The lack of transcriptional activation of the v-erbA oncogene is in part due to a mutation present in the DNA binding domain of the protein

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Received April 10, 1990; Revised and Accepted July 5, 1990

ABSTRACT

Using a transient co-transfection system we have demonstrated that response elements for estrogen (ER), thyroid hormone (TR) and retinoic acid receptors (RAR) are closely related. Thyroid hormone-induced activation of transcription was observed in CV1 cells and not in HeLa cells, suggesting the existence of cell-specific transcription factors necessary for the response. By contrast to its cellular counterpart (c-erbA/cTRα) the oncogene protein gag v-erbA is unable to activate gene transcription from different response elements derived from the rat growth hormone (rGH) gene promoter. A chimeric construct consisting of the ER in which the DNA binding domain has been replaced by that of cTRα was able to stimulate the reporter gene. In contrast, a construct in which ER DNA binding domain has been replaced by that of gag v-erbA did not activate gene transcription. These results lead us to the conclusion that the mutated DNA binding domain of v-erbA is in part responsible for the lack of transcriptional activation and in repression of gene expression. This is due in large part to the Gly73—Ser mutation which corresponds to the position of one of the three discriminating amino acids that are thought to interact with a specific base of the response element.

INTRODUCTION

The avian erythroblastosis virus (AEV) carries two oncogenes v-erbA and v-erbB transduced from the respective c-erbA and c-erbB proto-oncogenes (1–4). AEV causes erythroleukaemia in chickens and transforms haematopoietic cells of the erythroid lineage in cell culture (5). The functions of the v-erbA and v-erbB gene products in transformation were investigated by comparing the oncogenic properties of both mutants and variants of AEV (6). The v-erbA gene is both necessary and sufficient for oncogenesis. In contrast the v-erbB gene, which is expressed as a p75 gag v-erbB fusion protein, is not capable of oncogenic transformation but possesses the ability to potentiate the transformation of erythroid progenitors by a variety of other oncogenes, including v-erbB (7). Furthermore, expression of the v-erbA oncogene in chicken embryo fibroblasts stimulates their proliferation in vitro and enhances tumor growth in vivo (8). The cellular counterpart of the v-erbA gene of the AEV (c-erbA) has been shown to correspond to a thyroid hormone receptor (TR)(9,10). A comparison of the amino acid sequences of the chicken (c) TRα and the oncogene protein revealed a number of differences in v-erbA (9); a 9 amino-acid deletion and 11 amino-acid substitutions in the ligand binding domain, and two amino acid substitutions in the DNA binding domain (9 and see Figure 7). The v-erbA protein does not bind thyroid hormone (T3)(9,11). By analyzing the ligand-binding capacities of proteins representing chimeras between the normal receptor and p75 gag v-erbA, it has been shown that several mutations present in the carboxy terminal half of the protein cooperate in abolishing hormone binding (11,12). V-erbA is, however, localised to the nucleus and appears to bind DNA in vitro (9,13). Thus it can be postulated that v-erbA acts on gene transcription by a mechanism similar to its cellular counterpart, i.e. by binding to specific DNA sequences and stimulating or repressing gene expression. In this context, it has been demonstrated recently (14,15) that v-erbA can act as a constitutive repressor and, when coexpressed with the TR blocks activation by T3 from chimeric genes containing thyroid hormone response elements (TREs). Up to now, TREs have not been fully characterized. One has been localised in the region –202 to –148 of the rat growth hormone (rGH) promoter (16). This natural TRE (5'-GAATATCA-GGGACGTTACCAGGAGAGG-3') has also been shown to be a functional retinoic acid response element (RARE) in CV1 cells (17). Interestingly, this response element contains the sequence TGACC present in the estrogen response element (ERE: GTTCANNNTGACO; this sequence represents a common motif for the estrogen receptor (ER), the TR and the RAR. In this paper, we have compared the transcriptional activation properties of gag v-erbA with that of several nuclear receptors. For this purpose, we performed preliminary experiments to identify cis-acting sequences specifically activated by the different

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nuclear receptors, by testing different palindromic sequences derived from the natural rGH TRE/RARE for their ability to activate CAT when cotransfected with the TR, RAR, ER and glucocorticoid receptor (GR) in the presence or absence of their respective ligands. The different response elements thus identified were subsequently used to characterize the inhibitory effect of v-erb-A on transcription. Finally, using chimeric receptors in which the DNA binding domain of the ER has been exchanged with that of cTRα and v-erbA we have investigated the functional consequences of the two amino acid changes found in the DNA binding domain of gag v-erbA.

