Functional Interaction between the Two Zinc Finger Domains of the v-erb A Oncoprotein

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Abstract
The v-erb A oncogene of avian erythroblastosis virus is a mutared and virally transduced copy of a host cell gene encoding a thyroid hormone receptor. The protein expressed by the v-erb A oncogene binds to DNA and acts as a dominant negative inhibitor of both the thyroid hormone receptor and the closely related retinoic acid receptor. The v-erb A protein has sustained two amino acid alterations within its DNA-binding domain relative to that of c-erb A, one of which, at serine 61, is known to be important for v-erb A function in the neoplastic cell. We report here that the second alteration, at threonine 78, also plays an important, although more indirect, role: alteration of the sequence at threonine 78 such that it resembles that of c-erb A can act as an intragenic suppressor and can partially restore function to a v-erb A protein rendered defective due to a mutation at position 61. Threonine 78 lies within the D-box of the v-erb A protein, a region thought to mediate receptor-receptor dimerizations, and is not in physical proximity to the serine at position 61. It therefore appears that an indirect interaction occurs between these two sites and that this interaction is crucial for v-erb A function.

Introduction
AEV is a retrovirus which induces both erythroleukemias and fibrosarcomas in birds (1). Two distinct oncogenes are contained within the AEV genome: v-erb A and v-erb B, each of which represents transduced and altered copies of normal cellular loci (1). The v-erb B oncogene, derived from a host cell gene for the epidermal growth factor receptor, is both sufficient and necessary for oncogenic transformation (1, 2). The v-erb A oncogene, in contrast, is not itself sufficient for oncogenic transformation but instead acts in neoplasia by blocking differentiation of infected erythroid cells and by altering the growth requirements of infected fibroblasts (1, 3). The v-erb A oncogene is derived from c-erb Aα, a gene for a thyroid hormone (T3/T4-thyronine) receptor (4, 5). Thyroid hormone receptors, in common with the closely related retinoid, vitamin D3, and steroid receptors, act as hormone-regulated transcription factors, binding to specific DNA sequences (termed hormone response elements) and, in response to hormone ligand, modulating expression of adjacent target genes (6–8). All members of this nuclear hormone receptor family share a common modular structure consisting of a cysteine-rich “zinc finger” domain that confers DNA binding and a more COOH-terminal domain that binds the hormone ligand.

The DNA-binding “zinc finger” domain within these receptors consists of two α-helices folded and stabilized by interactions of conserved cysteines with coordinated zinc ions (9–11). The alteration of just three amino acids within the first zinc “finger,” termed the P-box, can change the DNA binding specificities of one receptor with another. In contrast, the amino acids in the D-box, within the second zinc finger, are thought not to directly contact the DNA, but to play a more indirect role in binding of the receptor to DNA (12–14). Hormone response elements have been identified for the thyroid hormone, glucocorticoid, estrogen, and retinoic acid receptors (7). Generally, they consist of multiple copies of a “half-site”: a consensus hexa- or octanucleotide DNA sequence. Both the actual nucleotide sequence of each half-site and the spacing and orientation between them are important for proper recognition of cognate hormone response elements by the nuclear hormone receptors (15, 16). It has been proposed that whereas the actual sequence of the half-site is recognized by the P-box of the receptor, the D-box is involved in receptor-receptor dimerization and therefore in recognition of the spacing or orientation between the half-sites in the response element (12, 14, 16).

The v-erb A allele is expressed as a viral gag-fusion protein and contains 13 internal missense mutations, as well as small amino- and carboxy-terminus deletions relative to the c-erb A progenitor (4). Although the v-erb A protein retains the ability to bind to DNA, the changes sustained within the v-erb A ligand-binding domain have seriously impaired its ability to bind thyroid hormone and to dictate transcription of target genes (17–20). As a consequence, in vertebrate cells, the v-erb A protein acts as a constitutive but somewhat promiscuous transcriptional repressor and can interfere in a dominant negative fashion with the actions of the estrogen, retinoic acid, and thyroid hormone receptors (18, 19, 21, 22). This ability of the v-erb A protein to interfere with the actions of retinoic acid and thyroid hormone receptors appears strongly linked, at least indirectly, to its mechanisms of action in the neoplastic cell (22–25).

