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v-*erbA* Acts on Retinoic Acid Receptors in Immature Avian Erythroid Cells

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The v-*erbA* oncogene of avian erythroblastosis virus (AEV) encodes an aberrant version of a gene for a thyroid hormone receptor (c-*erbA*) and functions in neoplasia by blocking erythroid differentiation and altering the growth properties of fibroblasts. The v-*erbA* gene has been proposed to act as a dominant negative allele, functioning by interfering with the actions of its normal cell homologs, the thyroid hormone receptors. The v-*erbA* protein can also, however, interfere with the actions of other members of the nuclear hormone receptor family, and it has been proposed that interference with a retinoic acid-mediated response may be a crucial determinant of v-*erbA*'s function in the cancer cell. Here we report that the ability of v-*erbA* to interfere with retinoic acid receptor (RAR) action extends to the neoplastic erythroid cell and that v-*erbA* can inhibit transcriptional activation by all three isoforms (α, β, and γ) of RAR. Overexpression of RAR-α was found to partially overcome the presumptive v-*erbA* block to transcription in the erythroleukemic cell. These results are consistent with our hypothesis that v-*erbA* can act in neoplasia by interfering with a retinoic acid-mediated signal transduction pathway.

Retinoid and thyroid hormones are important effectors of vertebrate cell homeostasis, growth, and differentiation. These hormones manifest their actions directly at the level of gene expression, acting through nuclear receptors that bind to specific DNA sequences and modulate the expression of adjacent target genes in response to hormone (5, 11, 16, 18, 26). The receptors for retinoic acid (RA) and thyroid hormone share significant structural and functional relatedness and belong to a larger family of ligand-regulated transcription factors collectively termed the nuclear hormone receptors (11, 16, 18). The DNA binding domains of the RA and thyroid hormone receptors, in particular, share important structural features and exhibit related DNA sequence recognition properties. The thyroid hormone receptors have been reported to preferentially recognize DNA sequences (called thyroid hormone response elements) consisting of direct repeats of an AGGTCA half-site with a four-base spacing (e.g., AGGTCAnnnnAGGTCA), whereas RA receptors (RARs) preferentially recognize similar DNA sequences (retinoic acid response elements [RREs]) but with a five-base spacing (AGGTCAnnnnnAGGTCA) (13, 24, 37). Despite this general model, obtained from studies of artificial promoter constructs, the actual hormone response elements in the cell frequently consist of complex mixtures of half-sites in differing orientations and spacings, suggesting that overlapping as well as independent RA and thyroid hormone control pathways may exist. Indeed, the distinction between different response elements does not appear to be absolute (for example, the same half-site oriented as an inverted repeat, AGGTCATGACCT, is strongly recognized by both classes of receptors), and certain genes, such as the avian carbonic anhydrase II (CAII) gene, appear to be responsive to induction by both RA and thyroid hormone (10, 25, 32).

As has proven true of other participants in signal transduction pathways, the nuclear hormone receptors can act as oncogenes. The v-*erbA* oncogene of avian erythroblastosis virus (AEV), a transduced and aberrant copy of a gene (c-*erbA*α) for a thyroid hormone receptor, blocks the differentiation of infected erythroid cells and alters the growth properties of infected fibroblasts (14, 17, 29, 33, 38). These actions, although detectable in cells expressing the v-*erbA* oncogene alone, are most clearly seen in cells transformed by both v-*erbA* and the second oncogene in the AEV genome, v-*erbB* (15, 17). The v-*erbA* protein differs from the normal thyroid hormone receptor progenitor in that it has sustained small deletions at both N and C termini as well as 13 internal amino acid substitutions (29). Because of these structural changes, the v-*erbA* polypeptide is severely impaired in its ability to bind thyroid hormone and activate transcription in animal cells (3, 30, 40). Instead, the v-*erbA* protein acts as a constitutive repressor and, in cotransfection experiments, blocks transcriptional activation by the normal thyroid hormone receptor (3, 30). The v-*erbA* oncogene has therefore been proposed to act as a dominant negative allele, functioning in neoplastic cells by interfering with the actions of the c-*erbA* protein (3, 30). A simple interference with thyroid hormone receptor action, however, does not appear to fully account for the oncogenic effects of v-*erbA*. Humans displaying generalized thyroid hormone resistance synthesize mutant forms of thyroid hormone receptors that, like the v-*erbA* product, can interfere in a dominant fashion with normal thyroid hormone receptor action, yet these patients do not display an elevated occurrence of neoplastic or hematopoietic disease (12, 28). Indeed, aberrant expression of RARs, not thyroid hormone receptors, has been found closely associated with a number of forms of human neoplasia, including promyelocytic leukemias and certain hepatocarcinomas (1, 4, 6, 19).