**MATERIAL AND METHODS**

**Plasmid constructions**

All DNA constructions were performed using standard procedures. The cTRα expression vector (cTRα0) was constructed from the pFI vector (9) by insertion of the open reading frame at the EcoRI site of the eukaryotic expression vector pSG1 (18). The gag v-erbA expression vector was constructed by insertion of the SacI-Apal fragment of the AEV clone pcR1 (19) at the EcoRI site of pSG5 (20). For the construction of the chimeric receptors, DNA encoding region C was exchanged as a cassette by insertion of KpnI and Xhol sites at the 5' and 3' ends of this region respectively in the chicken c-erbA and v-erbA sequences. The c-erbA and v-erbA cassettes were then exchanged in HE28 (which is identical to the wild type HEO except for the presence of the KpnI and Xhol sites which do not affect the function of the expressed protein)(21) to create HEcTRα.CAS and HEv-erbA.CAS chimeric receptors respectively (Figure 4A). HEcTRα(S).CAS and HEcTRα(T).CAS (Figure 5A) were created by mutation of HEcTRα.CAS using the oligonucleotides 5'-GAGGGCTGCACTTCCCTGAGGATC-3' and 5'-ACCCCTTGACTGAGGATC-3' respectively. The reporter gene rGH-tk-CAT was constructed by inserting the upstream sequence of the rGH promoter (−311 to −45) at the XbaI site of the pBLCAT8+ (22 and Fig. 2A). The rGH-tk-Globin plasmid was constructed by replacing the vitellogenin gene promoter sequence of the vit-tk-Globin plasmid (23) by the rGH sequence of rGH-tk-CAT the reporter gene (24). Extracts corresponding to five units of β-galactosidase activity were used for the CAT assay with 0.1 μCi [14C]chloramphenicol (50 μCi/mmol Amersham) for 1 h at 37°C (HeLa cells) or 4 h at 37°C (CV1 cells). The acetylated chloramphenicol was separated by chromatography on silica gel TLC plates (Merck). For quantitation of the results, the acetylated and non-acetylated forms were excised and counted in 5 ml scintillation fluid.

**Gel shift assays**

Cos cells were transfected with 10 μg expression vector. Hormone was added 2 hours before harvesting. After 48h cells were harvested, washed in PBS and lysed by freeze-thawing in 100 μl extraction buffer (20 mM Hepes pH 7.8, 20% glycerol, 2 mM DTT and 400 mM KCl). The extracts were centrifuged at 10 000g for 15 min at 4°C and the supernatants recovered. A 5 μl aliquot extract was adjusted to a final concentration of 100 mM KCl and incubated with 2μg of poly(dIdC) and 50 000 cpm (5 fmol) of the [32P]-end-labelled double strand oligonucleotide 5'-GTCCAAATGTGCTGAGGGATG-TGACCTGATCAAATTTG-3' for 15 mn at 20°C. Protein-DNA complexes were excised and counted in 5 ml scintillation fluid.

**SI nuclease mapping**

HeLa cells were transfected as above with 1 μg of the rGH-tk-Globin reporter plasmid together with 0.3 μg of the reference

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Figure 1. Transcriptional activation mediated by the different nuclear receptors on the rGH-tk-CAT gene. HeLa (A) and CV1 (B) cells were cotransfected with 1 μg of rGH-tk-CAT reporter gene, 3 μg of receptor expression vector and 2 μg of the internal control plasmid pCH110. CAT assays were performed as described in Table 1. The spots corresponding to acetylated and non-acetylated forms were excised and quantified by scintillation spectroscopy.
Western blot analysis of chimeric receptors

Extracts corresponding to 20 units of β-galactosidase activity were separated by electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose (Hoefer transphor apparatus for 3h at 0.2 mA and 4°C). The chimeric receptors were revealed by using anti-hER monoclonal antibody D75 followed by rabbit anti-rat serum and [125I]protein A (28).

