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Genetic Dissection of Functional Domains within the Avian Erythroblastosis Virus v-*erbA* Oncogene

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The avian erythroblastosis virus v-*erbA* locus potentiates the oncogenic transformation of erythroid and fibroblast cells and is derived from a host cell gene encoding a thyroid hormone receptor. We report here the use of site-directed mutagenesis to identify and characterize functional domains within the v-*erbA* protein. Genetic lesions introduced into a putative hinge region or at the extreme C-terminus of the v-*erbA* coding domain had no significant effect on the biological activity of this polypeptide. In contrast, mutations introduced within the cysteine-lysine-arginine-rich center of the v-*erbA* coding region, a DNA-binding domain in the thyroid and steroid hormone receptors, abolished or severely compromised the ability of the viral protein to function. Our results suggest that the mechanism of action of the v-*erbA* protein in establishing the neoplastic phenotype is closely related to its ability to interact with DNA, presumably thereby altering expression of host target genes by either mimicking or interfering with the action of the normal c-*erbA* gene product.

Avian erythroblastosis virus (AEV) induces both fibrosarcomas and erythroleukemia in susceptible animals (4, 13). The genome of the ES-4/R strain of AEV contains two loci, v-*erbA* and v-*erbB*, that are involved in virus-mediated oncogenesis. The AEV v-*erbB* oncogene is a virally transduced derivative of a cellular gene encoding the epidermal growth factor receptor and is absolutely essential for virus-mediated oncogenesis (6, 27, 31). In contrast, the AEV v-*erbA* gene is derived from one of a family of genetic loci which encode thyroid hormone receptors and is more distantly related to genes for steroid and retinoid receptors (9, 15, 22, 26, 33, 34). The viral *erbA* coding sequence is slightly truncated relative to that of its cellular progenitor (termed c-*erbA*), and 13 internal amino acid changes have also occurred between the chicken cellular and viral *erbA* protein sequences (26). As a consequence of these structural divergences, the viral polypeptide, unlike the cellular *erbA* protein, does not bind thyroid hormones (20, 26).

The v-*erbA* gene is not essential for AEV-mediated oncogenesis (6, 12). However, immature erythroid cells transformed by v-*erbB* in the absence of v-*erbA* tend to spontaneously differentiate along the erythroid pathway, demonstrate poor growth properties in culture, and require highly supplemented medium for propagation in vitro (6, 12, 35). In contrast, erythroid cells transformed by v-*erbB* in the presence of a functional v-*erbA* gene remain tightly blocked in an immature, highly proliferative state and can be propagated under relatively simple culture conditions (6, 12, 35). Subtle effects of v-*erbA* on the proliferative properties of fibroblasts have also been reported (7).

The actual mechanism of action of the v-*erbA* gene product is not well understood. If analogies can be drawn from the action of the steroid and thyroid hormone receptors, the v-*erbA* protein may function in oncogenesis by modulating transcription of specific target genes in the host cell, perhaps by mimicking the action of an activated receptor bound to hormone (11, 26, 34). However, alternative models of v-*erbA* action have also been proposed which suggest v-*erbA* may act as a disfunctional receptor interfering with, rather than mimicking, the action of its normal cellular homolog (1, 35). In addition, many of the details of thyroid receptor action at the molecular level remain poorly understood and are themselves largely drawn by extrapolation from the steroid receptor paradigm.

To better understand the relationship between the mechanism of action of the v-*erbA* protein and that of the steroid and thyroid hormone receptors, we have created a series of genetic lesions in the v-*erbA* coding region and tested the abilities of the resulting mutants to potentiate erythroid transformation and to modify the growth properties of fibroblasts. Our results indicate that the highly basic and cysteine-rich domains within the v-*erbA* protein, representing potential DNA-binding regions, are necessary for v-*erbA* function. Portions of the v-*erbA* protein derived from the T3-thyronine-binding domain of c-*erbA* also appear to be required for full v-*erbA* activity. These results favor a model in which the v-*erbA* protein functions in oncogenesis as a modulator of transcription, interacting with and presumably altering the expression of host cell target genes.

MATERIALS AND METHODS

Molecular clones, virus stocks, and cells. In addition to those described previously, two new plasmid constructs were created: a pBR322 subclone of the 2.8-kilobase (kb) *Eco*RI-*Sal*I fragment of the AEV DNA genome containing *gag* and N-terminal v-*erbA* sequences, and a version of the entire pAEV-11-3 infectious clone (referred to as pAEV-11-3L) which lacks the right-hand *Eco*RI site (created by filling in this restriction site and religating the DNA) (21, 27, 32).

In-frame insertions in v-*erbA* were created by the introduction of *Hpa*I hexanucleotide linkers (GTTAAC) into *Alu*I, *Hae*III, *Rsa*I, or *Eco*RV restriction sites in different regions of the v-*erbA* protein-coding domain (21, 29). The site of each insertion mutation was identified by restriction endonuclease mapping and was confirmed by DNA sequence analysis (21). Each of the insertion mutations, created within plasmid subclones of the v-*erbA* sequence, was reconstructed into an infectious form of the entire AEV genome (either pAEV-11-3R or pAEV-11-3L) containing the v-*erbB* gene (21). Infectious virus stocks were subsequently obtained by transfection of the mutant DNA clones into

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avian fibroblasts in the presence of a Rous-associated virus 1 (RAV-1) helper virus genome as described previously (21).

