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Ontogeny of the v-*erbA* Oncoprotein from the Thyroid Hormone Receptor: an Alteration in the DNA Binding Domain Plays a Role Crucial for v-*erbA* Function

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The avian erythroblastosis virus v-*erbA* oncogene is imprecisely derived from a cellular gene (c-*erbA*) encoding a thyroid hormone receptor: the v-*erbA* protein has sustained both small terminal deletions and internal amino acid sequence changes relative to c-*erbA*. We report here that one of these missense differences between v- and c-*erbA* proteins, located in a zinc finger DNA binding domain, has dramatic effects on the biological activities of the encoded protein. Back mutation of the viral coding sequence to resemble c-*erbA* at this site severely impairs erythroid transformation and produces subtle changes in DNA binding by the encoded protein, suggesting that differences in DNA binding by the viral and cellular proteins may be involved in the activation of v-*erbA* as an oncogene.

Avian erythroblastosis virus (AEV) transforms both fibroblasts and erythroid cells to an oncogenic state. The AEV genome contains two loci involved in establishing the neoplastic phenotype: v-*erbA* and v-*erbB* (16). The v-*erbB* gene product is a tyrosine-directed protein kinase and is absolutely essential for transformation of either cell type by AEV. The v-*erbA* oncogene, on the other hand, is not essential for oncogenic transformation, but instead alters the neoplastic phenotype by blocking the differentiation of infected erythroid cells and by altering the growth properties of infected fibroblasts (13, 16). v-*erbA* represents a transduced derivative of a host gene encoding a thyroid hormone (T_3/T_4-thyronine) receptor (38, 44).

Thyroid hormone receptors are members of a larger family of ligand-regulated transcriptional factors that includes the steroid and retinoic acid receptors (1, 11). All members of this nuclear receptor family have a modular structure consisting of a DNA binding domain, defined by two zinc finger motifs and accompanying flanking sequences, one or more nuclear localization signals, and a C-terminal hormone binding domain (Fig. 1A). Upon binding of cognate hormone, these nuclear receptors interact with specific sequences in the cell genome and modulate the transcription of adjacent target genes; the DNA binding sites for thyroid hormone receptor are referred to as thyroid hormone response elements (TREs).

The v-*erbA* locus is a transduced and altered viral copy of the host cell gene (c-*erbA*α) from which it is derived (Fig. 1A). The v-*erbA* protein is fused to retroviral *gag* sequences at its N terminus and has sustained small N- and C-terminal deletions and 13 internal amino acid changes relative to the chicken c-*erbA*α progenitor (38). As a consequence of these changes, the v-*erbA* protein has a severely diminished capacity to bind thyroid hormones, and although it is able to bind to TRE sequences and act as a transcriptional activator in yeast cells, v-*erbA* cannot activate transcription in animal cells (8, 27, 33, 37). Instead, the v-*erbA* gene in vertebrate cells acts as a repressor and inhibits activation of hormone-responsive genes by the normal thyroid hormone receptor. The v-*erbA* gene may therefore represent a novel form of oncogene that acts not by mimicking, but by interfering with, the function of its normal cell counterpart (8, 37, 46, 47).

It is principally the alterations sustained by the C terminus of the v-*erbA* protein, relative to the c-*erbA* progenitor, that appear responsible for the loss of hormone binding by the viral protein and for its conversion into a constitutive transcriptional repressor (8, 27, 37, 47). Nonetheless, there are additional sequence differences between the c- and v-*erbA* proteins that may be important. One of the most provocative of these is at the base of the first zinc finger: all known thyroid hormone receptors encode Cys-Glu-Gly-Cys-Lys-**Gly** at this site, whereas the viral *erbA* gene encodes Cys-Glu-Gly-Cys-Lys-**Ser** (38; Fig. 1). This domain plays a crucial role in recognition of, and discrimination between, the different hormone response elements by the different steroid and thyroid hormone receptors (2, 9, 26, 41). Intriguingly, the estrogen receptor, which in common with the v-*erbA* protein binds to but does not activate transcription at TRE sequences, diverges from the thyroid hormone receptor at this same site: Cys-Glu-Gly-Cys-Lys-**Ala** (15).

We report here that alteration of the serine in the first zinc finger of the v-*erbA* protein back to the c-*erbA*-encoded glycine results both in an alteration in the DNA binding properties of the viral protein and in a dramatic impairment in its ability to function in neoplasia. In contrast, a v-*erbA* gene matching the estrogen receptor at this site, encoding an alanine, acts in neoplasia in a manner very similar to that of the wild-type v-*erbA* gene. We suggest that, in addition to changes in the C-terminal hormone binding domain, alterations in the DNA binding properties of the viral protein play a critical role in the genesis of the v-*erbA* oncogene from the c-*erbA* hormone receptor.

MATERIALS AND METHODS

Construction of the S61G, S61A, and 82-t v-*erbA* mutations. Subclones of the molecularly cloned AEV genome, either a 1.7-kb *Sst*I-to-*Sal*I fragment for the S61A mutation or a 1.2-kb *Xho*I-to-*Sal*I fragment for the S61G mutation (43), were introduced into M13mp18 and subjected to site-directed mutagenesis utilizing the *dut ung* methodology of

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Kunkel (Bio-Rad Mutagene kit). The sequence of the complementary oligonucleotide was either 5′-GACGG AAAAA GCCCT TGCAG CCCTC-3′ for the S61G mutation or 5′-GGTCC GACGG AAAAA GGCCT TGCAG CCCTC-3′ for the S61A mutation (underscored bases are noncomplementary to the wild-type v-*erbA* sequence) (38). Mutant M13 vector clones were identified by DNA sequence analysis, the double-stranded replicative forms were isolated, and the portions of the mutagenized v-*erbA* sequence represented within a 349-bp *Sca*I-to-*Pvu*II fragment were engineered in a multistep process into the pAEV-11-3L infectious form of the cloned AEV genome. Correct transfer of the mutant lesions into the infectious plasmid vector was confirmed by a second round of DNA sequence analysis. A v-*erbA* null mutant, designated 82-t, was created by the introduction of a premature termination codon at the beginning of the v-*erbA* sequence (31).