Immunoprecipitation of the cTRα and gag v-erbA proteins

For immunoprecipitation of cTRα and gag v-erbA, subconfluent HeLa cells were transfected as above with 5 μg of receptor expression plasmid per 90 mm plate and grown in the absence of hormone for 40 h. The cells were then metabolically labelled with [35S]Methionine (10 μCi/ml medium; 1200 Ci/m mole, Amersham) in 5 ml serum-free medium. Cell extracts were prepared and processed for immunoadsorption with anti-erbA antiserum (kindly provided by J. Ghysdael), electrophoresed and visualized by fluorography as described earlier (29).

RESULTS

Activation of different response elements derived from the rGH promoter sequence by the different nuclear receptors

The different plasmids expressing cTRα (9), human (h)RARα (30), hER (29) and hGR (31) were cotransfected with the reporter plasmid rGH-CAT (see materials and methods and Fig. 2A) and a reference plasmid that contains the β-galactosidase gene under the control of the simian virus (SV) 40 early promoter (pCH110). The results are presented in Figure 1. Transfection in CV1 cells of an expression vector encoding the cTRα (cTR0) in the absence of T3 led to a 50% decrease of CAT activity compared to the control plasmid pSG1 (Fig. 1B compare lanes 1 and 8). This phenomenon has been observed by other authors (14,32) who found that the response of the rGH promoter with TR was due to a combination of a repression of basal (–T3) expression and an increase in hormone-induced expression. We therefore decided to express the effect of the different nuclear receptors as fold induction (ratio plus/minus hormone : see Table 1). Transfection of cTR0 in the presence of T3 led to a stimulation of CAT activity in response to T3 in CV1 cells (4.9-fold induction) but not in HeLa cells (Compare lanes 8 and 9 Fig. 1A with lanes 8 and 9 Fig. 1B and Table 1). When HeLa cells were cotransfected with the expression vector encoding the hRARα (hRAR0) and the reporter plasmid,
A 3.4-fold induction in CAT activity was observed in the presence of RA and a 1.9-fold induction was observed in cotransfected CV1 cells. Stimulation of transcription from the rGH-tk-CAT reporter gene was weak or absent in the presence of hER and E2 (2.0 and 0.8-fold induction in HeLa and CV1 cells respectively). Finally, no stimulation was observed in the presence of hGR and dexamethasone either in HeLa or CV1 cells (see Table 1), demonstrating that the GR interacts specifically only with its response element (TGACCC) common to TR, RAR and ER (33).

In order to test the effect of spacing on transcription by TR, RAR, and ER, HSVtk-CAT reporter plasmids driven by promoters containing different TGACC palindromes were constructed (Fig. 2). Insertion of the sequence GATCAGGGACGTGACC (oligo TRE1) that corresponds to the wild type rGH sequence (nucleotide -181 to -166) resulted in a 4.0-fold induction of CAT activity in CV1 cells in the presence of cTRa and T3 (see Table 1). Transformation of this sequence into a perfect palindromic sequence with a six base pair spacing GGTCAGGGACGTGACC (oligo TRE3) resulted in a slight increase in induction as compared to the wild type sequence (see Table 1). Transfection of constructs derived from recently published TRE sequences (17,34) (TRE6-tk-CAT construct contains the perfect palindromic sequence TGACC without spacing, TRE12-tk-CAT the perfectly palindromic sequence TGACCTGA with a spacing of six base pair and TRE13-tk-CAT contains the perfect palindromic sequence TGACCTGA without spacing) led to a same level of transcriptional activation in CV1 cells. RARa was able to stimulate transcription from all the tested response elements in HeLa cells. These results show that the gap between the different palindromic sequences is not critical for activation by the cTRα or hRARα. In contrast, the hER is able to stimulate transcription of the ERE-tk-CAT construct only.

![Figure 2](image1)

Figure 2. Structure of the hormone-responsive reporter genes. (A) The organisation of the different reporter genes is shown schematically. The start site of each gene is marked with an arrow. (B) List of the different oligonucleotides subcloned in front of the tk promoter (into pBLCAT8+) to create the different TRE-tk-CAT and ERE-tk-CAT constructs.