We are interested in the structural basis behind the different transcriptional properties of the v- and c-erb A proteins. Although the changes sustained by the carboxy-terminus of the v-erb A protein are thought to play the principal role in the generation of the dominant negative

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3 The abbreviations used are: AEV, avian erythroblastosis virus; SDS, sodium dodecyl sulfate; RA, retinoic acid; TRE, thyroid hormone-responsive element; CAT, chloramphenicol acetyltransferase.
phenotype of v-erb A, we have noted that two of the missense alterations between v- and c-erb A occur within very intriguing portions of the amino-terminal DNA-binding domain. One of these alterations occurs at position 61, a glycine codon in c-erb A but a serine codon in v-erb A, and lies within the crucial P-box sequence (Fig. 1). Consistent with the importance of the P-box domain in DNA recognition, we have previously demonstrated that the ability of v-erb A to function in the cancer cell is dependent on the viral serine at this P-box site, and that replacement of the serine with the c-erb A-encoded glycine results in a significant loss in erythroid colony formation (26). The second location that differs between the DNA-binding domains of v- and c-erb A lies within the D-box, at a site thought critical for dimerization between receptor molecules: codon 78 encodes a threonine in v-erb A and a lysine in c-erb A (Fig. 1). The functional significance of this alteration has not previously been explored. In this report, we investigate the role of this second alteration between v- and c-erb A proteins, with a special focus on its importance in transcriptional regulation.

Results
Alteration of the v-erb A DNA-binding Domain Sequence to That of c-erb A. Of the two amino acid sites that differ in the DNA-binding domains of the v- and c-erb A proteins, one, at codon 61 within the P-box, has already been shown to be critical for v-erb A function (22, 26). We began our investigation into the role of the second alteration, at codon 78 within the D-box, by using a site-directed mutagenesis approach. The threonine codon at the base of the second zinc finger of v-erb A was changed to a lysine (denoted T78K), making the v-erb A sequence identical to that of c-erb A at this site (Fig. 1). A similarly altered v-erb A gene, but bearing the c-erb A sequence at the P-box codon 61 (denoted S61G), was described previously (26). We also created a v-erb A allele combining these two single mutations to generate an S61G/T78K double mutation, resulting in a DNA-binding domain sequence identical at all positions to that of c-erb A.

Our first goal was to ascertain that the mutant AEV clones were both viable and capable of producing a stable v-erb A protein. Avian fibroblasts infected by the different v-erb A mutants were radiolabeled with 35S-amino acids and lysed, and the v-erb A proteins were subsequently immunoprecipitated with an erb A-directed polyclonal antibody. The immunoprecipitates were then analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The erb A-directed serum detected a protein of the appropriate size (M, 75,000, denoted p75) for the gag-v-erb A polypeptide in the lysates of cells infected with wild-type, S61G, T78K, or the S61G/T78K viral mutants (Fig. 2, Lanes 7 to 10).
levels of the p75 protein synthesized by the various v-erb A mutants was comparable, with accumulation of the S61G mutant v-erb A protein during the 3-h pulse perhaps slightly greater than that of the other alleles. In contrast, the p75 protein was not detected in the lysate of cells infected by the v-erb A 82-t null mutant (Fig. 2, Lane 6), nor was the p75 v-erb A protein detected by preimmune serum (Fig. 2, Lanes 1 to 5). The additional protein, approximately 48 kilodaltons in size, detected in high abundance in Lane 10, is also detectable at low levels in several of the immunoprecipitates utilizing preimmune serum; its relatively higher abundance in the wild-type protein sample shown here is not a reproducible feature of these immunoprecipitations.