Cells and virus. Chicken fibroblasts were isolated from embryonated SPAFAS eggs and were propagated in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth (Difco), and antibiotics (100 U of penicillin per ml, 1 mg of streptomycin per ml, and 2.5 μg of Fungizone [amphotericin B] per ml). The fibroblasts were transfected with wild-type and mutant forms of the pAEV-11-3L infectious molecular clone by calcium phosphate precipitation in the presence of pRAV-10R, an infectious molecular clone of the Rous-associated virus-1 (RAV-1) genome (29, 39). Bone marrow cells were isolated from the femurs of 6- to 8-day-old SPAFAS chicken neonates and were used in the methylcellulose medium assays (see below).

Biological assays. The ability of mutant viruses to induce erythroid colonies was assayed by a methylcellulose medium-bone marrow cell technique (31). The ability of infected fibroblasts to display anchorage-independent growth was assayed by colony formation in soft agar medium (29). Titers of AEV stocks were determined by serial dilution and colony formation in soft agar medium.

Biochemical assays. The v-*erbA* proteins synthesized in AEV-infected fibroblasts were analyzed by radiolabeling the cells for 2 h with ^{35}S-amino acids, followed by lysis and immunoprecipitation analysis; radiolabeled proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and were visualized by fluorography (32). The amount of each cell lysate subjected to immunoprecipitation was normalized relative to total protein synthesis as measured by hot trichloroacetic acid precipitation; normalization relative to total cell number yielded virtually identical results (data not shown).

Immunofluorescent localization of the v-*erbA* proteins in infected fibroblasts was performed on Formalin-fixed and detergent-permeabilized cells as described, using an polyclonal antibody raised against a bacterially synthesized portion of the v-*erbA* polypeptide (4–6).

DNA binding by mutant and wild-type v-*erbA* proteins was determined by a McKay-type immunoprecipitation assay with two modifications from our previous protocol: KCl was used instead of NaCl in the DNA binding buffer, and the agarose gels were usually dried and autoradiographed directly, rather than first subjected to capillary blot transfers (4).

The ability of v-*erbA* to repress transcription of a TRE reporter gene was tested in transient transfections of CV-1 cells, using the same expression and reporter vector constructs previously described (8). Chloramphenicol acetyltransferase (CAT) reporter gene expression was quantitated by a solvent partitioning protocol (30).

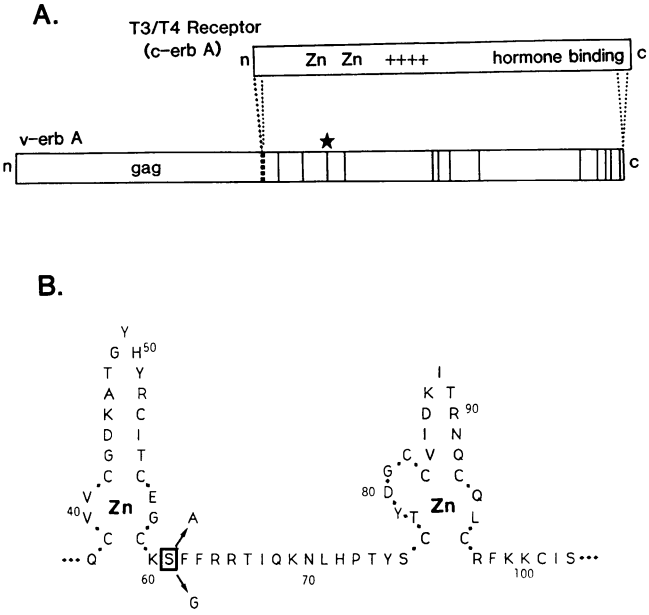

FIG. 1. Schematic of the v- and c-*erbA* proteins. (A) Comparison of the primary structures of the v- and c-*erbA* (T_3/T_4 receptor) proteins. The locations of two zinc finger domains (Zn), a highly basic domain (++++) that includes a nuclear localization signal, and the hormone binding domain are illustrated within the diagram of the c-*erbA* protein. Below, within the v-*erbA* protein schematic, are shown the locations of *gag* sequences, N- and C-terminal deletions, and internal amino acid sequence differences (vertical lines) relative to the c-*erbA* protein. The codon 61 position is starred. (B) Expanded schematic and sequence of the zinc finger domain of v-*erbA*. A highly schematic two-dimensional diagram of the two zinc fingers within the v-*erbA* protein is represented in single-letter amino acid code. The locations of the cysteines thought to be coordinated to zinc ions (Zn) are shown. Codon 61, a serine in the wild-type v-*erbA* protein, is boxed, and the two mutations analyzed in this report, serine to glycine (S61G), and serine to alanine (S61A), are indicated.

RESULTS

A sequence within the first zinc finger in v-*erbA* was altered to that of c-*erbA* and to that of the estrogen receptor gene. The serine-61 codon at the base of the first zinc finger in the v-*erbA* gene was changed to a glycine codon mimicking the c-*erbA* sequence at this site (designated S61G) or to an alanine codon mimicking the estrogen receptor sequence (designated S61A) (11; Fig. 1B). The genetic lesions were reconstructed into infectious clones of the full-length AEV genome and were introduced into chicken embryo fibroblasts in the presence of a RAV-1 genome; AEV is replication defective and requires an appropriate helper virus for production of infectious virus particles (17). Virus released by these primary transfectants was used to infect erythroid cells and secondary cultures of fibroblasts.

