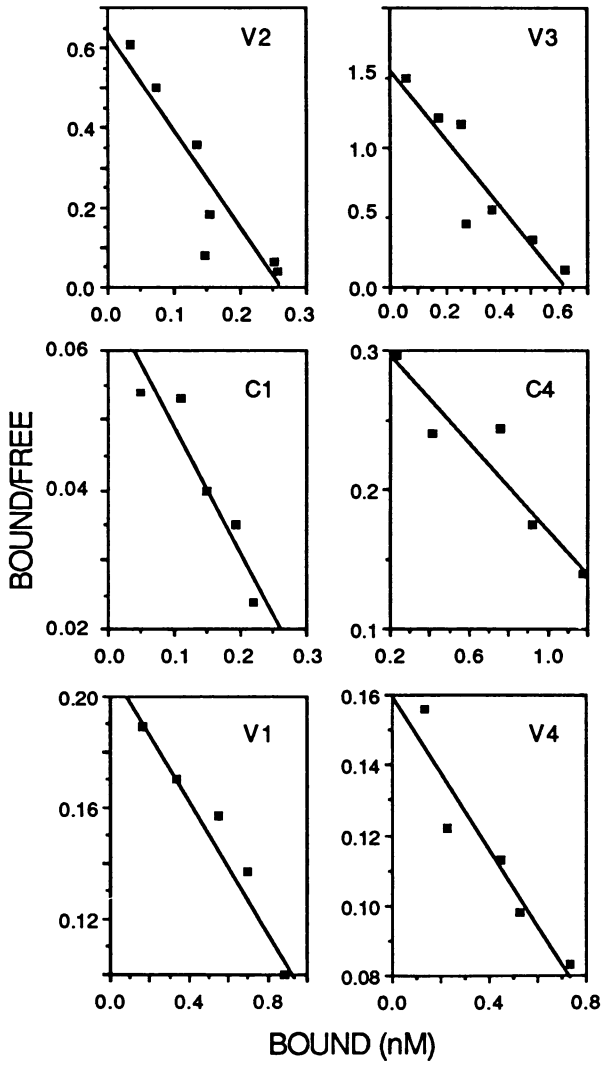

Fig. 3. Scatchard analyses of [^{125}I]T_3 binding to *erbA* proteins synthesized *in vitro*. Aliquots of the lysates were incubated overnight at 0–4°C with different concentrations of [^{125}I]T_3 (3000 mCi/mg). Radiolabeled hormone was dried under vacuum to remove ethanol. Note the different scales.

(Figure 3 and Table I), in which the internal region of the ligand-binding domain of c-*erbA* was replaced by the corresponding sequence of P75$^{gag\text{-}v\text{-}erbA}$ containing eight amino acid substitutions (Figure 1).

Concordant results were obtained for the corresponding *gag–erbA* constructs (Table I). Replacement of almost the entire viral *erbA* component of P75$^{gag\text{-}v\text{-}erbA}$ by the homologous cellular sequence (V_3) or by only the ligand-binding domain (V_2) resulted in fully active receptors with dissociation constants of 0.41 and 0.39 nM respectively. Finally, the presence in P75$^{gag\text{-}v\text{-}erbA}$ of either the extreme carboxy-terminal sequence (V_1, see Figure 1) or the adjacent ligand-binding region of c-*erbA* (V_4) conferred an intermediate affinity for the ligand binding, with K_d values of 8.6–9.2 nM (Table I, Figure 3). Taken together, the results suggest that both the point mutations and the deletion found in the C-terminal half of P75$^{gag\text{-}v\text{-}erbA}$ contribute to its lack of hormone binding.

Characterization of other domains in erbA

A biologically inactive mutant of v-*erbA*, denoted v-*erbA*^{td359}, was recently described (Damm *et al.*, 1987). The mutant protein has a single amino acid replacement which is responsible for the defectiveness. The lesion, a Pro to Arg change, is located (in the 'hinge' region) between the DNA and hormone-binding regions at amino acid position 144. To test if this amino acid replacement would affect hormone binding, a fragment of the v-*erbA*^{td359} gene, containing the lesion at position 144 as well as the upstream and two of the downstream v-*erbA*-specific amino acid replacements, was joined to a c-*erbA* fragment encoding the ligand-binding domain to yield the v-*erbA*^{H} construct (see Figure 1). The results of the subsequent T_3-binding studies and Scatchard analyses using *in vitro* synthesized protein showed that the mutant v-*erbA*^{H} protein bound T_3 with a K_d identical to that of the c-*erbA* and V_2 proteins (Table II), suggesting that neither the mutation at position 144, nor the next two downstream amino acid replacements contributed by v-*erbA*, affect hormone binding.