Chicken secondary cells consisting mainly of fibroblasts were obtained from 10-to-12-day-old SPAFAS embryos and were propagated in DME8+1 medium (Dulbecco modified Eagle medium supplemented with 10% tryptose phosphate broth, 8% fetal bovine serum, 1% heat-inactivated chicken serum, 1 mg of streptomycin per ml, 100 U of penicillin per ml, and 2.5 μg of amphotericin B per ml) in a 0.2% bicarbonate–5% carbon dioxide buffering system.

Assays for oncogenic transformation. The ability of our different mutants to transform fibroblasts to anchorage-independent growth was determined by a soft agar colony assay as described previously (21). The ability of the mutant viruses to transform immature erythroid cells was determined by a slight modification of a previously published protocol (14). Bone marrow cells were isolated from 6- to 10-day-old SPAFAS chickens, and erythrocytes were separated by and discarded after centrifugation (1,200 × *g* for 20 min) through a 1.077-density Histopaque (Sigma Chemical Co.) cushion. The erythrocyte-depleted bone marrow cells atop the Histopaque cushion were resuspended in DME8+1 medium containing Polybrene (4 μg/ml), the cells were mixed with appropriate stocks of mutant or wild-type AEV, and the mixtures were incubated at 37°C for 1 h with gentle rocking. The infected bone marrow cells (10^7 per assay) were subsequently concentrated by centrifugation (1,000 × *g* for 10 min), suspended in 1 ml of DME8+1 medium, and mixed with 4 ml of methylcellulose medium (2% methylcellulose, 1× Dulbecco modified Eagle medium, 5% tryptose phosphate broth, 2.5% bovine calf serum, 1% heat-inactivated chicken serum, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer, 0.1 mM 2-mercaptoethanol, 0.1% sodium bicarbonate, and 0.2% bovine embryo extract). Each assay mix was transferred into a 35-mm tissue culture dish and incubated at 39°C in a 5% CO_2 atmosphere. Cultures were usually fed with fresh methylcellulose medium every 7 to 10 days. Macroscopic erythroid colonies were scored after 10 to 21 days.

The oncogenicity of wild-type and mutant AEV stocks was determined by inoculating 1-day-old SPAFAS chickens with 0.1 ml of virus stocks obtained from the culture medium of AEV-infected fibroblasts. Animals were injected in the wing web, and development of disease was monitored by palpation (for detection of fibrosarcomas), necropsy, and periodic blood smears (for detection of erythroblastosis).

RESULTS

In-frame insertion mutations were created in different domains of v-*erbA*. The v-*erbA* coding region can be conceptually divided into several domains based on its structural relatedness to the steroid and thyroid hormone receptors: a cysteine-rich domain that has been hypothesized to contain two "zinc finger" DNA-binding motifs, a larger lysine- and arginine-rich domain that may also be involved in interactions of the v-*erbA* protein with nucleic acids, and a C-terminal domain which, in the c-*erbA* protein, appears to potentiate binding to the T3/T4 thyronine ligand (Fig. 1) (5, 15, 20, 26, 33, 34). In addition to sequences derived from the c-*erbA* locus, the 75,000-molecular-weight v-*erbA* protein also contains an N-terminal domain encoded by retrovirus-derived *gag* polypeptide sequences (Fig. 1).

To dissect the actual contribution of each of these presumptive domains to v-*erbA*-mediated erythroid transformation, we used the technique of oligonucleotide-linker mutagenesis to introduce small in-frame insertions at different points in the v-*erbA* sequence (Fig. 1). The resulting amino acid sequences introduced at these sites, predicted from DNA sequence analysis, are presented in Table 1. In two mutations analyzed, insertion of the GTTAAC linker introduced a premature termination codon (at positions 82 and 907). These latter termination mutations are denoted with a letter t in Fig. 1.

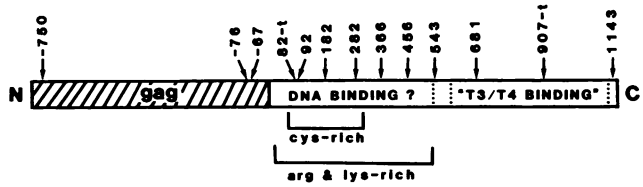

FIG. 1. Schematic representation of the *gag*-v-*erbA* protein. The 75,000-molecular-weight *gag*-v-*erbA* protein is depicted from N-terminus (N) to C-terminus (C). The domain contributed by retroviral *gag* sequences is indicated (cross-hatched), as are two nested domains within the v-*erbA* sequence which are high in cysteines and in arginines and lysines and which may constitute a DNA-binding site. Also indicated is a C-terminal domain which, in the thyroid hormone receptor, is thought to be involved in the binding of the T3/T4 thyronine hormone ligand. The sites of our various insertion mutations are indicated above, expressed as the distance in base pairs from the beginning of the v-*erbA*-specific sequence. Mutations in the *gag* domain were therefore assigned negative numbers. The suffix t indicates mutations that result in premature termination of translation at those sites (see Table 1).

All mutant viruses transformed avian fibroblasts. Each of our v-*erbA* insertion mutations was reconstructed into a complete copy of the AEV genome containing a functional v-*erbB* oncogene and transfected into avian fibroblasts in the presence of an RAV-1 genome. AEV is replication defective and requires a suitable helper virus, such as RAV-1, to produce infectious virus particles (13). Virus stocks obtained from these primary transfectants were subsequently used to infect fresh fibroblasts or erythroid cells.