Fibroblasts infected by AEV possessing a wild-type or mutant v-*erbA* gene were first analyzed for the synthesis of the appropriate polypeptides by immunoprecipitation with *erbA*-directed serum and SDS-polyacrylamide gel electrophoresis (Fig. 2). Abundant proteins of the expected size for

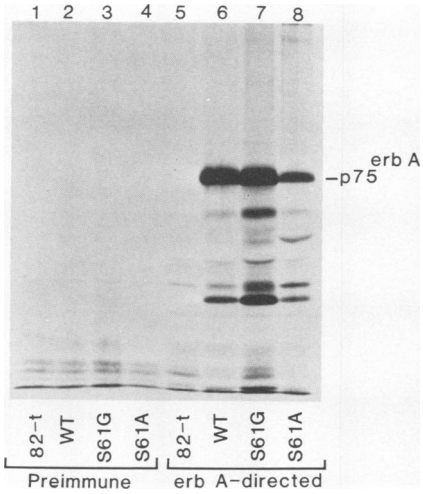

FIG. 2. Immunoprecipitation analysis of v-*erbA* proteins synthesized by the wild-type and codon 61 AEV mutants. Fibroblasts infected by the 82-t v-*erbA* null mutant (lanes 1 and 5), by wild-type (wt) AEV (lanes 2 and 6), by the S61G v-*erbA* mutant (lanes 3 and 7), or by the S61A v-*erbA* mutant (lanes 4 and 8) were metabolically radiolabeled with ^{35}S-amino acids for 2 h, washed, lysed by sonication, and analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Either preimmune serum (lanes 1 to 4) or *erbA*-directed antiserum (lanes 5 to 8) was used. The location of the 75,000-molecular-weight *gag*–v-*erbA* protein is indicated (p75^{erbA}). Each sample was normalized to total protein synthesis as determined by hot trichloroacetic acid precipitation.

the *gag*–v-*erb* A polypeptide (75,000 Da in apparent molecular size; designated p75$^{v\text{-}erbA}$) were detected in lysates of cells infected by the wild-type, S61G, and S61A viruses but not in cells infected by AEV bearing a null mutation in v-*erbA* (82-t; Fig. 2) or by the RAV-1 helper virus alone (data not shown). The 75,000-M_r proteins were not detected by preimmune serum (Fig. 2). The S61G and S61A mutant v-*erbA* proteins were synthesized at levels comparable to that of the wild-type protein; the slightly reduced level of the S61A protein seen in Fig. 2 was not a reproducible feature of our assays. Pulse-chase experiments demonstrated that the turnover rate of the mutant proteins was identical to or slightly slower than that of the wild-type protein (data not shown). These results indicate that neither the S61A nor the S61G mutation had adverse effects on the synthesis or the stability of the encoded polypeptides.

The mutant v-*erbA* proteins exhibited a subcellular localization identical to that of the wild type. Although the majority of the wild-type v-*erbA* protein is found in the nucleus of the infected cell, a consistent 30 to 40% of the v-*erbA* protein is found as a separate, nonexchangeable cytoplasmic subpopulation of unknown function (3, 5, 6). Analysis of linker insertion mutations has demonstrated that nuclear accumulation of the v-*erbA* protein is essential for its function in the neoplastic cell and requires both a nuclear targeting sequence, flanking codon 122 in the v-*erbA* sequence, and the integrity of the zinc finger (DNA binding) domains (5, 6). A similar phenomenon has been observed for the c-*erbA* protein (21).

Because of the close apparent linkage between DNA binding and nuclear accumulation, we tested the effects of our codon 61 mutations on the subcellular distribution of the v-*erbA* protein as visualized by immunofluorescence. As in previous studies, the majority of the wild-type v-*erbA* protein was found in the nucleoplasm of AEV-infected fibroblasts, with the remainder uniformly distributed in the cytoplasm (Fig. 3D). Both the S61G and S61A mutant v-*erbA* proteins exhibited a pattern virtually identical to that of the wild-type polypeptide (Fig. 3B and C), suggesting that neither genetic lesion had a significant effect on nuclear transport or retention. Cells infected by the 82-t null mutant and visualized with *erbA*-directed antibodies (Fig. 3A), or cells infected by the wild-type virus but visualized with preimmune serum (data not shown), demonstrated very low background levels of fluorescence.

Both the alanine and glycine v-*erbA* mutants exhibited altered DNA binding properties. We tested the effects of the S61G and S61A v-*erbA* mutations on DNA binding by use of a coupled DNA binding-immunoprecipitation (McKay-type) procedure. In this assay, a mixture of radiolabeled DNA restriction fragments was incubated with an unlabeled lysate of AEV-infected cells. The v-*erbA* protein, and any DNA fragments bound by it, were subsequently immunoprecipitated by *erbA*-directed antibodies and washed, and the bound DNA fragments were resolved by gel electrophoresis and visualized by autoradiography. As the substrate we used a restriction digest of the rat growth hormone gene, a thyroid hormone-responsive locus that contains several thyroid hormone receptor (c-*erbA* protein) binding sites (4).

As demonstrated in our previous work, the wild-type v-*erbA* protein bound most strongly to three particular fragments derived from the rat growth hormone gene: C, F, and H (Fig. 4). These three fragments contain known or near-consensus binding sites for the c-*erbA* protein, and we have previously shown that the binding of all three can be specifically abolished by use of an artificial TRE oligonucleotide. No DNA binding was seen with extracts of cells infected by the v-*erbA* 82-t null mutant (Fig. 4A), indicating that c-*erbA* proteins, expressed at very low levels in these cells, do not contribute to the observed DNA binding pattern.

Both the S61A and S61G mutant v-*erbA* proteins exhibited DNA binding patterns that were related, but not identical, to that displayed by the wild-type protein (Fig. 4). The S61A protein was most divergent in this assay from the wild-type protein, binding fragments C and F but not H. In addition, the S61A mutant protein strongly bound fragments A and B (derived largely from plasmid vector sequences) as well as weakly binding fragment D; none of these latter fragments were bound at significant levels by the wild-type v-*erb* A protein (Fig. 4). In contrast to the S61A mutation, the S61G mutation demonstrated a more subtle effect on DNA binding, exhibiting a consistent increase in the binding of fragment D and a reproducible decrease in the ability to bind fragments E and H relative to the wild-type v-*erbA* (Fig. 4). These alterations in the DNA binding patterns of the v-*erbA* mutant proteins most likely reflect a shift in their affinities for different subsets of binding sites rather than a general increase in non-sequence-specific DNA binding: the mutations do not lead to a general increase in the affinity of the v-*erbA* protein for all input DNA fragments, the DNA binding observed was stable to salt concentrations that eliminate nonspecific DNA binding by other v-*erbA* mutant proteins (6), and the observed binding was not readily abolished by the introduction of lambda DNA in the binding mixtures (data not shown). In addition to the qualitative changes detailed above, observable over a range of KCl concentrations, the S61G mutant appeared to display a higher salt optimum overall for DNA binding than did the wild-type protein (Fig. 5). Taken together, these results