The T78K Mutation Alone Does Not Have a Significant Effect on v-erb A-Mediated Erythroid Transformation. But, Unexpectedly, Is Able to Partially Suppress the Defect Resulting from the S61G Mutation. The transforming properties of each v-erb A mutant were evaluated using a methylcellulose growth assay: chicken bone marrow cells were infected with AEV carrying the various v-erb A mutations, the cells were then placed into methylcellulose media, and colony formation was monitored. We used relatively simple medium in these assays, un-supplemented by anemic chicken serum, tumor growth factors, insulin, or erythropoietin. Since a functional v-erb A gene is required for erythroid cell proliferation under these conditions, the number of erythroid colonies produced by each virus reflects the activity of the different v-erb A alleles (26). To compensate for possible differences in viral titer among the different mutant and wild-type virus stocks, we have normalized the erythroid-transforming ability of each of our mutant viral stocks to the fibroblast-transforming ability of the same stock (a property of the unaltered v-erb B locus). However, our overall conclusions would be essentially the same whether the normalized or original data were analyzed: comparable numbers of transformed fibroblast colonies were obtained for all of the AEV clones, indicating that the v-erb B gene was intact and that viral replication was not affected by the v-erb A mutations (Table 1, and data not shown).

Consistent with previous results, virus bearing the wild-type v-erb A gene generated a large number of erythroid colonies under these conditions, whereas the v-erb A 82-t null mutant was totally inactive in the assay (Fig. 3). Virus carrying the single S61G v-erb A lesion yielded an approximately 5-fold reduction in its ability to induce erythroid colonies compared to wild-type, in keeping with the significantly impaired function previously reported for this mutant allele (26). In contrast, the T78K mutant produced erythroid colonies at a normalized efficiency near that of wild-type levels, indicating that the substitution of the c-erb A lysine for the v-erb A threonine had only a minor effect on v-erb A function. On this basis, we anticipated that the double lesion, S61G/T78K, would exhibit a defect equal to, or perhaps slightly more severe than, that of the S61G lesion alone. Unexpectedly, the S61G/T78K double mutant consistently showed improved erythroid cell-transforming capabilities (41% of wild-type levels) when compared to the S61G mutation alone (20% of wild-type levels). Thus, although the T78K alteration by itself had little or no direct effect on erythroid transformation, combination of the T78K lesion with the defective S61G mutation appeared to partially restore the oncogenic capabilities of v-erb A. This apparent intragenic suppression was reproducible and was observed as a 2-fold enhancement in three separate assays, each performed in duplicate.

Table 1 Erythroid-transforming capabilities of v-erb A alleles

<table>
<thead>
<tr>
<th>Construct</th>
<th>Average no. of erythroid colonies</th>
<th>Ratio erythroid/fibroblast</th>
<th>% compact erythroid colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>0</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>S61G</td>
<td>29.5</td>
<td>0.176</td>
<td>&gt;95</td>
</tr>
<tr>
<td>T78K</td>
<td>65.3</td>
<td>0.621</td>
<td>&gt;95</td>
</tr>
<tr>
<td>Double</td>
<td>65.8</td>
<td>0.358</td>
<td>85</td>
</tr>
<tr>
<td>Wild-type</td>
<td>126.3</td>
<td>0.875</td>
<td>&gt;95</td>
</tr>
</tbody>
</table>

*See Fig. 3 for details.
* Average of the number of colonies determined in three separate assays, each done in duplicate.
* Calculated by dividing the number of erythroid colonies in each experiment by the fibroblast-transforming capabilities of each viral stock (as determined in "Materials and Methods"), to compensate for any potential variation in viral titer.
* Compact versus diffuse morphology was determined by microscopic evaluation of the methylcellulose medium assays. Examples of the differing morphologies are shown in Fig. 4.