Table II. Effect of the specific *td*359 mutation on thyroid hormone binding

Protein	Specific binding[a]		Dissociation constant[b] (nM)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
V_2	7.3	7.4	0.40	0.40
v-*erbA*^{H}	3.0	4.3	0.41	0.48
v-*erbA*^{td359}	1.2	2.6	NM[c]	NM
v-*erbA*^{wt}	1.2	1.2	NM	NM

[a,b,c] as in Table I.

To test if the lesion in the *td*359 protein instead affects DNA binding, nuclear extracts were made from erythroblasts transformed by either *wt* or *td*359 AEV, and were chromatographed on DNA cellulose columns. We chose to elute the proteins by stepwise elution with increasing concentrations of KCl, since pilot experiments had shown that the P75$^{gag\text{-}v\text{-}erbA}$ elutes in a broad peak betwen 0.15 and 0.35 M KCl (not shown). Eluted proteins were then immunoprecipitated with anti-*erbA* antiserum, and analyzed by SDS–PAGE. To identify the eluted 75-kd protein as P75$^{gag\text{-}v\text{-}erbA}$, the immobilized immune complexes were treated with the viral protease P15, yielding the two specific fragments F45$^{v\text{-}erbA}$ and F30^{gag} (Figure 4A). Figure 4B and C shows clearly that the *wt* and *td*359 v-*erbA* proteins dissociated from the DNA at similar concentrations of salt, suggesting that their affinities for at least non-specific DNA sequences are similar, and that the mutation at position 144 affects other functions of the *erbA* protein than DNA and ligand binding. In addition, control experiments employing normal cellulose instead of DNA cellulose showed that P75$^{gag\text{-}v\text{-}erbA}$ does not bind to the former matrix (data not shown).

Discussion

The P75$^{gag\text{-}v\text{-}erbA}$ protein contains several mutations and is defective in binding ligand, but nevertheless represents a stable protein with distinct biological activities. This allowed us to use a novel approach for identifying the T_3-binding domain in the thyroid hormone receptor. By making chimeric v-/c-*erbA* genes we could avoid introduction of *in vitro* generated mutations into c-*erbA* that potentially could lead to a destabilization or inactivation of the resulting mutant protein.

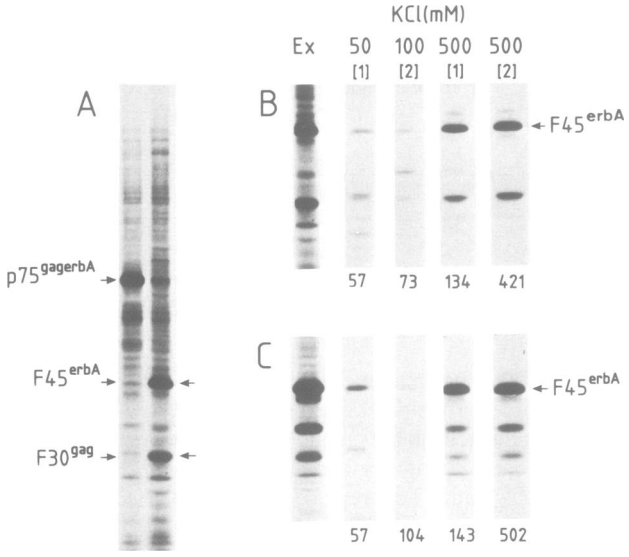

Fig. 4. The v-*erbA* proteins of *wt*AEV and *td*359AEV bind to DNA (**A**) Nuclear extract from *wt*AEV-transformed erythroblasts after immunoprecipitation with anti-*erbA* serum before (**left lane**) and after (**right lane**) cleavage of the immunoprecipitated *erbA* protein ($P75^{gag\text{-}v\text{-}erbA}$) into $F45^{erbA}$ and $F30^{gag}$. Nuclear extracts from *wt*AEV (**B**) and $erbA^{td359}$ $erbB^{wt}$ (**C**) erythoblasts were diluted and applied to a DNA cellulose column. Bound proteins eluted in a stepwise fashion with 50, 100 and 500 mM KCl as described in Materials and methods. Shown are the immunoprecipitated and p15-cleaved erbA proteins obtained from the extract (Ex), first 50 mM KCl eluate (50,[1]), second 100 mM KCl eluate (100,[2]) and first and second 500 mM KCl eluates (500,[1]; 500,[2]). Small numerals below the lanes indicate the actual salt concentration present in the eluate as determined by conductivity measurements.