v-*erbA* can specifically interfere with the actions of other members of the nuclear hormone receptor family, and we have suggested that interference with RAR actions might play a crucial role in v-*erbA*'s function in neoplastic cells (34). These observations, however, were largely correlative in nature and were based on experiments performed in a cultured human cell line rather than in the true erythroid cell

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target of v-*erbA*-mediated neoplasia. With these observations in mind, we have investigated the interactions between RARs and the v-*erbA* oncoprotein in more detail. Here we demonstrate that the ability of v-*erbA* to interfere with RAR action extends to neoplastic erythroid cells and that v-*erbA* can inhibit transcriptional activation by all three isoforms of RAR. Consistent with our hypothesis, overexpression of RAR-α was found to partially overcome the presumptive v-*erbA* block to transcription in these erythroleukemic cells.

MATERIALS AND METHODS

Cells and plasmids. A chicken erythroid cell line transformed by infection with the wild type ES-4/R strain of AEV was isolated and maintained as suspension cultures at 39°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 1% heat-inactivated chicken serum, streptomycin (1 mg/ml), penicillin (100 U/ml), and amphotericin B (Fungizone; 2.5 μg/ml) (all supplied by GIBCO), using a 1.2% bicarbonate–5% CO_2 buffering system. HeLa cells were propagated as monolayer cultures at 37°C in the same medium but lacking chicken serum and tryptose phosphate broth. Serum was depleted of hormones when indicated by treatment with activated charcoal and dextran resin (35).

The *tk*-CAT, IR-RRE-*tk*-CAT, DR-4-*tk*-CAT, DR-5-*tk*-CAT, and β-RRE-*tk*-CAT plasmid constructs are all derivatives of the same pBLCAT2 plasmid and contain the thymidine kinase (*tk*) promoter of herpes simplex virus driving expression of a chloramphenicol acetyltransferase (CAT) reporter gene (22). The *tk*-CAT construct lacks known hormone response elements. The IR-RRE-*tk*-CAT plasmid contains a single copy of an inverted-repeat thyroid/retinoid response element oligonucleotide (5′-TCGAGTTC TCAGG TCA TGACCTGA GAAC-3′) introduced at the unique *Hin*dIII site upstream of the *tk* promoter (35). The DR-4-*tk*-CAT and DR-5-*tk*-CAT constructs contain the same half-sites, but oriented as direct repeats with 4-bp (5′-TCGACTCAGGTC ACAGGAGGTCAGAG-3′) and 5-bp (5′-TCGACTCAGGTC ACCGAAAGGTCAGAG-3′) spacers, respectively. The β-RRE-*tk*-CAT plasmid contains a single RRE derived from the human RAR-β gene (5′-AGCTGGGTAGGGTTCA CCG AA AGTTCA CTCG-3′) (35). The pRS-v-*erbA* expression vector contains a full-length copy of the *gag*–v-*erbA* protein (designated wild type) expressed under the control of the Rous sarcoma virus promoter and was generously provided by C. C. Thompson and R. M. Evans (3). A mutant v-*erbA* allele bearing a premature termination codon near the beginning of the coding sequence (82t; designated null) was introduced into the same vector (34). Expression vectors for human RAR-α, -β, and -γ, utilizing the early promoter of simian virus 40 in a pSG5 vector background, were kindly provided by P. Chambon (2, 7, 20). A similar expression vector for the retinoid X receptor (RXR) was created by introduction of the avian RXR-γ gene (the generous gift of Paul Brickell) into the *Eco*RI site of the pSG5 vector (27). The pRS-*lacZ* construct was used as an internal standard in the HeLa cell transfections (35).