![Fig. 3. Erythroid colony formation by v-erb A mutant viruses. AEV stocks, bearing the different v-erb A alleles, were tested for the ability to induce erythroid colonies. Virus recovered from transfected fibroblasts was used to infect chicken bone marrow cells, and subsequent colony formation in un-supplemented methylcellulose medium was monitored. The number of erythroid colonies induced by each viral variant was normalized to the fibroblast-transforming titer determined on the same virus stock. Null, the 82-t premature termination mutant; dbl, the S61G/T78K double mutant; wt, the wild-type virus. Results represent the average of three independent experiments.](image-url)
Intragenic Suppression within v-erb A

A. wild type

B. S61G

C. T78K

D. double

E. double

Fig. 4. Morphology of representative erythroid colonies. Erythroid cell colonies induced by the different AEV mutants were photographed through a phase microscope. Shown are typical colonies induced by the wild-type virus (A), the S61G mutant (B), and the T78K mutant (C), as well as two colonies of differing morphology induced by the S61G/T78K double mutant (D and E).

contrast, the S61G single mutant yielded very diffuse colonies surrounded by a halo of sparse cells, with less than 10% of the colonies exhibiting the condensed, wild-type appearance (Table 1; Fig. 4B). Intriguingly, the colonies induced by the S61G/T78K double mutant were largely of the compact, wild-type morphology (Fig. 4E), with only a low percentage (approximately 15%) of the more diffuse phenotype characteristic of the S61G allele alone (Table 1; Fig. 4D). These results again intimate that T78K mutation may act to significantly repair the functional defect in the S61G mutant gene.

The T78K Mutation Restores the Ability of the S61G Mutant to Interfere with Transcription. A fundamental aspect of v-erb A protein function appears to be its ability to act as a transcriptional repressor and to interfere with the actions of other nuclear receptors (18, 19, 22). The ability of v-erb A to repress the actions of the retinoic acid receptors, in particular, may be linked to v-erb A function in the neoplastic cell (22-25). Therefore, we next tested the ability of our different v-erb A mutant alleles to interfere with retinoic acid receptor action in a transient transfection assay. Expression plasmids bearing the different v-erb A mutant alleles were introduced into MCF-7 cells together with a retinoic acid-responsive reporter gene (RRE₉₅-tkCAT). RA was then added, or not,
to the medium, and the cells were subsequently assayed for reporter gene expression.

As expected, the endogenous retinoic acid receptors in the MCF-7 cells mediated a strong RA-dependent induction of reporter gene expression that was not affected by introduction of a plasmid bearing the 82-t v-erb A-null gene (Fig. 5). Introduction of the wild-type v-erb A allele, on the other hand, strongly inhibited this RA activation, consistent with previous work (22). The T78K mutant appeared to function as well as, if not slightly better than, wild-type v-erb A in this assay, efficiently interfering with RA activation of the reporter, whereas the single S61G lesion significantly impaired the ability of the v-erb A protein to repress RA receptor action. In contrast to the S61G single lesion, the S61G/T78K double mutant was able to effectively inhibit RA action in the MCF-7 cells, repressing reporter gene expression virtually as well as the wild-type v-erb A protein. Our results suggest that in this transcriptional repression assay, as in the erythroid colony assay, the T78K mutation appears to compensate for the defect due to the S61G lesion. Parallel aliquots of the transiently transfected cells were also radiolabeled with 35S-amino acids and subjected to an immunoprecipitation analysis using erb A-directed antibodies. This analysis revealed that detectable levels of v-erb A protein were synthesized by all of the constructs, with the expected exception of the 82-t null mutant, and that the relative ability to repress RA activity was not related to simple differences in the amount of v-erb A protein expressed by the different mutants (data not shown).

The Subcellular Localization of v-erb A Does Not Appear to Be Altered by the S61G or T78K Mutations. Subcellular localization of wild-type v-erb A is bimodal, with 60 to 70% localized to the nucleus in AEV-infected cells and the remaining 30 to 40% distributed evenly throughout the cytoplasm (27). It has been demonstrated previously that nuclear accumulation of v-erb A protein is necessary for v-erb A function (28). To verify that the S61G and T78K mutations did not achieve their effects through altering the subcellular localization of the v-erb A protein, immunofluorescent staining was performed on AEV-infected fibroblasts. Preimmune sera yielded only low background fluorescence for cells infected by any of the v-erb A mutant forms of AEV (data not shown). Cells infected with the 82-t null mutant also showed only a low level of background fluorescence, as expected (Fig. 6A). The wild-type v-erb A gave intense staining, with most of the signal localized to the nucleus, and the rest distributed evenly throughout the cytoplasm (Fig. 6B; Ref. 27). The S61G, T78K, and S61G/T78K mutants all gave staining patterns virtually indistinguishable from that of wild-type v-erb A (Fig. 6, C to E). Therefore, the differing abilities of our v-erb A mutants to transform erythroid cells and repress retinoic acid receptor function are probably not due to alterations in the subcellular localization of the v-erb A polypeptide. It should be noted that our immunofluorescence localization experiments were performed in avian fibroblasts; our previous analyses, however, have indicated that there are no detectable differences between v-erb A protein localization in erythroid and in fibroblast cells (27).