![Figure 3](image2)

Figure 3. Functional properties of the gag v-erbA oncogene protein. (A) Immunoprecipitation of the cTRα and gag v-erbA proteins after transfection in HeLa cells. See Materials and methods for details. M : Molecular weight markers. (B) TRE-binding properties of cTRα and gag v-erbA. A [32P]-labelled double strand oligonucleotide representing the TRE was incubated with nuclear extracts from COS cells and analysed in a band-shift assay as described in Materials and Methods. The free oligonucleotide is at the bottom of the gel. Arrows indicate the two major retarded complexes. (C) Comparison of the transcriptional activity obtained with cTRα and gag v-erbA in CV1 cells. The quantity of the different transfected plasmids (activator and competitor) is indicated.
In order to determine whether nuclear receptors are capable of interacting cooperatively with multimeric response elements, we constructed reporter plasmids with three copies of the TRE1, TRE3 and ERE sequences, denoted (TRE1)3-k-tk-CAT, (TRE3)3-k-tk-CAT and (ERE)3-k-tk-CAT (Fig. 2) and compared the fold induction obtained with monomeric and trimeric constructs. It is clear that additive effects were obtained for the cTrα in CV1 cells and for hRARα in Hela cells. By contrast a synergistic effect was obtained with these trimers in the presence of hER in Hela cells.

The oncoprotein p75 gag v-erbA acts as a constitutive repressor on the different tested TREs

In order to investigate the activity of gag v-erbA on different TREs we introduced the entire gag v-erbA coding sequence into the expression vector pSG5. Expression and DNA-binding of the oncogene protein were verified by immunoprecipitation of the protein (Fig. 3A) and gel retardation after transfection (Fig. 3B). When the expression plasmid was cotransfected in CV1 cells with reporter genes containing TREs, either without spacing (TRE13), with a spacing of six base pairs (TRE3), or with a trimer of the latter element (TRE3×3), a decrease in basal CAT activity was observed without any difference in the presence or absence of T3 (Fig. 3C). Interestingly, the repression observed with gag v-erbA was reproducibly lower than that obtained with cTrα in the absence of T3. To examine the effect of v-erbA on cTrα function, we performed transcription competition experiments. Transfection in CV1 cells using the cTrα0 together with a 10-fold excess of gag v-erbA0 gave rise to a decrease of transcription from the TRE3, TRE12 and TRE13-k-tk-CAT reporter genes (data not shown).

Chimeric estrogen receptors containing the cTrα DNA binding domain, but not those containing the v-erbA DNA binding domain, activate T3 responsive reporter genes in the presence of E2

From the results described above and by others (14 and 15) it is clear that cTrα and gag v-erbA can bind to the same DNA sequences but only cTrα can activate transcription. To determine the effect of the two amino acid substitutions found in the DNA binding domain of v-erbA on transcriptional activation, we exchanged this domain of hER in HE28 for that of cTrα or v-erbA to create the chimeric receptors HEcTrα.CAS or HEv-erbA.CAS respectively (Fig. 4A). Expression of the different receptors HE28, HEcTrα.CAS and HEv-erbA.CAS was detected on a Western blot using the hER-specific monoclonal erbA.CAS respectively (Fig. 4A). Specific binding measurements with [3H]oestradiol confirmed that the receptors were expressed at similar levels (data not shown). When Hela cells were cotransfected with the expression plasmid encoding HEcTrα.CAS and the rGH-tk-CAT reporter plasmid, a 4.8 fold induction was observed in the presence of E2 (see Table 1). S1 nuclease mapping of cytoplasmic RNA transcribed from the reporter gene rGH-tk-Globin (where the CAT gene was replaced by a promoterless β globin gene, Fig. 2A) confirmed the stimulation and demonstrated that the initiation of transcription occurred at the correct site (Fig. 4C). As expected, this chimera conferred E2-responsiveness on genes normally activated by T3. This stimulation occurred in Hela cells, whereas the stimulation obtained with the wild type cTrα was obtained specifically in CV1 cells (Fig. 1 and Table 1). The ability of the chimeric receptor HEv-erbA.CAS to stimulate transcription was also measured in HeLa cells by cotransfection of the receptor with the reporter plasmid rGH-tk-CAT in the absence and presence of E2. Although the chimeric receptor HEv-erbA.CAS was expressed in HeLa cells at similar levels and bound E2 with approximately the same affinity as HEcTrα.CAS, it was unable to stimulate rGH-tk-CAT or rGH-tk-Globin in the presence of E2 (Table 1 and Fig. 4C). Similar results were obtained when HEv-erbA.CAS was cotransfected with the different TRE-tk-CAT constructs (Table 1).