Transient transfections. HeLa cells were harvested by trypsin treatment, collected into medium, and recovered by centrifugation at 12,000 × *g* for 5 min; AEV-infected erythroid cells were harvested directly by centrifugation. In both cases, the cells were subsequently washed once with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4) and resuspended at 1.2×10^7 cells per ml in PBS. For the AEV-transformed erythroid cells, 8 μg of the appropriate reporter plasmid (or 8 μg each of the reporter plasmid and the pSG5-RAR-α expression plasmid, as indicated) was mixed with 0.42 ml of cell suspension and placed in a 0.4-cm electroporation cuvette, and electroporation was carried out at 340 V and 960 μF. For the HeLa transfections, 7.5 μg of reporter plasmid, 6 μg of pRS-v-*erbA* (null or wild type), 3 μg of the appropriate RAR expression plasmid, and 5 μg of the pRS-*lacZ* plasmid were mixed with 0.42 ml of cell suspension, and electroporation was carried out in a 0.4-cm cuvette at 250 V and 960 μF. The cells from each electroporation were diluted into 20 ml of hormone-depleted medium and divided into two equal aliquots; each aliquot was seeded into a 60-mm-diameter tissue culture plate. After an 18-h incubation, hormone (either all-*trans* RA, 13-*cis* RA, retinol, corticosterone, estradiol, or 3,3′,5′-triiodothyroacetic acid [triac], all prepared in ethanol) was added to one of the cell aliquots. The other aliquot received an equivalent volume of ethanol without hormone. After an additional 24 h of incubation, the cells were washed once with Tris-buffered saline (10 mM Tris-Cl [pH 7.6], 100 mM NaCl), scraped into the same buffer, and collected by centrifugation. The cells were resuspended in 100 μl of 0.25 M Tris-Cl (pH 7.8) per sample and were lysed by three freeze-thaw cycles. After clarification by a 5-min centrifugation at 16,000 × *g*, the cell lysates were assayed for CAT activity by a solvent partition system (34).

Northern (RNA) hybridizations. AEV-transformed erythroid cells were transfected with the pSG5-RAR-α expression vector and were subsequently treated with 5 μM all-*trans* RA, 10 μM triac, or no hormone for 24 h. AEV-transformed erythroid cells never subjected to electroporation were maintained, hormone treated, and analyzed in parallel. The cells were subsequently harvested, washed twice with PBS, and collected by centrifugation. The cells were resuspended in STE (0.1 M NaCl, 20 mM Tris [pH 7.4], 10 mM EDTA) and were lysed by addition of sodium dodecyl sulfate (SDS) to 0.5% and proteinase K to 300 μg/ml. Lysates were passed through a 21-gauge needle three to five times to reduce their viscosity and were incubated at 37°C for 1 h. Approximately 30 mg of oligo(dT)-cellulose (New England Biolabs) in 1 ml of binding buffer (0.4 M NaCl, 20 mM Tris [pH 7.4], 10 mM EDTA, 0.2% SDS) was next added to each lysate, and the resulting suspension was stirred for 2 h at room temperature. The oligo(dT)-cellulose was then washed with 10 ml of binding buffer and 5 ml of washing buffer (0.1 M NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 0.2% SDS), and the polyadenylated mRNAs were eluted with 3.5 ml of distilled water. The mRNAs were concentrated by ethanol precipitation, and recovery was determined as optical density at 260 nm.

Denaturing agarose gel electrophoresis using a formamide-formaldehyde buffer system was performed with approximately 10 μg of polyadenylated RNA per lane (23). The RNA was transferred to Hybond membranes (Amersham) by capillary blotting; after prehybridization, the RNAs on the membrane were hybridized to α-^{32}P-radiolabeled probes (23) representing the human RAR-α, -β and -γ cDNAs, an avian carbonic anhydrase cDNA (pRR108; a gift from J. Rogers, Physiological Laboratory, Cambridge University, Cambridge, United Kingdom), or a human β-actin cDNA. The membranes were subsequently washed for 20 min at room temperature in 2× SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 7.7])–0.1% SDS, for 10 min in the same buffer at 55°C, and finally for 10 min at 55°C in 0.2× SSPE–0.1% SDS.