The T78K Mutation Yields a DNA Binding Pattern Similar to That of Wild-Type v-erb A. The DNA binding capabilities of the v-erb A mutants were determined using a combined DNA binding/immunoprecipitation (McKay-type) assay. AEV-infected fibroblast lysates were incubated with radiolabeled DNA fragments derived from the rat growth hormone gene; this gene has a number of binding sites for v- and c-erb A proteins. The v-erb A proteins, as well as any associated DNA fragments, were subsequently immunoprecipitated with erb A-directed antisera, the immunoprecipitates were washed, and the bound DNA fragments were released and resolved by agarose gel electrophoresis/autoradiography.

Only faint background binding, principally of the DNA fragments a and b, was seen with the 82-t null mutant or by use of preimmune serum (Fig. 7A, Lanes 1 and 2). In contrast, the wild-type v-erb A protein (Lane 6) exhibited strong binding to DNA fragments c, f, and h, each of which contains a TRE binding site and has previously been shown to be bound by the v- and c-erb A polypeptides (20). The T78K mutant demonstrated a DNA binding pattern essentially identical to that of wild-type v-erb A in this assay (Lane 4). In contrast, the S61G mutant exhibited a consistently, if subtly, altered DNA binding pattern, with fragments h and f bound more weakly and fragment d bound somewhat more strongly than is seen for the wild-type protein (Fig. 7A, Lane 3; Ref. 26). This is most clearly observed in a quantitation by densitometry (Fig. 7B). The S61G/T78K double mutant appeared to display a mixture of traits in this assay, more closely duplicating the stronger binding of fragments f and h exhibited by the wild-type protein, but retaining the higher binding of fragment d seen for the single S61G mutant (Fig. 7A, Lane 5, and 7B). It is unlikely that these results reflect differences in the ability of the antisera to recognize the mutant v-erb A proteins, rather than genuine differences in DNA binding by the mutant v-erb A polypeptides: all mutant and wild-type v-erb A proteins interacted strongly with the anti-erb A antibody, and
Intragenic Suppression within v-erb A

A. null

B. wild type

C. S61G

D. T78K

E. double

Fig. 6. Subcellular localization of v-erb A protein by immunofluorescence analysis. Avian fibroblasts infected by the different AEV mutants were fixed with formalin, permeabilized with Triton X-100, and incubated with antisera directed against a bacterially synthesized portion of the v-erb A protein. The cells were washed and incubated with rhodamine-conjugated secondary antibodies and washed again, and the subcellular localization of the v-erb A protein was visualized by epifluorescent microscopy. Photographs of representative microscopic fields are presented: cells infected by the S2-t null mutant (A), the wild-type virus (B), the S61G mutant (C), the T78K mutant (D), and the S61G/T78K double mutant (E).

antibody is used in excess in these assays (Fig. 2, and data not shown). We conclude that in the double mutant, the T78K lesion appears to have altered the DNA binding properties to more closely approximate those of the wild-type v-erb A protein.

Discussion
The v-erb A protein is closely related structurally to the c-erb A protein. Functionally, however, the two proteins differ dramatically. The c-erb A protein, as a thyroid hormone receptor, regulates both positive and negative
transcription of target genes in response to thyroid hormone, allowing a cell to respond to its environment and to function appropriately in the scheme of a whole organism. The v-erb A protein, on the other hand, does not function in regulating appropriate cell behavior, but rather acts as a constitutive repressor in vertebrates and mediates inappropriate cell growth and differentiation. How do the relatively few structural differences between v-erb A and c-erb A confer such significantly different phenotypes? Our work seeks to address this question by investigating the significance of the structural differences in the DNA-binding domains of v- and c-erb A proteins and the role of these changes in the origin of the v-erb A oncprotein from the c-erb A hormone receptor. We focus here on two amino acids that differ in v- and c-erb A, codons 61 and 78, and that appear able to interact to alter the function of the encoded polypeptide.