The v-*erbA* locus of AEV is not required for the oncogenic transformation of fibroblasts, although effects of v-*erbA* on

TABLE 1. Changes in the v-*erbA* polypeptide sequence at insertion mutation sites

Site of insertion[a]	Resulting amino acid sequence[b]
−76	Glu-Glu-(VAL-ASN)$_5$-Leu-Ala
−67	Ala-Ser-(VAL-ASN)$_6$-Thr-Gly
82-t	Gly-CYS-TAA
92	Pro-ARG-(LEU-THR)$_9$-Cys-Leu
182	Cys-Lys-ARG-(LEU-THR)$_3$-Phe-Phe
282	Cys-Gln-(VAL-ASN)$_4$-Leu-Cys
366	Arg-Lys-(VAL-ASN)$_3$-Leu-Ile
456	Trp-Glu-(VAL-ASN)$_5$-Leu-Ile
543	Glu-Asp-VAL-ASN-Ile-Gly
681	Ser-Glu-(VAL-ASN)$_{10}$-Leu-Pro
907-t	Glu-Val-GLY-TAA
1143	Thr-Glu-(VAL-ASN)$_2$-Leu-Ser

[a] The position of each insertion is expressed as the distance in base pairs from the start of the v-*erbA* sequence; insertions in the *gag* domain are therefore assigned negative numbers.

[b] The number of GTTAAC hexanucleotide linkers inserted at each site and their position relative to the wild-type v-*erbA* sequence were determined by DNA sequence analysis. The resulting alterations to the v-*erbA* protein sequence at the site of each insertion mutation are indicated. New amino acid codons (or TAA termination codons) introduced into the v-*erbA* protein by the linker insertions are indicated in all uppercase letters. The number of dipeptide linkers inserted at each site is indicated by the numerical subscript. The wild-type v-*erbA* sequence was obtained from Damm et al. (3).

TABLE 2. Ability of v-*erbA* mutant viruses to transform bone marrow and fibroblast cells in vitro

Virus stock	Domain mutated	Erythroid transformation[a] (no. of colonies)	Fibroblast transformation[b] (no. of colonies)
RAV-1 only		0	0
v-*erbA* deletion[c]		0	197
Wild type		64	79
Insertion mutants			
−750	*gag*	41	29
−76	*gag*	103	47
−67	*gag*	29	18
82-t	DNA binding	0	37
92	DNA binding	0	67
182	DNA binding	2 (s)	57
282	DNA binding	1	68
366	DNA binding	2	43
456	DNA binding	0	61
543	Hinge	27	87
681	T3/T4 binding	12 (s)	20
907-t	T3/T4 binding	16 (s)	69
1143	C-terminus	69	65

[a] Erythroid transformation was measured as the ability of each virus stock to induce erythroid colony formation in the unsupplemented methylcellulose medium–bone marrow assay. The average number of erythroid colonies per assay is presented (two to six assays were performed on each virus). AEV mutants that yielded colonies significantly smaller in size than those induced by the wild-type virus are indicated (s).

[b] Fibroblast transformation was determined by using the same virus stocks used to measure erythroid transformation. The ability of each virus stock to induce anchorage-independent growth of fibroblasts (colony formation in soft agar) was assayed; the number of soft agar colonies induced per 0.01 ml of each virus stock is presented (average of two to six assays).

[c] A stock of AEV bearing a large deletion within the *gag*-v-*erbA* sequences (referred to as the *erbA* dl mutation in Sealy et al. [28]) was used as a v-*erbA*-negative control.

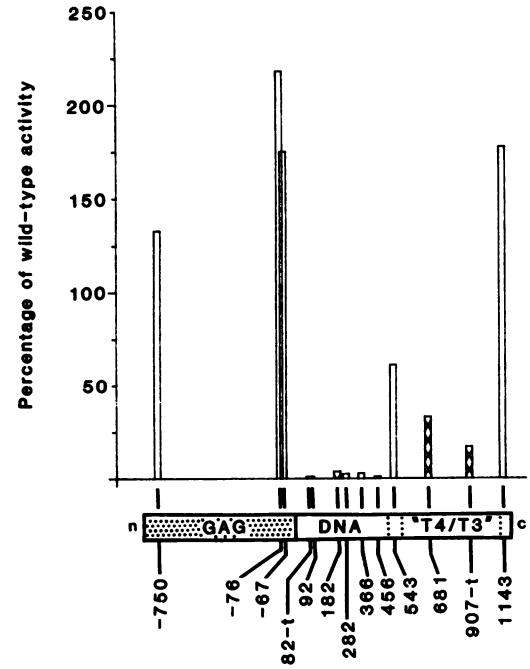

FIG. 2. Ability of the v-*erbA* insertion mutants to potentiate erythroid transformation. The biological activity of each of our insertion mutants was assayed by measuring the ability to induce erythroid colony formation in an unsupplemented bone marrow assay. The number of erythroid colonies induced by each mutant was normalized to the fibroblast-transforming titer of the same virus stock (erythroid colonies/fibroblast colonies). In this manner the effects of slight variations in the titers of the individual recovered virus stocks (Table 2) were taken into account. This normalized erythroid-transforming ability of each viral mutant, a measure of v-*erbA* activity, was subsequently expressed as a percentage of the wild-type activity determined in the same assay in the same manner. The results shown represent a compilation of from two to six separate assays with each viral mutant. Cross-hatched bars indicate mutants that yield erythroid colonies significantly smaller than those induced by wild-type virus (see Fig. 3). The position of each insertion mutation is displayed below the graph and is plotted relative to a schematic of the v-*erbA* protein-coding domain. Potential DNA-binding (DNA) and *gag*-derived (GAG) domains are indicated, as are sequences derived from the T4/T3 thyronine-binding domain of the c-*erbA* protein (T4/T3).

the growth properties of these cells have been reported (6, 7, 12, 27, 28). All of our AEV v-*erbA* mutants possessed a functional v-*erbB* gene and, as expected, produced a transformed morphology in infected fibroblasts and conferred anchorage-independent growth on these cells (Table 2). These properties confirmed that each of our reconstructed AEV genomes was infectious and that the function of the v-*erbB* oncogene was preserved.