Electrophoretic mobility shift assays. The v-*erbA* gene was engineered into a baculovirus transfer plasmid (pVL1392) under the control of the polyhedron promoter, and the recombinant virus was obtained by in vivo recombination and used to infect Sf9 cells (2a). RARs were analyzed from MCF-7 cells. In both cases, nuclear extracts were prepared, mixed with 15 μl of binding buffer (10 mM Tris-Cl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 5% glycerol, 2 μg of bovine serum albumin per ml), and incubated for 5 min at 25°C. Approximately 40,000 cpm (1 to 5 ng) of the appropriate radiolabeled oligonucleotide probe was next added to each binding reaction, and the samples were incubated for an additional 20 min at 4°C. The samples were subsequently resolved by electrophoresis in a 4.5% polyacrylamide gel–0.5× Tris-borate buffer system (23) at room temperature at 200 V for 75 min. The gels were then dried and autoradiographed.

RESULTS

All three species of RARs are inhibited by coexpression of the v-*erbA* oncogene protein. We have previously demonstrated that the v-*erbA* protein is able not only to inhibit its normal cellular homolog, the thyroid hormone receptor, but also to act promiscuously and inhibit the actions of RARs and estrogen receptors (34). The ability of mutant alleles of the v-*erbA* protein to interfere with RAR function appeared to closely correlate with their ability to participate in erythroid cell transformation (34). Three distinct receptors for all-*trans* RA, RAR-α, -β, and -γ, have been identified (2, 7, 20). We therefore first tested whether all three of these RAR species were sensitive to v-*erbA*-mediated inhibition or whether the effects of v-*erbA* were selective. Each RAR species, in the form of a simian virus 40 promoter expression vector, was introduced into HeLa cells by electroporation together with a second expression plasmid encoding either a wild-type or an inactive (null) copy of the v-*erbA* gene. The activities of the RARs were assayed by the cointroduction of a reporter construct containing an inverted-repeat RRE (IR-RRE) linked to a *tk*-CAT gene, and the accumulation of CAT enzyme in the presence and absence of RA was determined (Fig. 1).

The HeLa cells conferred slight RA responsiveness on the IR-RRE-*tk*-CAT reporter even in the absence of ectopically introduced receptors (Fig. 1, None), presumably reflecting the presence of low levels of endogenous RARs in these cells. The response of the reporter plasmid to RA was significantly enhanced, however, by the introduction of expression vectors encoding the cloned RAR-α, -β, or -γ (Fig. 1). All RAR species induced comparable levels of CAT activity in the presence of RA, assuming that all receptors were expressed at equal levels by the expression construct. However, the three different RAR species appeared to differ somewhat in the ability to induce CAT expression in the absence of added hormone; RAR-α displayed only a slight activation of the reporter in the absence of RA, whereas both the RAR-β and RAR-γ appeared to mediate greater induction under parallel conditions. Largely as a result of these different effects on the basal expression of the reporter, the extent of hormone induction (activity plus hormone/activity minus hormone) mediated by the three receptor isoforms ranged from 4- to 15-fold.

Cointroduction of the wild-type v-*erbA* gene with the RAR construct led to a clear inhibition of RA-mediated CAT (Fig. 1, WT). The extents of the v-*erbA*-mediated inhibition were similar for all three receptor isoforms (approximately 75%

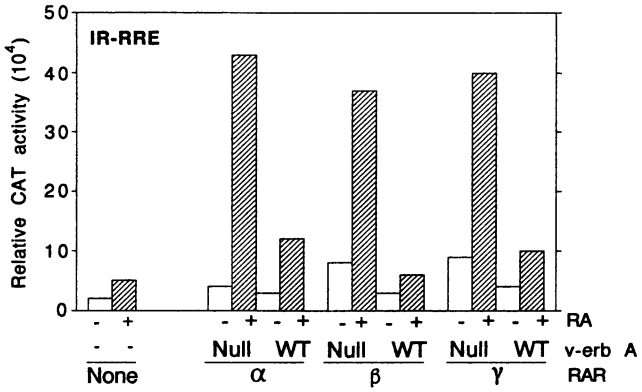

FIG. 1. Repression by the v-*erbA* oncogene of all three RAR isoforms. A pSG5 expression vector, containing the human RAR-α, -β, or -γ gene, was introduced into HeLa cells by electroporation in the presence of either a wild-type (WT) or a null mutant (Null) allele of v-*erbA*. The IR-RRE-*tk*-CAT construct was introduced at the same time, along with a pRS-*lacZ* construct as an internal control. After an 18-h recovery period, the cells were subsequently treated (+) or not (−) with 5 μM all-*trans* RA, harvested 24 h later, and assayed for CAT and β-galactosidase activities. Levels of CAT expression, expressed relative to the β-galactosidase activity determined for the same cell extracts, are indicated.