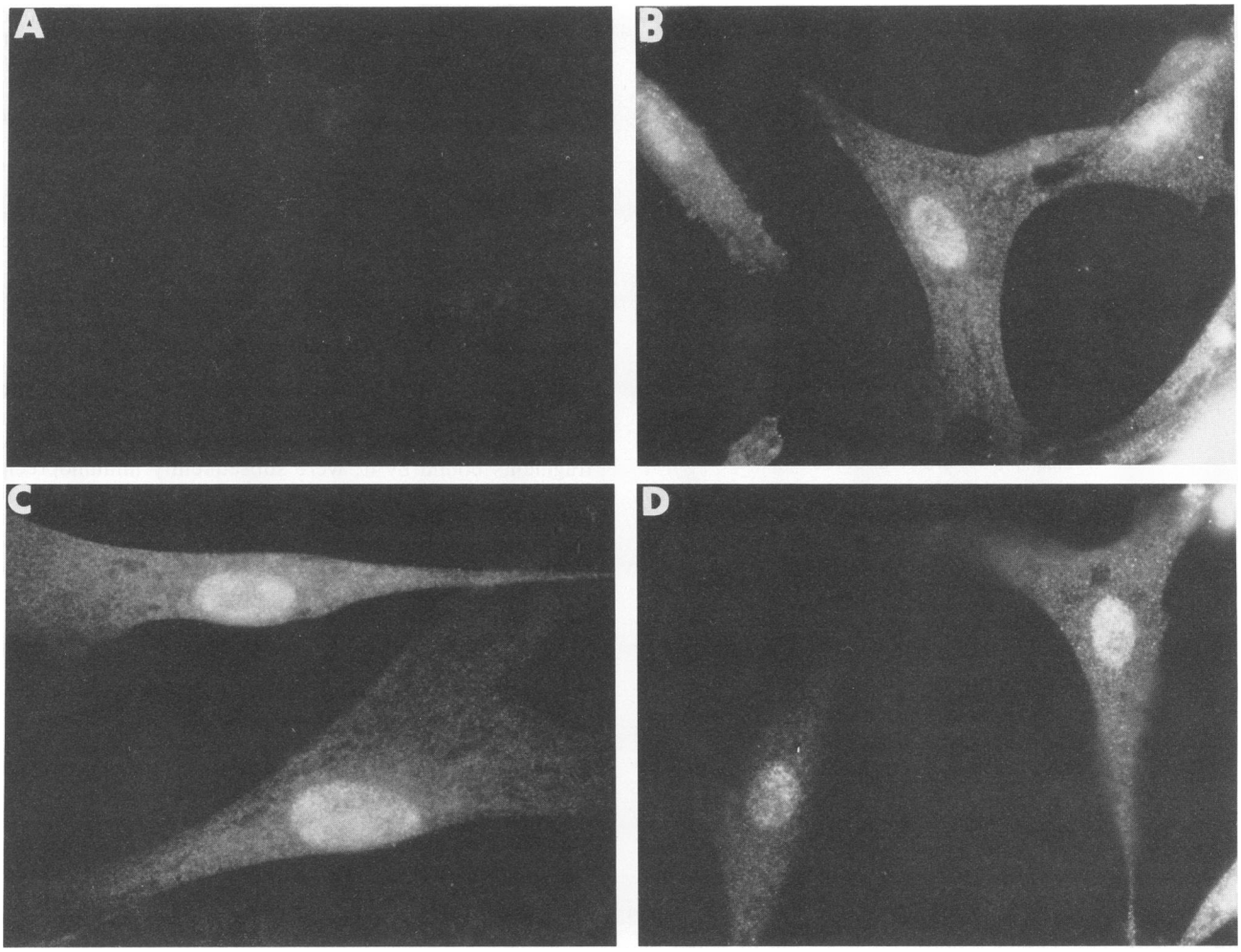

FIG. 3. Immunofluorescence analysis of the subcellular location of mutant and wild-type v-*erbA* proteins. Infected fibroblasts, grown on coverslips, were fixed with Formalin and permeabilized with Triton X-100. The location of the v-*erbA* protein was subsequently visualized by incubation of the fixed cells with *erbA*-directed antisera, followed by incubation with rhodamine-conjugated secondary antibody and photomicroscopy with epifluorescent illumination. (A) Cells infected by the 82-t (v-*erbA* null) mutant; (B) cells infected by the S61G mutant; (C) cells infected by the S61A mutant; (D) cells infected by wild-type AEV.

suggest that our mutant v-*erbA* proteins retain the ability to recognize DNA in a sequence-specific manner and that alteration of serine 61 has subtle but detectable effects on the DNA recognition properties of the v-*erbA* protein.

The S61G back mutation, but not S61A, severely impairs v-*erbA* function in transformed erythroid cells. The ability of our codon 61 mutant v-*erbA* proteins to function in erythroid transformation was tested by a methylcellulose colony assay: chicken bone marrow cells were infected by the different AEV viruses and were plated in medium supplemented only with fetal bovine serum, chicken serum, and tryptose phosphate broth. Only cells transformed by virus bearing a functional v-*erbA* gene, in addition to the v-*erbB* gene, can propagate and form a macroscopic colony under these conditions (7, 12, 23, 31). The wild-type virus gave rise to visible erythroid colonies in this assay, whereas the RAV-1 helper virus alone, or AEV bearing the v-*erbA* 82-t null mutation, yielded no colonies (Fig. 6 and 7).

The S61G back mutant was sharply impaired in its ability to function in erythroid transformation, producing approximately 15-fold-fewer colonies in our bone marrow assay than did the wild-type v-*erbA* virus (Fig. 6). The results of three independent assays are presented. The rare erythroid colonies that did appear in bone marrow assays of the S61G mutant were more diffuse and more loosely structured than those induced by the wild-type virus (Fig. 7). In contrast to erythroid transformation, fibroblast transformation by AEV is conferred principally by the v-*erbB* oncogene, and the v-*erbA* gene has only modest effects detectable on long-term propagation of fibroblasts in low-serum medium (12, 13, 16). Consistent with this observation, in high-serum medium the S61G AEV mutant induced the same transformed morphology and anchorage-independent growth properties in fibroblasts as did wild-type AEV and exhibited titers, measured as fibroblast-transforming units, equal to or greater than that of the wild-type virus (Fig. 6). These results confirm that the impairment of erythroid colony formation by the S61G mutation was due to a specific effect on v-*erbA* function and did not reflect an inhibition of viral replication or of v-*erbB* activity.