The testing of the chimeric v-/c-*erbA* proteins identified a large C-terminal region required for hormone binding. Although our experiments do not precisely define the upstream end of the ligand-binding domain, they lead to several conclusions. First, they demonstrate that the viral *gag* region or the mutations present in the DNA-binding region of $P75^{gag\text{-}v\text{-}erbA}$ do not affect the affinity for the ligand. Secondly, both the structure of the ultimate C terminus and at most seven of the nine C-terminal amino acid replacements (of which three can be considered conservative) in $P75^{gag\text{-}v\text{-}erbA}$ contributed to the complete loss of affinity for T_3. This suggests that the overall conformation of this whole domain is essential for binding thyroid hormone. Finally, a point mutation located close to the putative DNA-binding region and known to abolish the biological activity of $P75^{gag\text{-}v\text{-}erbA}$ had no effect on hormone binding or subcellular localization, suggesting that the 'hinge' region is not involved in association with ligand but perhaps in the capacity of $P75^{gag\text{-}v\text{-}erbA}$ to function as a transcriptional regulator. Mutations in the same region of steroid receptors can also abolish biological activity possibly by affecting nuclear transport (Kumar *et al.*, 1986; Giguère *et al.*, 1986); however, the mutation at position 144 in the $v\text{-}erbA^{td359}$ protein is located outside the region which in steroid receptors confers nuclear transport (Picard and Yamamoto, 1987).

The fact that several mutations, present in the ultimate C terminus as well as in internal regions, have accumulated in $P75^{gag\text{-}v\text{-}erbA}$ and co-operate in abolishing hormone binding suggests that hormone-independent action by v-*erbA* provided a selective advantage to AEV during its selection as a highly and acutely oncogenic strain of virus (Rothe-Meyer *et al.*, 1933; for a recent review, see Beug *et al.*, 1986).

The homology between *erbA* and receptor genes for steroid hormones essentially covers two regions (Weinberger *et al.*, 1985; Conneely *et al.*, 1986; Green *et al.*, 1986; Krust *et al.*, 1986). The first is the cysteine-rich DNA-binding region, which is separated by a poorly conserved sequence (the 'hinge' region) from the second homologous region, which has been shown to mediate ligand binding (Giguère *et al.*, 1986; Kumar *et al.*, 1986; Godowsky *et al.*, 1987). Our results demonstrate that the thyroid hormone receptor has its ligand-binding region in the second homologous region as has been described for steroid receptors, thus further emphasizing the functional homology between the two classes of receptors. However, the ligand-binding domain of the thyroid hormone receptor is distinct from those of both the glucocorticoid and estrogen receptors: the former steroid receptor is very sensitive to structural alterations in its C terminus, the latter is comparatively insensitive (Kumar *et al.*, 1986; Godowsky *et al.*, 1987), whereas the T_3 receptor appears intermediate in this respect.

Thyroid hormone receptors are known to either up- or down-regulate the expression of specific target genes as a response to binding of ligand (Ivarie *et al.*, 1981; Oppenheimer *et al.*, 1983). Recently, cDNAs for at least two distinct thyroid hormone receptors have been cloned (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Thompson *et al.*, 1987), confirming an earlier observation that the genomes of higher vertebrates contain at least two *erbA*-related genes (Jansson *et al.*, 1983). Their tissue-specific expression, target gene specificity and mode of action is still unclear. However, the recent demonstration (M.Zenke *et al.*, 1987) that *band 3* and *ALA-S* expression in erythroblasts is constitutively down-regulated by $P75^{gag\text{-}v\text{-}erbA}$ contrasts the effects of the normal thyroid hormone receptor, which has not been reported to affect the expression of these genes, and also not to confer a constitutive repression of transcription. It is therefore likely that the mutations in v-*erbA*, possibly in conjunction with the high level of expression, are responsible for the aberrant regulatory function of $P75^{gag\text{-}v\text{-}erbA}$ in erythroblasts. It seems probable that the ligand independence confers the constitutivity; the mutations in the ligand-binding domain, or elsewhere, and the addition of *gag* may all contribute to the repression of transcription. The availability of chimeric v-/c-*erbA* genes with known hormone-binding properties will make it possible to assess the influence of the various mutations on transcriptional regulation of target genes for $P75^{gag\text{-}v\text{-}erbA}$ activity, such as *band 3*.