Multiple TGACC motifs are able to promote stimulation of transcription by the chimeric receptor HEcTrα.CAS

The absence of stimulation found with the rGH promoter sequence or with the monomeric constructs (see Table 1) does not imply that the DNA binding domain of v-erbA is not functional. Indeed a very high stimulation was obtained (94-fold induction) with the trimeric construct (TRE3)3-tk-CAT in the presence of HEv-erbA.CAS (Table 1), demonstrating that this chimeric receptor is able to stimulate transcription in the presence of multiple response elements.

The mutation Gly—Ser (position 73) abolishes the ability of HEcTrα.CAS to stimulate transcription from the rGH promoter

The DNA binding domain of cTrα differs from that of gag v-erbA in only 2 amino acids (Fig. 5A and 7). To evaluate the relative influence of these two substitutions on the impaired transcriptional activation observed with v-erbA, we constructed two new chimeric receptors by site directed mutagenesis: HEcTrα(S).CAS has a glycine—serine mutation at amino acid 73 and HEcTrα(T).CAS has a lysine—threonine mutation at amino acid 90 (Fig. 5A). The former mutant was not able to stimulate rGH-tk-CAT whereas the latter was still able to stimulate rGH-tk-CAT, although at a slightly lower level than HEcTrα.CAS (Fig. 5B and 4C), demonstrating that the glycine—serine mutation is deleterious for transcriptional activation.

Competition experiments between the different chimeric receptors

In order to determine if the DNA binding domain of v-erbA by itself is able to compete with its homologous counterpart in cTrα, we performed in vivo transcription competition experiments with the chimeric receptors. The rGH-tk-CAT construct was used because transcription from this reporter gene was stimulated by HEcTrα.CAS and not by HEv-erbA.CAS in Hela cells (Fig. 5C and Table 1). Cotransfection of HEv-erbA.CAS with HEcTrα.CAS led to a drastic decrease in the stimulation by E2 compared to the stimulation obtained with the transfection of HEcTrα.CAS alone (Fig. 6A). Changing the receptor/reporter ratio (by increasing the quantity of transfected HEcTrα.CAS plasmid while the reporter plasmid was unchanged) led, in all cases to a 50% inhibition by the same quantity of HEv-erbA.CAS. HE28 which stimulates rGH-tk-CAT slightly (1.8—2.5 fold induction, see Table 1) does not inhibit the stimulation by HEcTrα.CAS to the same extent.
DISCUSSION

Response elements for estrogen, thyroid hormone and retinoic acid receptors are closely related

Our study on transcriptional activation of different nuclear receptors demonstrated that both TR and RAR can activate transcription from a series of reporter genes containing the palindrome TGACC with different spacings (gaps of zero, three or six nucleotides long). Another TRE localised in the Moloney murine leukaemia virus (MoMLV) contains an imperfect palindrome with a gap of five nucleotides (15). The recently described retinoic acid response element (RARE) of the laminin B1 gene (−477 to −432) contains two imperfect palindromes (AGACAAGGTGACC and TAACCTAGCTCACC) with a gap of three and four nucleotides that are important for the RA responsiveness of the RARE (35). All these different response elements demonstrate that there is no spatial requirement between the two motifs for TR and RAR to promote transcription. This phenomenon has been also described for the cAMP response element (CGTCA motif)(36). By contrast, the ER is only active on the ERE indicating that, in this case, the spacing of the half-palindromes is essential for correct positioning of the structure.
Cell specific transcriptional activation of nuclear receptors

Using an estrogen chimeric receptor containing the DNA binding domain of cTRα, we confirmed that region C of cTRα is responsible for specific recognition of a T3 responsive target gene; this chimeric receptor conferred E2 responsiveness on genes normally activated by T3. Chimeric TR/GR with hybrid functional properties have also been described recently (39). Interestingly the TR was active on all the reporter genes tested in CV1 cells but not on simple TGACC-containing palindromes in Hela cells. By contrast the chimeric receptor HEcTRα.CAS (TR binding domain in an ER backbone) was active in Hela cells. These observations indicate that a cell-specific factor(s) which is absent or very limiting in Hela cells is required for TR mediated transcriptional activation. It is noteworthy that this factor is not required when the chimeric receptor is used instead of the wild type TR suggesting that this factor may interact with domains outside the DNA binding domain. Similar observations on specific target gene activation of nuclear receptors have already been made (40 and references therein). It will be interesting to define the domain of the TR which confers this cell specificity.