Replacement of the Threonine 78 of v-erb A with the c-erb A Lysine Exhibits Little or No Phenotypic Effect in a Wild-Type Background but Can Partly Reverse the Defect Induced by a Second Site Mutation. All known c-erb A alleles encode a lysine within the D-box element, at a site where the v-erb A allele encodes a threonine (6, 8). To address the question of the relationship between this change and v-erb A function in the cancer cell, we replaced the threonine at this position in the v-erb A protein with the c-erb A-encoded lysine. This single "back" mutation, T78K, had little or no observable effect on the encoded protein. Virus bearing the T78K mutation induced erythroid colonies nearly identical in number and morphology to those induced by wild-type v-erb A; the T78K-encoded protein exhibited a subcellular distribution, DNA binding properties, and transcriptional repression capabilities virtually indistinguishable from those of the wild-type v-erb A protein. The T78K mutant was therefore dramatically different in its properties from our serine to glycine mutation at position 61 within the P-box, an alteration which leads to significant disruptions of the transcriptional and oncogenic functions of the v-erb A protein.

Our results initially suggested that the difference between v- and c-erb A protein at codon 78 might be a neutral mutation, the result of genetic drift occurring from the time of divergence of the viral from the cellular allele. However, extension of our analysis to a S61G/ T78K double mutant demonstrated that the T78K lesion does have significant effects on phenotype in this genetic background. The double mutant consistently exhibited an erythroid colony-transforming potential closer to that of wild-type, both in terms of colony number and morphology, than did the S61G single mutation, suggesting that the T78K lesion was, in effect, an intragenic suppressor of the S61G defect. This ability of the T78K lesion to restore function to the S61G defect was also demonstrated by the capacity of the S61G/T78K double mutant to repress retinoic acid receptor action in transient transfections, an assay in which the S61G single mutant possessed only severely limited activity.

The S61G/T78K double mutant, encoding a glycine at position 61 and a lysine at position 78, creates a DNA-binding domain identical to that of the c-erb A thyroid hormone receptor. It is important to note, therefore, that the S61G/T78K mutant does not appear to fully mimic all of the activities of the wild-type v-erb A gene in our assays, suggesting that the presence of a serine at position 61 and a threonine at position 78 confers on the wild-type v-erb A protein detectably greater function in the neoplastic cell than the c-erb A sequence from which it is derived. This conclusion is supported by evidence obtained in other systems that the DNA-binding domains of v- and c-erb A are not functionally equivalent (21, 29).

We conclude that (a) the amino acids in the P- and D-box must be compatible with one another for v-erb A

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Fig. 7. Coupled DNA binding-immunoprecipitation assay on mutant and wild-type v-erb A proteins. A, agarose-gel electrophoretic analysis. Avian fibroblasts infected by the different AEV mutants were lysed, and the lysates were incubated with 32P-radiolabeled restriction fragments derived from the rat growth hormone gene (input). The v-erb A proteins, and any bound DNA fragments, were subsequently immunoprecipitated and washed, and the radiolabeled DNA was released by treatment with SDS. The DNA fragments were resolved by agarose-gel electrophoresis and visualized by autoradiography. The lysates analyzed in this manner included fibroblasts infected by the 82-t null mutant (null, Lane 2), the S61G mutant (Lane 3), the T78K mutant (Lane 4), the S61G/T78K double mutant (dbl, Lane 5), and wild-type virus (wt, Lanes 1 and 6). Preimmune serum was used for Lane 1, and anti-v-erb A antisem for Lanes 2 to 6. a to i, the rat growth hormone restriction fragments used as input DNA (Bonde and Privalsky, 1990). B, quantitation of A. The relative intensity of the DNA fragments bound by the different v-erb A mutant proteins was determined by scanning densitometry and is presented relative to the intensity of band c (defined as 1). Abbreviations are as in Fig. 3.
function, and (b) both sites contribute to the ability of the viral allele to participate fully in oncogenesis.