Effect of insertions into *gag*, hinge, and C-terminus regions. One of the principal effects of v-*erbA* expression is to block transformed erythroid cells in an immature differentiation state. One important phenotypic consequence of this phenomenon is that erythroid cells transformed in the presence of v-*erbA* retain high proliferative capacity and can readily propagate in culture in a relatively simple medium. Erythroid cells infected by AEV lacking a functional v-*erbA* gene tend to terminally differentiate and are compromised in their ability to propagate in culture, requiring carefully defined pH and a variety of medium supplements (3, 6, 12, 17, 35). We used these properties as the basis for our initial screening of the phenotype of our mutants.

Chicken bone marrow cells were infected by stocks of our different v-*erbA* AEV mutants and suspended in methylcellulose medium containing serum and tryptose phosphate broth but no other supplements. It has been reported that the ability of AEV-infected erythroid cells to propagate under these conditions is closely linked to the presence of a functional v-*erbA* gene product (3, 6, 12, 17). Bone marrow cells infected either by the RAV-1 helper virus alone or by a deletion mutant of AEV which is unable to synthesize a detectable v-*erbA* protein failed to form visible colonies in our assay, as predicted from the work cited previously (Table 2). In addition, a linker insertion which introduced a termination codon at position 82, able to express only 27 residues of the 387-amino-acid-long *erbA* domain, also failed to induce detectable erythroid colonies under our assay conditions (Table 2 and Fig. 2). Both of these mutant viruses contained fully functional v-*erbB* genes and transformed fibroblasts to anchorage-independent growth at near wild-type levels (Table 2).

Three separate insertion mutations in the *gag*-specific domain of the *gag*-v-*erbA* fusion polypeptide produced viruses fully capable of inducing erythroid colonies in our assay (Table 2, Fig. 2 and 3), confirming that the *gag*-derived sequences do not appear to be required for v-*erbA* activity. These results are in contrast to those obtained with the *gag* fusion proteins encoded by the v-*abl* and v-*fps* oncogenes, in which *gag*-related sequences do appear to be necessary for function, possibly by stabilizing the oncogene polypeptide or by contributing necessary subcellular localization signals (24, 29). An insertion at position 543, within a domain that has been described as a putative hinge region between the