Materials and methods

Construction of chimeric erbA genes

Chimeric v-/c-*erbA* genes were constructed in plasmids pTZ or pGEM by exchanging homologous fragments between a chicken c-*erbA* cDNA (clone pF1Δ, a derivative of pF1 lacking most of the 5′ untranslated sequences; Sap *et al.*, 1986) and the cloned *gag*-v-*erbA* gene (Vennström *et al.*, 1980; sequenced by Debuire *et al.*, 1984, and Damm *et al.*, 1987) utilizing restriction sites common to both genes.

Recombinant V_1 was constructed by replacing the sequences 3′ to the *Sac*I site in v-*erbA* with the corresponding fragment of c-*erbA*, thus removing the nine-amino-acid deletion and one-amino-acid substitution of v-*erbA* and reintroducing the proper C terminus. In recombinant V_2 the *Sac*I–*Bst*XI fragment of v-*erbA* (the sites are located immediately upstream of the initiating AUG, and at position 321 respectively) was joined to a *Bst*XI–*Apa*I fragment of c-*erbA*. V_3 contains a *Sac*I–*Dra*III fragment (the former site is located before *gag*, the latter is at nucleotide position 11 in v-*erbA*) join-

ed to the subsequent *Dra*III−*Apa*I fragment of c-*erbA* (*Apa*I site is located after the coding region). V_4 was made by replacing the C-terminal *Sac*I−*Apa*I fragment of V_3 with the corresponding fragment of c-*erbA*. The C series of chimeras were constructed by inserting into pF1Δ fragments of v-*erbA* using the same restriction sites as was used for the V series above. Finally, v-$erbA^H$ was made by recombining *gag*-v-*erbA* from *td*359AEV with c-*erbA* at a unique *Eco*RV site. The resulting chimeras are shown in Figure 1. Sites unique to either v- or c-*erbA* provided the means for unambiguous confirmation of the resulting constructs. Nucleotides in c-*erbA* are numbered according to Sap *et al.* (1986) and those in v-*erbA* according to Debuire *et al.* (1984).

In vitro transcription and translation

Plasmids containing the chimeric genes under the control of the phage T7 promoter were linearized with *Eco*RI or *Xba*I (v-*erbA*), extracted with phenol/chloroform and precipitated. Templates were transcribed with T7 RNA polymerase (100 U/ml, Biolabs) as described previously (Sap *et al.*, 1986). Transcripts were translated in micrococcal-nuclease-treated rabbit reticulocytes lysates (Amersham) in the presence of 1.2 mCi/ml [^{35}S]methionine under conditions suggested by the manufacturer. Proteins were analyzed by electrophoresis on 10% SDS−containing polyacrylamide gels followed by fluorography and autoradiography.

Hormone-binding assay

Binding and Scatchard analyses were done as previously described (Inoue *et al.*, 1983; Sap *et al.*, 1986).

Assay of P75$^{gag\text{-}v\text{-}erbA}$ binding to DNA cellulose

*wt*AEV erythroblasts (clone E3) or erythroblasts transformed with a recombinant v-$erbA^{td359}$/v-*erbB* virus (Damm *et al.*, 1987, clone B1) were labeled with [^{35}S]methionine at 0.5 mCi/ml for 2 h. Nuclei were prepared as described (Sap *et al.*, 1986) and ~5 × 10^7 nuclei extracted with 50 μl 0.3 M KCl, 10% glycerol in extraction buffer for 90 min at 4°C. After centrifugation, the supernatant was diluted to 50 mM KCl and applied to a DNA cellulose column (0.2 ml). The columns were stepwise eluted with: 50 mM KCl (2 × 200 μl), 100 mM KCl (2 × 200 μl) and 500 mM KCl (3 × 200 μl). After addition of concentrated RIPA buffer (Beug *et al.*, 1981) aliquots of the nuclear extract, the flow-through and the eluted fractions were immunoprecipitated with anti-*erbA* antiserum and analyzed by SDS−PAGE and fluorography as described previously. To identify unambiguously immunoprecipitated P75$^{gag\text{-}v\text{-}erbA}$, an aliquot of the washed immune complexes immobilized on Protein A containing *Staphylococcus aureus* was digested with the viral protease P15 to yield specific F45^{erbA} and F30^{gag} fragments (Beug *et al.*, 1981).

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