**Nature of the Functional Interaction between the P- and D-Boxes in v-erb A.** It is intriguing that amino acids located at two separated sites in the v-erb A primary sequence (codons 61 and 78) can have such profound and interconnected influences on protein function. Studies on other nuclear hormone receptors have provided additional evidence for complementary interactions between amino acids in the D-box and the P-box participating in the DNA binding and functional properties of these receptors. For example, altering the DNA binding specificity of the glucocorticoid receptor to that of the thyroid hormone receptor requires changes to both the P- and D-box sequences, whereas either change alone leads to an inactive receptor (14). Conversely, joining the P-box sequence from a glucocorticoid receptor with a D-box (and flanking region) derived from the estrogen receptor produces a novel receptor with a promiscuous ability to recognize both glucocorticoid and estrogen response elements (12).

What are the roles of the P- and D-box elements, and how might they functionally interact? Although three-dimensional structural data are not available for either the c-erb A or v-erb A protein, reasonable predictions can be made based on structural studies of the closely related glucocorticoid and estrogen receptors (9-11). The DNA-binding domain of the nuclear hormone receptors is thought to be dominated by two \( \alpha \)-helices. The first helix, which includes the P-box, appears to lie within the major groove of the DNA binding site and is able to make base-specific contacts with the hormone response element. One P-box amino acid in particular, equivalent to codon 61 in v- and c-erb A, is in a position to make a critical base pair contact with the DNA response element and appears to play a central role in the ability of the receptor to discriminate among different DNA sequences (11). This structural analysis strongly confirms our genetic evidence and highlights the importance of differences in the codon 61 position for v- and c-erb A function. The second \( \alpha \)-helix (nearer the carboxy-terminus of the receptor) is oriented along the DNA axis and can make several nonspecific contacts with the phosphodiester backbone of the DNA molecule.

The D-box, in contrast, does not directly contact the DNA but instead forms a portion of an important interface that appears to mediate receptor-receptor dimerization, consistent with biochemical evidence indicating that the nuclear receptors bind to their DNA sites as dimers (11, 30). It has been further proposed that the D-box-mediated interactions between receptor dimers may determine the ability of the receptors to recognize the spacing between DNA half-sites (7, 16). It is provocative that the second amino acid difference in the DNA-binding domains of v- and c-erb A, at position 78, occurs in this D-box region.

How do the P- and D-box elements interact, and how can we account for the intragenic suppression phenomenon reported here? The predicted tertiary structure of the nuclear receptors suggests that the P- and D-box elements are not in close physical proximity to one another. Instead, we propose an indirect interaction. Evidence indicates that binding of the v-erb A protein to the DNA is necessary for its ability to interfere with the functions of other nuclear hormone receptors. We suggest that the wild-type v-erb A protein binds to one of the half-sites in a hormone response element and prevents, by protein-protein interactions, the functional binding of a retinoic acid or thyroid hormone receptor to the other half-site. Alterations in the P-box, such as the S61G mutation described here, would lead to changes in the interaction between the v-erb A protein and the DNA; these may be manifest either as a change in the tertiary structure of the v-erb A protein itself, or perhaps as an alteration in the precise orientation of the v-erb A protein on the DNA. The S61G v-erb A protein would therefore be unable to make the protein-protein contacts necessary for interfering with the actions of a second, functional hormone receptor on the adjacent half-site. Mutation of the D-box threonine 78 to a lysine would, by changing the dimerization interface, restore the ability of v-erb A to act as a repressor, thereby reinstating both biochemical and biological function.