Unlike the S61G mutant, the S61A mutant was virtually fully functional in the erythroid assay, yielding bone marrow

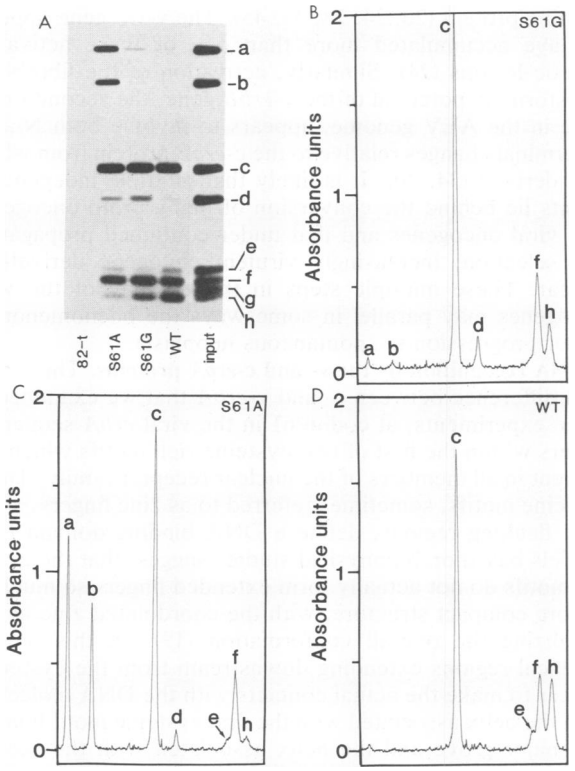

FIG. 4. DNA binding by mutant and wild-type v-*erbA* proteins. (A) Coupled DNA binding-immunoprecipitation (McKay) analysis. Lysates of fibroblasts infected by mutant or wild-type AEV were incubated with ^{32}P-labeled restriction fragments derived from the rat growth hormone gene (input). The v-*erbA* proteins, and any DNA fragments bound to them, were subsequently immunoprecipitated with *erbA*-directed antiserum and washed, and the bound DNA fragments were released and analyzed by agarose gel electrophoresis. An autoradiogram of the gel is presented; the different rat growth hormone DNA restriction fragments are labeled a to h, as in our previous report (4). Equal numbers of total ^{32}P counts were loaded for each sample. (B) Scanning densitometer analysis, in arbitrary absorbance units, of the DNA fragments bound by the S61G v-*erbA* protein. (C) Scanning densitometer analysis of the DNA fragments bound by the S61A v-*erbA* protein. (D) Scanning densitometer analysis of the DNA fragments bound by the wild-type v-*erbA* protein.

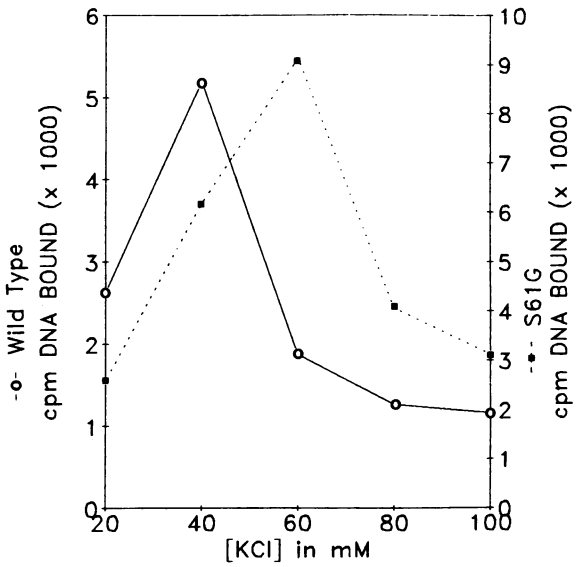

FIG. 5. Effects of KCl on DNA binding by the wild-type and S61G v-*erbA* mutant proteins. The McKay assay described in the legend to Fig. 4 was repeated with different KCl concentrations in the binding and wash buffers, and the total amount of DNA bound at each salt concentration was determined by a Cerenkov method. Data for DNA binding by the wild-type protein (—), and by the S61G protein (---) are shown.

colonies nearly equal in number and indistinguishable in appearance from those induced by the wild-type v-*erbA* protein (Fig. 6 and 7). Fibroblast transformation by the S61A mutant was also identical to that induced by wild-type virus (Fig. 6).

The S61G mutant is able to repress transcription despite its loss of function in erythroid transformation. The ability of the v-*erbA* protein to act as a transcriptional repressor has been proposed as the probable mechanism through which the viral oncoprotein blocks erythroid differentiation (8, 37, 47). We therefore tested the ability of our mutant proteins to serve as transcriptional repressors, and to inhibit the function of the c-*erbA* protein, in transient transfections assays (8). As previously noted, in the absence of hormone, both the c-*erbA* protein and the wild-type v-*erbA* protein functioned as repressors and inhibited basal expression of a TRE-linked reporter gene (Fig. 8A). Addition of thyroid hormone converted the c-*erbA* protein into a strong transcriptional activator, greatly stimulating expression of the TRE reporter gene, whereas the wild-type v-*erbA* protein remained a repressor independent of hormone status (Fig. 8A). Introduction of the wild-type v-*erbA* gene together with the c-*erbA* gene abolished c-*erbA*-mediated stimulation of reporter gene expression (Fig. 8B).