inhibition over those levels seen in cells cotransfected with the null v-*erbA* allele). Expression of v-*erbA* appeared to interfere with the basal as well as the RA-induced activities of all three RARs. These experiments indicate that the actions of the viral protein are relatively broad and do not appear to detectably distinguish between the differing RAR subtypes in the experiments performed.

Neoplastic erythroid target cells express high levels of mRNA for RAR-α and RAR-γ. Our working hypothesis, that v-*erbA* acts in erythroleukemic cells by interfering with an RA signal transduction pathway, requires that erythroleukemic cells normally express RARs and that the actions of these receptors be blocked by v-*erbA*. We therefore next examined AEV-transformed erythroid cells for expression of RARs by Northern analysis. Since the transcription of RARs in several other cell systems is known to be under hormonal regulation (8, 9, 36), we analyzed these cells both in the presence and in the absence of RA and in the presence and absence of thyroid hormone (Fig. 2). The AEV-transformed erythroid cells expressed significant levels of RAR-α mRNA (Fig. 2A, lane 1) but no detectable mRNA for the RAR-β species (data not shown). Two bands (at 3.2 and 4.4 kb) were detected when filters were probed with the human RAR-γ cDNA (Fig. 2B); however, at a higher wash stringency, only the 4.4-kb species was detected (data not shown). The 3.2-kb band in Fig. 2B therefore likely reflects cross-reactivity of the human RAR-γ probe with the avian RAR-α mRNA, although an alternatively spliced RAR-γ mRNA cannot be ruled out. The relative levels of the RAR-α and RAR-γ mRNAs were unaffected by either thyroid hormone (Fig. 2A and B, lane 2) or RA treatment (lane 3), and no RAR-β mRNA expression was detected even after treatment with RA (data not shown), a process that induces RAR-β receptors in other cell lines. The same blots were also stripped and hybridized to a human β-actin probe as a measure of RNA recovery and loading (Fig. 2C).

The RARs expressed in AEV-transformed erythroid cells are functional on the β-RRE element. We next wished to test

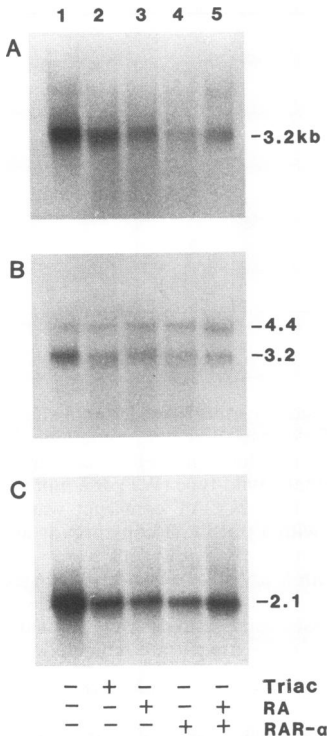

FIG. 2. Expression by neoplastic erythroid target cells of high levels of RAR-α and RAR-γ mRNA. Polyadenylated RNAs were isolated from chicken erythroid cells infected with AEV and were resolved by denaturing agarose gel electrophoresis. The mRNAs were subsequently transferred to nylon membranes and probed by nucleic acid hybridization. The RNAs were isolated from untreated cells (lane 1), cells treated for 24 h with 10 μM triac (lane 2), cells treated for 24 h with 5 μM all-*trans* RA (lane 3), cells transfected with pSG5-RAR-α (lane 4), or cells transfected with pSG5-RAR-α and treated for 24 h with 5 μM RA (lane 5). (A) The mRNAs were hybridized to a human RAR-α cDNA probe; (B) the mRNAs were hybridized to a human RAR-γ cDNA probe; (C) the mRNAs were hybridized to a β-actin cDNA probe. Autoradiography was for 24 to 48 h.