The mutated DNA binding domain in v-erbA plays a role in the lack of transcriptional activation and in repression of gene expression

Using different chimeric receptors we have been able to demonstrate the effect of one of the two amino acid differences between cTRα and v-erbA in the DNA binding domain. It is clear that the mutation Gly—Ser at position 73 greatly decreased or abolished (depending on the reporter gene used) the ability of the chimeric receptor to stimulate transcription. It is interesting to note that this amino acid 73 corresponds to one of the three discriminating amino acids that are thought to interact directly on the DNA helix. Three amino acids located at the C-terminal side of the ER or GR first finger play a key role in target gene specificity (33,37). It is interesting to note that cTRα and hRARα, which have the same three discriminating amino acids (see Fig. 7), recognize the same response element with little gap requirement. ER which differs in one out of the three discriminating amino acids has a strict gap requirement: it does not recognize TRES with spacings other than three base pairs. It would be interesting to test if the vitamin D3 receptor (38), which has the same three discriminating amino acids as cTRα and hRARα, is able to bind to the same response elements.
correlates well with the in vivo situation, since viral infection of the c-erbA/TR gene products could block differentiation by interfering with the normal function of cell differentiation, including the erythrocyte avian transporter binding to the response element. The large excess of oncogene could directly compete for the cTRs and the oncogene protein, could occur in vivo. Alternatively, the oncogene protein could generate a chimeric receptor whose behavior is virtually identical to that of v-erbA (14).

Interestingly, HEcTRa.CAS is more strongly inhibited by HEV-erbA.CAS than cTRα is by v-erbA. This is probably due to the formation of heterodimers since the same inhibition (50%) was found for the different ratios receptor/reporter tested (Fig.6). This mechanism of inhibition, although not demonstrated between TRs and the oncogene protein, could occur in vivo. Alternatively, the oncogene protein could directly compete for binding to the response element. The large excess of oncogene protein found to be necessary for the repression of transcription correlates well with the in vivo situation, since viral infection leads to a high level of expression of the protein. In this respect, Zenke et al. (41) have shown recently that the v-erbA oncogene specifically suppresses transcription of genes involved in erythroid cell differentiation, including the erythrocyte avian transporter (band 3) gene and less efficiently, the S-aminolevulinic acid synthase (ALAS) gene. As the c-erbA (TR) genes are preferentially expressed during late stages of erythrocyte differentiation (42), and may play a role in the differentiation of chicken erythrocytes, it is possible that the v-erbA oncogene could block differentiation by interfering with the normal function of the c-erbA/TR gene products.

ACKNOWLEDGEMENTS

We are grateful to S. Green for the gift of HE28, to J. Sap and B. Vennstrom for the cTR and gag v-erbA plasmids, M. Petkovich for hRARα, V. Kumar for HGO, Abbot laboratories for anti-hER antibodies and J. Ghysdael for anti-erbA antiserum. We are grateful to the cell culture group and to J.M. Bornert for technical assistance and to A. Stauber and F. Ruffenach for preparing the oligonucleotides. We thank C. Werlé and B. Boulay for help in preparing and reproducing the figures, the secretarial staff for typing the manuscript, J. White and S. Ali for critical reading of the manuscript. We thank P. Chambon for continuous support and encouragement. This work was supported by grants from the CNRS, the INSERM (CNAMTS), the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale and the Ligue Nationale Française contre le Cancer.

H. de V. was supported by the Association pour la Recherche sur le Cancer for the year 1988. D.M. was supported by a fellowship from the University Louis Pasteur.

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Figure 7. Homology between different nuclear receptors within the DNA binding domain and v-erbA. The conserved cysteine residues are in bold type. Dashes, spaces introduced into the sequences to obtain maximal alignment. The two asterisks show the two amino acid substitutions found in v-erbA when compared to the cTRα sequence. The position of the three ‘discriminating’ amino acids (33) are indicated by vertical bars.

with the specific bases of the TGACC motif (see fig. 7 and ref. 33 and 37). However, comparison of the results obtained between HEV-erbA.CAS and gag v-erbA with the (TRE3)-3-k-CAT construct (high stimulation vs. no stimulation) lead us to conclude that the lack of transcriptional activation by v-erbA is not only due to the mutation in the DNA binding domain. It has been demonstrated that substitution of the hormone binding region of cTRs for that of v-erbA generates a chimeric receptor whose behavior is virtually identical to that of v-erbA (14).