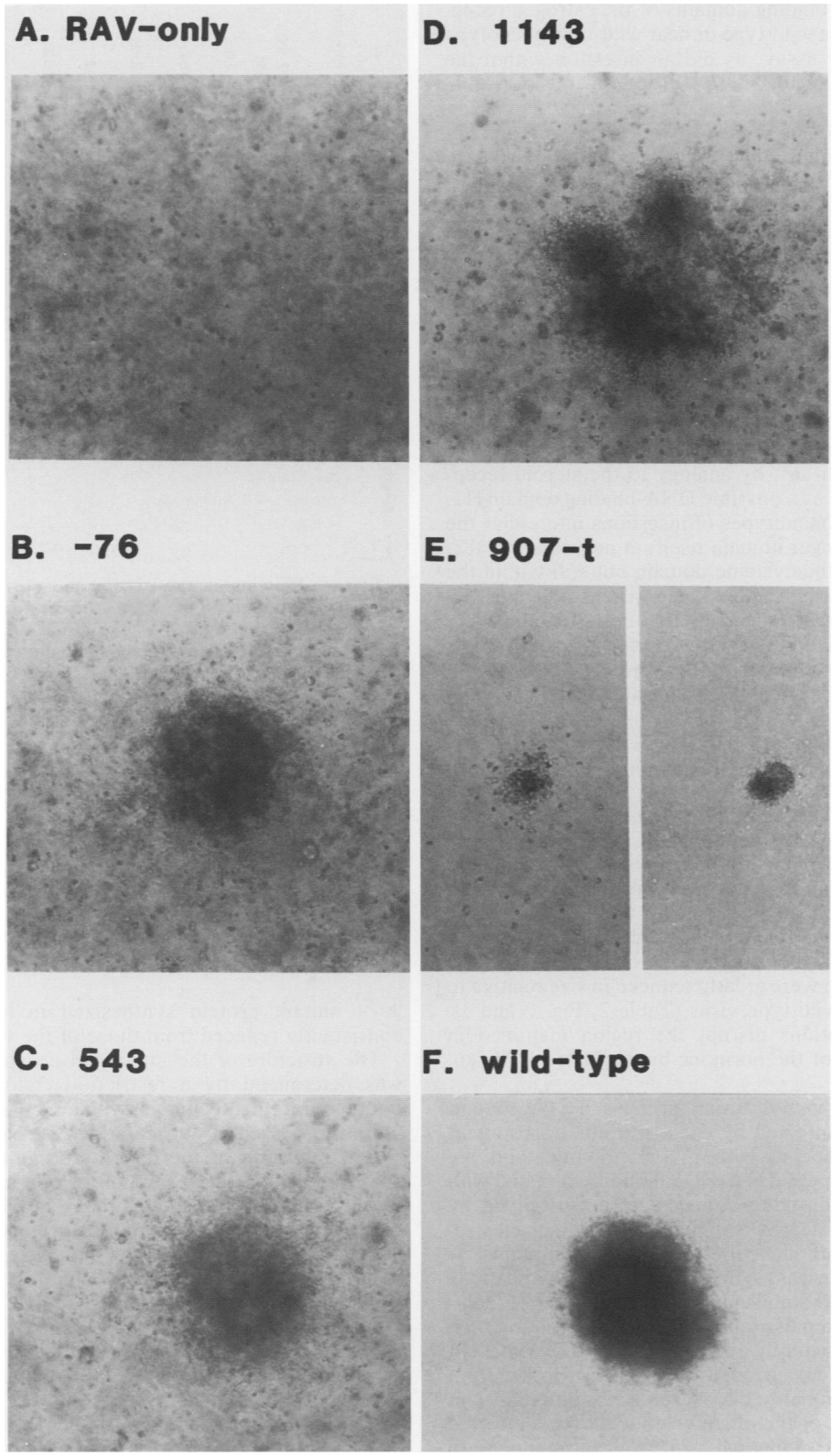

FIG. 3. Erythroid colonies induced by v-*erbA* mutant viruses in unsupplemented methylcellulose medium. Representative photomicrographs are presented of erythroid colonies induced by wild-type AEV or by AEV genomes bearing various insertions in the *gag*-v-*erbA* coding domain. Included are colonies induced by viruses bearing insertions in the *gag* domain (at position −76), in the putative hinge region between the DNA-binding and hormone-binding domains of v-*erbA* (at position 543), near the extreme C-terminus of v-*erbA* (at position 1143), and at position 907. The latter mutation (907-t) results in a prematurely truncated v-*erbA* polypeptide lacking 84 C-terminal amino acids; two fields are presented to illustrate the significantly reduced size of these colonies. Representative fields from an RAV-1-only assay (a negative control) and a wild-type AEV-infected cell assay (a positive control) are also shown for comparison.

DNA- and hormone-binding domains of the estrogen receptor (18), also yielded a wild-type or near wild-type phenotype in our bone marrow assay, as did an insertion within the extreme C-terminus of the v-*erbA* protein at position 1143 (Table 2 and Fig. 2).

The erythroid colonies induced by these v-*erbA* mutant viruses were indistinguishable in morphology and in size from those induced by wild-type virus (Fig. 3), and cells picked from these colonies propagated in liquid medium with growth properties similar to those displayed by erythroid cells transformed by wild-type virus (data not shown), suggesting that the integrity of these domains was not essential for v-*erbA* function.

Insertions into the DNA-binding and the remnants of the hormone-binding domains. Five different in-frame insertions, at postions 92, 182, 282, 366, and 456, abolished or severely impaired the ability of the mutated viruses to induce erythroid colonies in the unsupplemented assay. These five distinct genetic lesions all mapped within a domain of the v-*erbA* protein which has, by analogy to the steroid receptors, been implicated as a possible DNA-binding domain (11, 15, 16, 18, 33). The phenotypes of insertions into either the cysteine-rich, zinc finger domain itself, at positions 92, 182, and 282, or outside the cysteine domain but still within the lysine- and arginine-rich regions, at positions 366 and 456, were virtually identical, suggesting that both domains were necessary for potentiation of erythroid transformation. Cells derived from the rare colonies induced by these DNA-binding domain mutants failed to propagate in liquid medium (data not shown).

Although derived from a cellular gene for a thyroid hormone receptor, both point and deletion mutations appear to render the viral *erbA* protein incapable of binding thyroid hormone (20, 26). Despite this apparent lack of hormone binding by the viral polypeptide, the region of v-*erbA* derived from the hormone-binding domain of c-*erbA* still appears to be important for full v-*erbA* protein funcion. Both the in-frame insertion at position 681 and the premature termination mutation at position 907 produced relatively low numbers of bone marrow colonies in our assay, and the colonies that did form were greatly reduced in size relative to those produced by wild-type virus (Table 2, Fig. 2 and 3). Both of these mutations disrupt the region identified in c-*erbA* as a portion of the hormone-binding site (8, 11, 16, 18).

Production of *gag*-v-*erbA* fusion proteins by the mutant viruses. Fibroblasts infected by each of the v-*erbA* mutant viruses were radiolabeled with [^{35}S]methionine, and the v-*erbA*-encoded polypeptides were immunoprecipitated with antisera directed against the *gag* domain and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 4A). All of the in-frame insertion mutants, as well as the wild-type virus, synthesized roughly comparable amounts of the 75,000-molecular-weight *gag*-v-*erbA* polypeptide during the 2-h pulse label (the modest variation seen in Fig. 4 was not consistently detected in other experiments). The v-*erbA* polypeptides encoded varied slightly in size from mutant to mutant, probably due to the slight differences in the number and sites of the oligonucleotide linkers inserted in each mutant gene. Identical p75$^{gag\text{-}erbA}$ proteins were detected in mutant-infected cells by antisera specific for the v-*erbA* protein domain, but were absent in cells infected by RAV-1 alone or by the 82-t mutant (Fig. 4B). The premature truncation mutant at position 907-t encoded an appropriately truncated polypeptide of 66,000 apparent molecular weight (Fig. 4B). Unlike the in-frame insertions, the levels of the 907-t mutant protein synthesized in infected cells were consistently reduced from those of the wild-type protein.