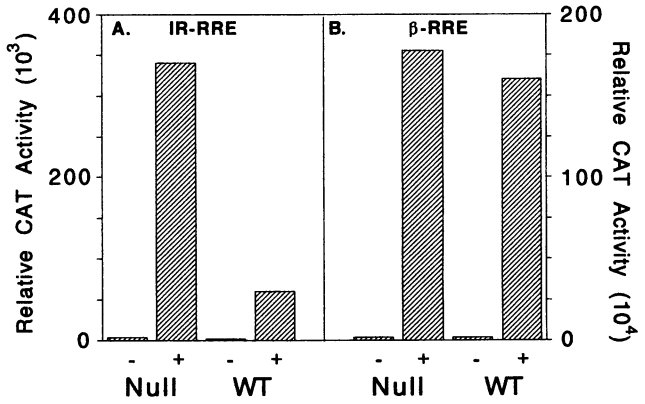

FIG. 3. Evidence that the v-*erbA* protein exhibits differential interference on two distinct classes of RRE. MCF-7 human breast carcinoma cells were transfected with a pRS-v-*erbA* expression vector bearing either a wild-type v-*erbA* allele (WT) or an inactive copy of the viral gene (Null), together with reporter constructs containing either the IR-RRE or β-RRE inserted upstream of the ΔMTV promoter (34). The cells were subsequently treated (+) or not (−) with 5 μM all-*trans* RA, and CAT expression was determined relative to the activity of a pRS-*lacZ* plasmid cointroduced as an internal standard.

whether the RAR mRNAs detected in the AEV-transformed erythroid cells were actually expressed as functional RARs. To do so, we made use of a previous observation: although the v-*erbA* protein is a strong repressor of RAR function on an RRE consisting of an inverted repeat of an AGGTCA half-site (IR-RRE), the v-*erbA* protein does not interfere with RAR activity on the β-RRE element (an RRE composed of GGTTCA and AGTTCA half-sites oriented as a direct repeat with a 5-bp spacing) (34). Figure 3 illustrates this marked difference. In the absence of a functional v-*erbA* gene, a CAT reporter containing either the IR-RRE element or the β-RRE element exhibits strong RA-induced transactivation in MCF-7 human mammary tumor cells, mediated by the high levels of endogenous RARs in this cell line (Fig. 3, Null). Cointroduction of a wild-type v-*erbA* gene specifically inhibited gene expression from the IR-RRE, while no effect was seen on the β-RRE (Fig. 3, WT). These results are paralleled by in vitro DNA binding studies; the v-*erbA* protein strongly binds to the IR-RRE motif but demonstrates no significant recognition of the β-RRE sequence, whereas the RARs bind to both with virtually equal affinities (Fig. 4). The differential interference of these two distinct classes of response element enabled us to assay for both v-*erbA*-independent and v-*erbA*-dependent RAR activities in a single cell line.

We therefore introduced the β-RRE-*tk*-CAT reporter into the AEV-transformed erythroid cells to test for RAR function in a v-*erbA*-independent context (Fig. 5). The erythroleukemic cells conferred strong, RA-dependent activation on this reporter element, with maximal induction occurring at approximately 1 μM all-*trans* RA, suggesting that the receptor expression detected by Northern analysis was reflected in an actual accumulation of functional RARs in these cells. The activation of the β-RRE reporter (approximately 20-fold) in the AEV-transformed cells was comparable to that seen in other cell lines, such as MCF-7 cells, that are known to express high levels of RARs (34). The induction of the β-RRE reporter was specific for RA, either the all-*trans* isomer (lanes 2 and 3) or the 13-*cis* form (lane 4); no activation of the reporter was detected in response to vitamin A (retinol), triac, glucocorticoid, or estrogen (lanes 5 to 8).

v-*erbA* binds to a subset of RREs and blocks RAR action on this class of RREs in neoplastic cells. We next tested the IR-RRE-*tk*-CAT reporter in otherwise identical experiments (Fig. 6A). As previously demonstrated, this response element is recognized by both the RAR and the v-*erbA* proteins, and RAR activation mediated by the IR-RRE sequence is strongly repressed in the presence of the v-*erbA* polypeptide. Under parallel conditions in which the β-RRE reporter exhibited extremely high RA-induced activation, the IR-RRE reporter was completely silent in the AEV-transformed erythroid cells (compare the left panel in Fig. 6A with lane 3 in Fig. 5). We interpret these results as indicating that the v-*erbA* protein acts to suppress the actions of the RARs on this class of response elements in the erythroid target cells as it does in transiently transfected MCF-7 and HeLa cells.