Both the S61G and S61A mutants functioned much like the wild type in the transcription assay, repressing basal expression of the TRE reporter gene both in the presence and in the absence of thyroid hormone (Fig. 8A). The S61A mutant behaved in a manner indistinguishable from that of the wild-type protein, whereas the S61G mutant exhibited a slight reduction in its ability to repress reporter gene expression relative to the wild-type protein. Both the S61A and S61G mutants were able to inhibit c-*erbA* action when viral and cellular genes were cointroduced into the CV-1 cells (Fig. 8B and data not shown). Inhibition by either the S61G mutant or the wild-type v-*erbA* gene demonstrated a very similar concentration dependence (Fig. 8B). These results indicate that the ability of the v-*erbA* protein to repress c-*erbA* function in transient transfection assays does not parallel the ability of v-*erbA* protein to function in the transformed erythroid cell and suggest that a more complex mechanism of v-*erbA* action may be operative in neoplasia.

DISCUSSION

Structural alterations involved in the etiology of the v-*erbA* oncogene protein. v-*erbA*, an altered host cell gene for a thyroid hormone receptor, blocks the differentiation of erythroid cells and alters the growth properties of fibroblasts in low-serum conditions (13, 16). What is the molecular basis behind the conversion of a thyroid hormone receptor into the v-*erbA* oncogene protein? It has been previously demonstrated that changes in the C terminus of the v-*erbA* polypeptide, consisting of a deletion and five amino acid alterations relative to the c-*erbA* protein, have impaired the viral

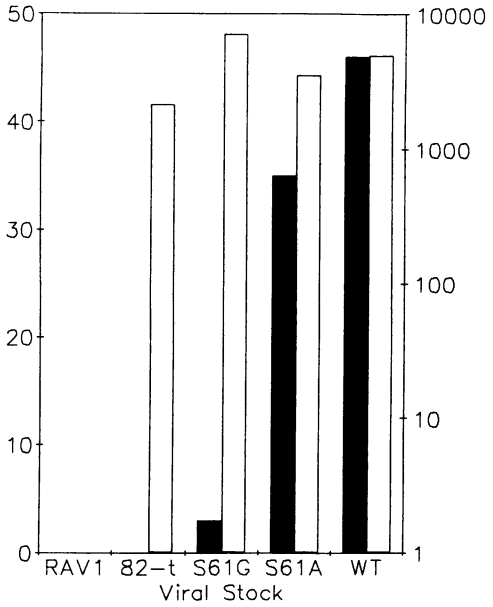

FIG. 6. Transforming properties of mutant and wild-type AEVs. Erythroid cell-transforming activity (■) was assayed by a methylcellulose medium-bone marrow assay; presented are the number of erythroid colonies induced by each virus stock per 35-mm dish (5×10^6 bone marrow cells). Fibroblast-transforming activity (□) was determined by serial dilution of each virus stock, followed by a soft agar colony–anchorage-independent growth assay; presented are the fibroblast-transforming titers (soft agar colonies per milliliter) for the same virus stocks as used in the erythroid assays. Both fibroblast and erythroid data represent the averages of two (S61A and 82-t) or three (RAV-1, S61G, and wild-type AEV) independent assays.

protein's ability to bind hormone (27). Unable to respond to thyroid hormone but able to bind to TRE sequences, the v-*erbA* protein functions as a constitutive repressor in animal cells (8, 37). This ability of v-*erbA* to act as a transcriptional repressor appears to be closely linked to its role in blocking erythroid differentiation: restoration of the c-*erbA* C terminus to the v-*erbA* protein restores hormone-stimulated transactivation to the chimeric protein and renders the *erbA*-mediated block to differentiation reversible by addition of thyroid hormones (47).

The work described here demonstrates that, in addition to the alterations sustained by the v-*erbA* C terminus, a single amino acid difference between v- and c-*erbA* proteins at the base of the first zinc finger motif has critical effects on the ability of v-*erbA* to participate in establishing the transformed phenotype. Thus, at least two structural changes, involving alterations in hormone response and perhaps in DNA recognition, appear crucial for the viral protein's actions in the neoplastic cell.

A clear precedent for multiple structural changes contributing to the activation of a proto-oncogene comes from analysis of the c-*src* protein. Although loss of a regulatory phosphotyrosine at position 527 in the c-*src* protein is sufficient to convert the relatively quiescent c-*src* polypeptide into an activated oncoprotein (reviewed in reference 22), additional changes at a number of different sites within the c-*src* coding region can either autonomously activate the oncogenic potential of c-*src* or act synergistically with the tyrosine 527 alteration to generate a more strongly transforming protein (20, 24, 28, 35, 45). The v-*src* gene appears to have accumulated more than one of these activating genetic lesions (24). Similarly, activation of the fibroblast-transforming potential of the v-*erbB* gene, the second oncogene in the AEV genome, appears to involve both N- and C-terminal changes relative to the c-*erbB* protein from which it is derived (34, 40). It is likely that multiple independent events lie behind the conversion of many proto-oncogenes into viral oncogenes and that under continued propagation and selection, increasingly virulent oncogene derivatives appear. These multiple steps in the etiology of the viral oncogenes may parallel in some ways the phenomenon of tumor progression in spontaneous neoplasia.

DNA recognition by the v- and c-*erbA* proteins. The amino acid difference between c- and v-*erbA* that we examined in these experiments, at codon 61 in the viral *erbA* sequence, occurs within the first of two cysteine-rich motifs which are present in all members of the nuclear receptor family. These cysteine motifs, sometimes referred to as zinc fingers, along with flanking regions, define a DNA binding domain (11). Models based on biophysical studies suggest that the cysteine motifs do not actually form extended fingers so much as a more compact structure, with the coordinated zinc atoms stabilizing the overall conformation (19). In this model, α-helical regions extending downstream from the cysteines appear to make the actual contacts with the DNA molecule, with the helix associated with the first cysteine motif lying in the major grove and the helix associated with the second cysteine motif contacting the DNA backbone.

Three amino acids in the first cysteine motif, which include the serine 61 of interest here, appear to have the strongest effects on the specificity of DNA recognition by the nuclear receptors; in the proposed three-dimensional structure of the cysteine motif domain, these three amino acids are in an appropriate position to make base-specific contacts with the DNA (9, 19, 26, 41). Thus, it is perhaps not surprising that alteration of serine 61 in the v-*erbA* protein can have measurable effects on the DNA binding properties of the polypeptide. In our hands, back mutation of the v-*erbA* serine to the c-*erbA*-encoded glycine is associated with a higher salt optimum for DNA binding. This elevated salt optima may not necessarily reflect an increased affinity constant for DNA but is consistent with a previous observation that c-*erbA* protein binds TREs with higher apparent affinity than does the wild-type v-*erbA* protein (37).