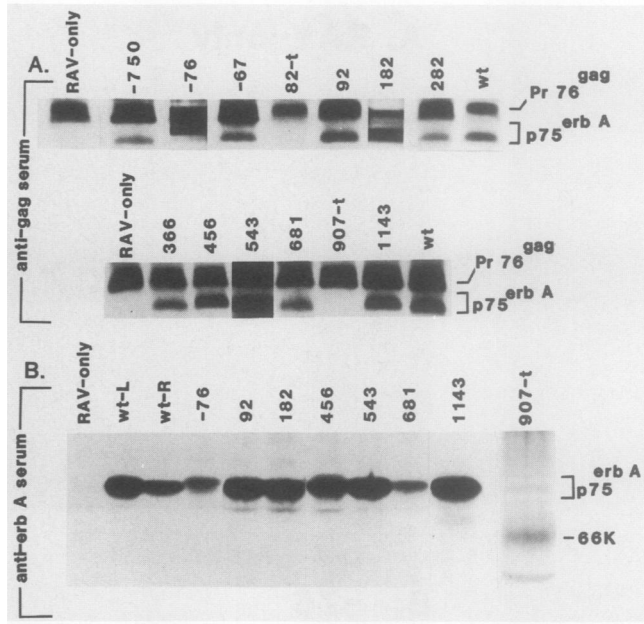

FIG. 4. Proteins synthesized by the v-*erbA* insertion mutants. Fibroblasts infected by the RAV-1 helper virus alone, by wild-type AEV, or by the different AEV v-*erbA* mutants were radiolabeled for 2 h with a mixture of [^{35}S]methionine and cysteine (New England Nuclear Translabel), the cells were sonicated, and the lysates were immunoprecipitated with either anti-*gag* (A) or anti-*erbA* (B) antisera (2, 23). Proteins in the immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and visualized by fluorography (23). The positions of the 75,000-molecular-weight *gag*-v-*erbA* fusion protein (p75$^{erb\ A}$) and the prematurely terminated 66,000-molecular-weight polypeptide synthesized by the 907-t mutant (66K) are indicated. The RAV-1 helper virus encodes a 76,000-molecular-weight *gag* precursor polypeptide (Pr 76^{gag}) that also reacts with the *gag*-directed antisera used in panel A. Cells infected by the pAEV-11-3L (wt-L) and by the pAEV-11-3R (wt-R) constructs were analyzed in panel B to demonstrate that both forms of infectious AEV plasmid synthesize comparable amounts of v-*erbA* protein.

The structure of the integrated proviruses in these cells was determined by a restriction endonuclease mapping-Southern blotting technique with an *erbA*-specific hybridization probe. Each of our mutations introduced a novel *Hpa*I site at the point of the insertion, simplifying this form of analysis. Within the resolution of this method, each of the v-*erbA* in-frame insertion mutant proviruses retained the genetic lesion introduced into the original molecular clones, and no cross-contamination with wild-type viruses or generation of recombinant genomes could be detected (data not shown). This technique, of course, only confirms the retention of at least one linker at the site of each insertion and does not prove that the original number of linkers at each site is retained in each mutant in a stable manner.

Ability of the mutant viruses to modify the growth properties of fibroblasts. In addition to the previously noted effects of v-*erbA* on erythroid cells, expression of v-*erbA* can also alter the growth properties of avian fibroblasts (7). One effect of v-*erbA* on fibroblasts is a significant increase in the growth rate and life span of these cells in medium with low serum. Our experiments confirmed that fibroblasts infected with AEV containing a wild-type v-*erbA* gene continued to propagate for over 6 weeks in medium with 0.5% serum and

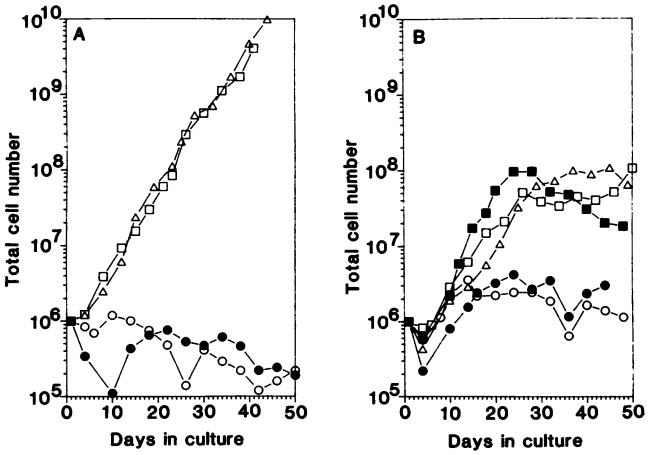

FIG. 5. Growth properties of fibroblasts infected by v-*erbA* mutant AEV: propagation in medium containing low serum. Fibroblasts infected by the various mutant viruses were seeded at 10^6 cells per 60-mm culture dish in F-10 medium containing 0.5% fetal bovine serum. The cells were incubated at 39°C and periodically trypsinized and counted. If the cells had increased in number, they were reseeded at 10^6 per dish; if the cells had not increased in number, they were reseeded at the preexisting cell density. Total (theoretical) cell yield was calculated cumulatively by dividing the cell count per dish by the ratio of the cell population that was replated at the previous passage, as described previously (7). (A) Growth curves of fibroblasts infected by the RAV-1 helper virus alone (○), by AEV containing a wild-type v-*erbA* gene (△), and by AEV v-*erbA* mutants bearing an insertion at 543 (□) or an insertion at 82-t (●). (B) Growth curves of fibroblasts infected by AEV v-*erbA* mutants bearing insertions at 182 (■), 282 (○), 456 (●), 681 (□), and 907-t (△).