**Materials and Methods**

**Construction of Mutants.** A 1.2-kilobase XhoI fragment from the molecularly cloned AEV genome was inserted into the M13 mp18 vector to serve as a single-stranded template for the mutagenesis protocol (26). The v-erb A threonine codon at position 78 was changed to the c-erb A lysine codon by the dutilung method of Kunkel (Bio-Rad Mutagenic kit), using a 5'-CACC ATCGT ACTTG CAGGA GTAGG-3' oligonucleotide containing a single base mismatch (underlined) relative to the wild-type sequence. The resulting v-erb A mutation, denoted T78K, was identified by DNA sequence analysis of individual M13 clones. The genetic lesion was next transferred to a pUC vector to permit easy isolation of double-stranded DNA and, by use of intermediate constructs, was ultimately reconstructed into a molecular clone of the entire avian erythroblastosis virus genome (denoted pAEV-11-3L) for the generation of infectious virus stocks (26, 31). The T78K mutation was also engineered into a pRSV-v-erb A expression vector for transient transfection studies (18). Generation of the S61G mutants has been previously described, and the creation of the S61G/T78K double lesion was achieved by simultaneous use of both mutant oligonucleotides in the M13 mutagenesis protocol. The 82-t null mutation is the result of introduction of a termination codon after base pair 82 (codon 27) in the v-erb A coding sequence (28). The identities of the final pAEV-11-3L and pRSV-v-erb A vector constructs were confirmed by DNA sequence analysis.

**Cells and Virus.** Chicken fibroblasts and MCF-7 human mammary carcinoma cells were isolated and propagated as previously described (22, 26). The avian fibroblasts were transfected with wild-type, or mutant forms of pAEV-11-3L by a calcium phosphate precipitation method, together with an infectious molecular clone of Rous-associated virus-1 (RAV-1) to serve as a helper virus (31). Virus stocks isolated from these primary transfections were used in subsequent studies. The effects of the viral mutations on erythroid colony formation were determined by infection of chick bone marrow cells in a methylcellulose medium colony assay (26). Three separate assays were performed, with duplicate plates in each. Fibroblast-transforming titers of the AEV stocks were determined by serial dilution and measurement of an-
choragie-independent colony formation in a soft agar growth assay (26).

**Biochemical Assays.** The ability of the v-erb A mutants to interfere with retinoic acid receptor function was assayed in MCF-7 cells, using the previously described prSV-v-erb A expression construct and a reporter gene composed of the palindromic retinoic acid response element linked to a thymidine kinase promoter and the CAT gene (pRRE<sub>μ</sub>-kCAT) (22). The expression and reporter plasmids, together with a prSV-lacZ plasmid to serve as an internal standard, were introduced into the MCF-7 cells by electroporation, and the cells were subsequently exposed or not (as indicated) to retinoic acid and harvested 24 to 30 h later (22). CAT activity was determined and was normalized to the β-galactosidase activity assayed in parallel (22).

Analysis of v-erb A-encoded protein synthesis was performed by radiolabeling cells for 3 to 4 h with 32P-amino acids; either stably infected avian fibroblasts or transiently transfected MCF-7 cells were used. The v-erb A-related polypeptides were isolated by immunoprecipitation of the radiolabeled cell lysates, subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography (26, 27). The amount of each lysate subjected to immunoprecipitation was normalized to total protein synthesis, as determined by a hot trichloroacetic acid precipitation procedure.

Immunofluorescent subcellular localization of the v-erb A protein was performed on formalin-fixed and detergent-permeabilized fibroblasts, using a rabbit polyclonal antibody directed against a bacterially produced fragment of v-erb A (26, 27).

The DNA binding properties of the mutants were assayed using a McKay-type immunoprecipitation assay as previously described (20), except that the gels were dried directly onto filter paper rather than blotted onto membranes. Briefly, unlabeled cell extracts containing the v-erb A protein were incubated with 32P-radiolabeled DNA fragments; these were derived from the rat growth hormone gene and contain appropriate binding sites for the v-erb A protein (TREs). The v-erb A proteins and any associated DNA fragments were then immunoprecipitated with erb A-directed antiserum, the DNA fragments were released from the v-erb A protein by SDS treatment, and the DNA molecules were resolved by 2% agarose-0.1% SDS-gel electrophoresis.

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**References**