The ability to bind to individual rat growth hormone restriction fragments also appears to differ, if subtly, between the wild-type and codon 61 mutant v-*erbA* proteins. Naturally occurring TREs, such as in the rat growth hormone gene used in our studies, share common sequence motifs but nonetheless diverge in their exact sequences and in their relative affinities for the thyroid hormone receptor. The disparities in the binding patterns of our position 61 mutant and wild-type proteins are presumably reflections of these differences in the individual rat growth hormone TRE sequences and their relative abilities to interact with the different amino acids introduced at position 61. The low abundance of c-*erbA* protein in most cells has made it difficult to directly test whether the DNA binding pattern manifested by the S61G back mutation resembles that of the c-*erbA* protein, and further studies using a baculovirus expression system are under way. Intriguingly, however, one effect of the S61G mutation is an enhanced binding of fragment D from the rat growth hormone gene; the c-*erbA* protein is known to bind to sites within fragment D, whereas

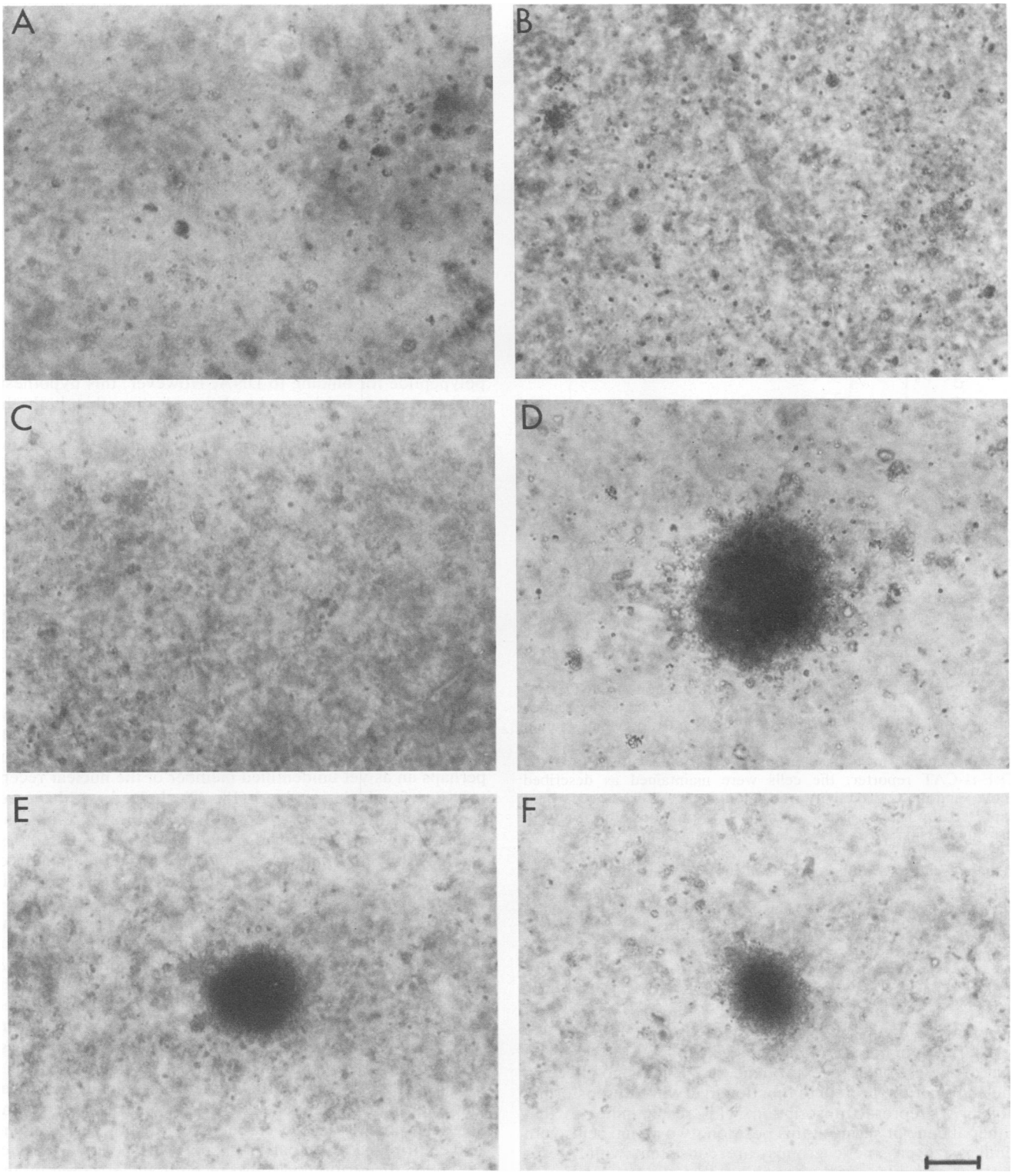

FIG. 7. Erythroid colonies induced by mutant and wild-type AEV. Micrographs of bone marrow-methylcellulose assays performed by using mutant or wild-type AEV stocks are shown. (A) Typical field resulting from infection with the RAV-1 helper alone; (B) typical field resulting from infection with the 82-t v-*erbA* null mutant; (C) typical field resulting from infection with the S61G mutant; (D) rare, diffuse erythroid colony resulting from infection with the S61G mutant; (E) typical erythroid colony resulting from infection with the S61A mutant; (F) typical erythroid colony resulting from infection with wild-type AEV.