eventually produced a theoretical yield of over 10^{10} cells from an inoculum of 10^6 cells (Fig. 5A). In contrast, fibroblasts from the same embryo infected by the RAV-1 helper virus alone (Fig. 5A) or by the 82-t truncation v-*erbA* mutant never increased in cell number above the initial inoculum size of 10^6 cells and eventually declined in cell number. The 82-t mutant failed to propagate under these low-serum culture conditions despite the presence of a fully functional v-*erbB* gene, indicating that growth under low-serum conditions is an autonomous property conferred by the v-*erbA* locus.

In general, the ability of our v-*erbA* insertion mutants to confer fibroblast growth in low serum paralleled their ability to potentiate erythroid transformation in our previous assay. Thus, fibroblasts transformed by our 543 insertion mutant, which has a lesion in the putative hinge region of the v-*erbA* protein, grew with kinetics and an ultimate yield indistinguishable from those of cells infected by wild-type virus (Fig. 5A). Fibroblasts infected with the 282 or 456 insertion mutants of v-*erbA*, representing two different mutations within the putative DNA-binding domain, were dysfunctional in the erythroid assay and failed to confer growth in low serum on fibroblasts (Fig. 5B). Fibroblasts infected by the 681 and 907-t v-*erbA* mutants, with insertions within sequences derived from the c-*erbA* hormone-binding domain, displayed an intermediate phenotype in the fibroblast assay, growing more slowly in low serum and producing a total cell yield somewhat reduced from that produced by wild-type fibroblasts (Fig. 5B).

Unexpectedly, viruses bearing an insertion at position 182 in v-*erbA*, a lesion near the center of the putative DNA-binding domain which severely compromised the ability of the virus to potentiate erythroid transformation, were capable of mediating growth of fibroblasts in the low-serum assay (Fig. 5B). Although not as effective in this property as the wild-type or 543 mutant AEV, the phenotype conferred by the mutation at position 182 could clearly be distinguished from that induced by viruses totally lacking a functional v-*erbA* gene (such as the 82-t and 282 mutants).

TABLE 3. Induction of leukemias by v-*erbA*-negative and v-*erbA*-positive AEVs

Virus stock	No. of animals with erythroblastosis/no. inoculated	Time to appearance of erythroblastosis[a] (days)	Time to death[b] (days)
RAV only	0/7		
82-t	7/12	8, 11, 12, 12, 18, 21, 21 (15)	26, 19, 27, 30, 21, 23, 24 (24)
Wild type	4/8	8, 12, 16, 37 (18)	12, 19, 30, 48 (27)

[a] Days after inoculation that first abnormal erythroblasts were detected in peripheral blood. Data for individual animals and the numerical average (in parentheses) are presented.

[b] Days after inoculation until death. Data for individual animals and the numerical average (in parentheses) are presented.

Leukemogenic potential of v-*erbA*-defective viruses. It has been reported that v-*erbA*$^-$ v-*erbB*$^+$ viruses either fail to induce eythroleukemias or induce an atypical, more differentiated erythroleukemia with a greatly delayed onset and lower incidence compared with that induced by wild-type AEV (3, 6, 28). To test the effect of our v-*erbA* mutations on in vivo oncogenesis, we compared stocks of virus containing the RAV-1 helper virus alone, the highly truncated 82-t v-*erbA* mutant, and the wild-type virus for the ability to induce neoplasia in neonatal SPAFAS chickens. In contrast to the previously published studies, both wild-type and v-*erbA* mutant viruses yielded similar incidences and time courses of erythroblastosis in these animals, and the diseased animals had very similar blood profiles (Table 3).

Transformed erythroid cells isolated from animals infected by the v-*erbA*-defective 82-t mutant virus appeared to have slightly slower growth in culture and demonstrated a slightly higher incidence of differentiated benzidine-positive cells than the erythroid cells derived from wild-type virus-infected animals, but these effects proved too subtle to serve as definitive assays for v-*erbA* function (data not shown). Immunoprecipitation with *gag*-directed sera demonstrated that the v-*erbA*-defective erythroid cells were infected but, as expected from the nature of the mutation, failed to synthesize a detectable v-*erbA* protein (data not shown). These results rule out reversion or recombination as a possible explanation for the erythroleukemias induced by these v-*erbA*-defective mutants.

We concluded from these preliminary results, obtained by using a v-*erbA* null mutant, that leukemogenicity would not provide a well-defined means of differentiating between the potentially more subtle phenotypic effects of our other, in-frame insertion mutations, and we therefore did not test our remaining mutants with this assay. There are several possible explanations for the apparent discrepancy between our results and those of other researchers, including slight differences in the method of inoculation and possible genetic differences between the flocks from which the animals were obtained (6, 25, 28).