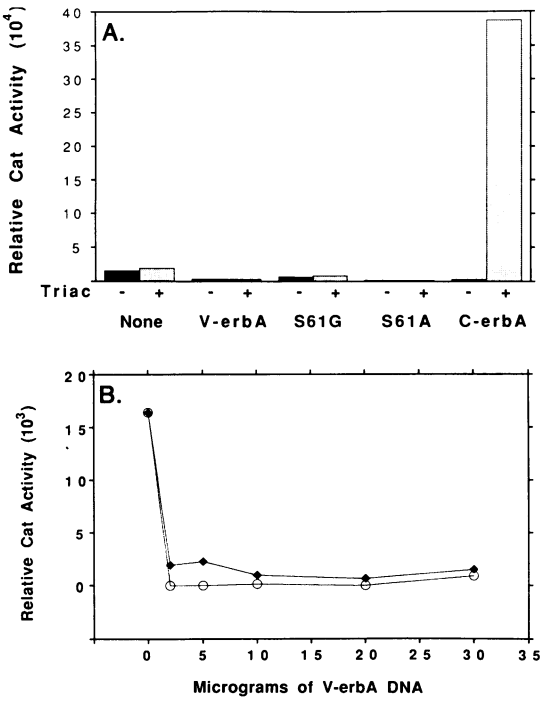

FIG. 8. Transcriptional repression mediated by wild-type and mutant v-*erbA* proteins. Molecular clones of c-*erbA* or v-*erbA* were introduced into CV-1 cells together with a TRE-*tk*-CAT reporter gene by a calcium chloride technique (8). After 12 h, the cells were transferred to hormone-stripped medium (−) or to medium containing 10 μM triac (+), incubated for an additional 36 h, and assayed for CAT activity as described in Materials and Methods. CAT activity is expressed relative to that of a Rous sarcoma virus-*lacZ* construct cotransfected into the same cells as an internal control. (A) Effects of different c- and v-*erbA* constructs on expression of the TRE-*tk*-CAT reporter gene. Fifteen micrograms of either carrier DNA alone (None), the wild-type v-*erbA* gene, the serine 61-to-glycine mutant (S61G), the serine 61-to-alanine mutant (S61A), or the c-*erbA* gene were individually introduced into CV-1 cells along with 7.5 μg of the TRE-*tk*-CAT reporter, the cells were maintained as described above, and the CAT activity was determined. All *erbA* genes were expressed by using a Rous sarcoma virus-promoter construct (8). (B) Suppression of c-*erbA* action by cotransfection of v-*erbA*. A fixed amount of c-*erbA* DNA (5 μg) was introduced into CV-1 cells in the presence of various amounts of cotransfected wild-type (○) or S61G (◆) v-*erbA* DNA. All cultures were treated with 10 μM triac hormone, the cells were harvested 32 h later, and the CAT activity, relative to that of an internal Rous sarcoma virus-*lacZ* control, was determined.

the wild-type v-*erbA* protein recognizes this fragment poorly or not at all (4, 14, 25).

Alteration of the v-*erbA* sequence at codon 61 to resemble that of the estrogen receptor gene had little or no effect on the ability of the protein to function in erythroid transformation or to repress transcription. This result indicates that either alanine or serine at this position, two amino acids with similar-size prosthetic groups, are consistent with v-*erbA* function and rules out the possibility that phosphorylation of the wild-type v-*erbA* serine is in some manner responsible for its biological activity. It is perhaps intriguing that both the estrogen receptor and the v-*erbA* protein appear to share the ability to bind to TREs but not to activate transcription from these sites (15). The relationship of this observation, however, to structure of these receptors and to regulation of transcription remains unclear; despite the resemblance of the biological phenotype of the S61A mutation to that of the wild-type gene, its DNA binding properties in our McKay-type assay are different in detail from those of the wild-type v-*erbA* protein.

It is perhaps important to note that DNA binding by the v-*erbA* protein may not be a fully autonomous function: sequence-specific DNA binding by the v-*erbA* protein appears to be enhanced by the presence of other, host cell factors (4); the effects of our v-*erbA* mutations on on DNA binding may reflect changes in protein-protein interactions as well as direct effects on DNA recognition.

What is the target of v-*erbA* action in the neoplastic cell? It is intriguing that efficient v-*erbA* function in the transformed cell depends, in part, on a change in the sequence of its DNA binding domain from that of the c-*erbA* gene. One possible explanation is that the serine in the wild-type v-*erbA* protein confers a higher affinity for TREs and thereby permits the viral protein to more effectively compete with the c-*erbA* polypeptide for binding to DNA. However, this hypothesis does not agree with results demonstrating that both the c-*erbA* protein and the S61G v-*erbA* back mutant can actually bind certain TRE sequences more tightly than can the wild-type v-*erbA* protein and that the S61G mutant efficiently interferes with c-*erbA* action in transient transfections.

We suggest that the change of v-*erbA* codon 61 to a serine may have altered the DNA binding characteristics of the v-*erbA* polypeptide to permit it to promiscuously recognize an alternative hormone response element. Thus, the v-*erbA* protein may operate in neoplasia not by blocking the action of c-*erbA* proteins (thyroid hormone receptors) but by interfering with the actions of some other member of the steroid/thyroid hormone/retinoic acid receptor family of transcription factors. Because of their known roles in differentiation and close similarities to the thyroid hormone receptors, the retinoic acid or vitamin D receptors may represent plausible targets for this promiscuous interference by v-*erbA*, or perhaps an as yet unidentified member of the nuclear receptor superfamily is involved (18, 42). Indeed, we have recently determined that the wild-type v-*erbA* protein can efficiently block the action of retinoic acid receptors, whereas the S61G mutant cannot, suggesting that the target of v-*erbA* action in the neoplastic cell may be a retinoic acid-mediated differentiation process (39a).

Our data and general conclusions are consistent with the observations of Zenke et al. (47), who demonstrated that chimeric constructs of the c-*erbA* gene containing the DNA binding domain of v-*erbA* induced a partial block to erythroid differentiation distinct in phenotype from that induced either by authentic c-*erbA* or by authentic v-*erbA*. The analyzed construct contained v-*erbA* sequences encompassing both the site of our own mutation, at codon 61, and a second site, codon 87, at the base of the second zinc finger that also differs in sequence between v- and c-*erbA*. It was not determined in these earlier experiments which amino acid contributed to the partial phenotype. The importance of the codon 61 position was also recently underscored by the results of de Verneuil and Metzger, demonstrating that a thyroid hormone receptor-estrogen receptor chimera bearing a glycine at this location functioned as an efficient transcriptional activator, whereas substitution of a serine into this position inhibited the transcriptional activity of the chimera (10).

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