DISCUSSION

Does linker insertion mutagenesis accurately map functional domains? The results of any form of mutagenesis must be

viewed with caution in the absence of specific knowledge of the effects of the genetic lesions on the overall conformation and stability of the polypeptide. However, evidence so far indicates that the linker insertion method reported here can indeed serve to accurately identify and dissect functional domains within a polypeptide. A nearly identical method has served to successfully map domains within the closely related estrogen receptor, as well as a number of other oncogenes, including v-*fps*, v-*erbB*, v-*fms*, and v-*myc* (8, 19, 21, 29, 30). Although we cannot directly assay the effects of our different insertions on the overall conformation of the mutant v-*erbA* polypeptides, we note that each of our in-frame insertion mutants synthesized a detectable v-*erbA* polypeptide in amounts roughly comparable to those synthesized by the wild-type virus, indicating that none of these lesions drastically destabilized the encoded polypeptide.

We believe the effect of each of our insertion mutations on the phenotype of the encoded v-*erbA* polypeptide is indicative of the site rather than the nature of the insertion. Eight of the insertions we analyzed resulted in the introduction of multiples of a Val-Asn dipeptide pair, amino acids generally similar in hydrophobicity and size to those already existing at the point of the insertion. Two of these mutations introduce Leu-Thr pairs and a single arginine codon at the site of the insertion; both of these lesions are in a region of the polypeptide that is already relatively basic (3). Although the number of linkers inserted at each site varied from mutant to mutant, no consistent correlation was detected between the number of linkers introduced at a site and the effect of the genetic lesion on the phenotype. For example, the two insertions analyzed within the *gag* domain introduced 10 or 12 amino acids, yet these mutants produced fully functional v-*erbA* polypeptides, whereas two different insertions within the putative DNA-binding domain completely abolished v-*erbA* erythroid activity, although each introduced only 6 amino acids. A similar lack of correlation between phenotype and size of insertion was also found in related studies on the v-*erbB* protein (21).

Is function in erythroid transformation related to ability to bind to DNA and modulate transcription? Our genetic dissection of v-*erbA* strongly underscores the involvement of the lysine-, arginine-, and cysteine-rich domain, a potential DNA-binding region, in erythroid transformation. Five different insertions within this domain drastically inhibited the ability of the v-*erbA* protein to cooperate with v-*erbB* in the transformation of bone marrow cells, whereas mutants carrying insertions both immediately upstream and downstream possessed wild-type activities. The wild-type v-*erbA* polypeptide is present in both the nuclei and cytoplasm of infected cells and is capable of binding to DNA in vitro (2). We are presently investigating the biochemical properties of the polypeptides encoded by the different insertion mutants. One mutant already investigated, with an insertion at position 366, appears to be defective in both DNA binding and nuclear localization, confirming the importance of these properties in the biological function of the viral polypeptide (2). Our evidence therefore strongly indicates that the ability of the v-*erbA* protein to interact with the chromatin of the infected cell is a prerequisite for the function of this polypeptide in erythroid transformation, analogous to the actions of the thryoid and steroid hormone receptor gene family.

It was recently reported that a proline-to-arginine codon mutation at position 437 in the v-*erbA* sequence abolishes v-*erbA* function (3, 20). This result agrees well with our own data; our nearby insertion at position 456 also disrupted v-*erbA* function. Munoz et al. (20) conceptually place their 437 mutation within the hinge domain of the v-*erbA* protein; we suggest instead that neither the 437 lesion nor our own 456 mutation properly maps to the hinge region but that they are within a separate, highly basic domain of the v-*erbA* protein probably important for DNA binding and nuclear localization (2).

Two different genetic lesions within the C-terminal third of the v-*erbA* protein, a region thought to mediate T3/T4 thyronine binding by the c-*erbA* polypeptide, yielded an intermediate phenotype, inducing minute bone marrow colonies. These results were unanticipated in light of the observation that, unlike the c-*erbA* polypeptide, even wild-type v-*erbA* protein lacks the ability to bind thyroid hormones (20, 26). Apparently the C-terminus of the v-*erbA* protein serves an important function distinct from that directly related to hormone binding. Perhaps these results reflect a requirement for retention in the viral polypeptide of C-terminal sequences involved in the "activation" that the c-*erbA* protein undergoes upon hormone binding and which alters the receptor into an activator of transcription.

Is the mechanism of action in fibroblasts identical to that in erythroid cells? In addition to its actions in erythroid cells, the v-*erbA* protein also has a variety of effects on the growth properties of fibroblasts (7). Our results confirmed that fibroblasts expressing the wild-type v-*erbA* protein grew at faster rates in low-serum medium and reached significantly higher overall cell yields before culture senescence than fibroblasts containing no AEV genome at all or those containing an AEV genome expressing a highly truncated version of the v-*erbA* protein (the 82-t mutant).

In general, our v-*erbA* mutants exhibited parallel effects in erythroid and fibroblast cells. One exception is our position 182 insertion mutant, which demonstrated divergent properties in erythroid and fibroblast assays, exhibiting extremely low function in the erythroid assay and near wild-type levels in the fibroblast assay. This result may be purely a quantitative effect; if the levels of v-*erbA* activity necessary for function in the fibroblast growth assay are lower than those necessary for v-*erbA* function in our erythroid assay, the partially crippled 182 insertion mutant might function adequately in fibroblasts but fall below the minimum threshold required for establishing the erythroid phenotype. Alternatively, the wild-type c-*erbA* protein appears to act by recognizing and binding to specific target DNA sequences and modulating the transcription of nearby genes (10, 35). Perhaps the v-*erbA* protein recognizes different target genes or different transcription factors in fibroblasts and in erythroid cells, and the divergent effects of our v-*erbA* mutants in fibroblasts and in erythroid cells reflect these different interactions in the two cell types.

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