We next extended our observations to an additional series of response elements consisting of direct repeats of the AGGTCA half-site separated by 4 (DR-4) or 5 (DR-5) bp. The DR-4 motif, like the IR-RRE, is recognized by both the

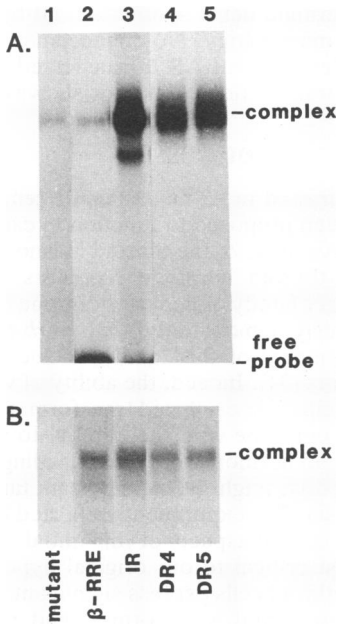

FIG. 4. Binding of the v-*erbA* and RAR proteins to hormone response elements in vitro. The relative abilities of the v-*erbA* protein and the RAR to bind to different hormone response elements were determined by an in vitro electrophoretic mobility shift assay. Each radiolabeled oligonucleotide was incubated in the presence of nuclear extracts of Sf9 cells infected by a v-*erbA*-recombinant baculovirus (A) or MCF-7 cells (which express high levels of RAR-α and RAR-γ) (B). Probe bound to protein (complex) was resolved from free probe by native polyacrylamide gel electrophoresis and was visualized by autoradiography. The identities of the complexes were confirmed by use of specific antisera in supershift experiments (data not shown). The greater intensity of the v-*erbA* complexes relative to those seen with the RARs is due to the higher levels of expression of the viral protein in the baculovirus system (data not shown).

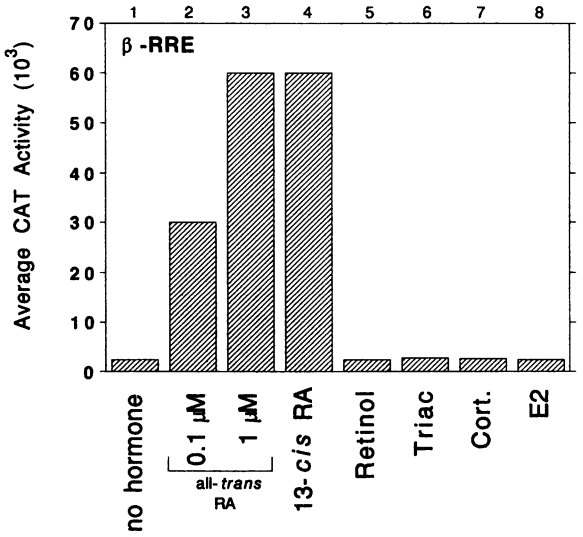

FIG. 5. Evidence that RARs expressed in AEV-transformed erythroid cells are functional on the β-RRE-containing element. AEV-transformed erythroid cells were transfected with the β-RRE-*tk*-CAT reporter, treated with hormone or not, as indicated, for 24 h, harvested, and assayed for CAT activity. Averages of three independent experiments are presented. Treatment included no hormone (column 1), 0.1 μM all-*trans* RA acid (column 2), 1 μM all-*trans* RA (column 3), 1 μM 13-*cis* RA (column 4), 1 μM vitamin A (retinol; column 5), 10 μM triac (column 6), 0.1 μM corticosterone (Cort.; column 7), or 10 μM estradiol (E2; column 8).

v-*erbA* protein and the RARs in DNA binding studies (Fig. 4) and in cell transfection assays (2a, 37). Consistent with this dual tropism, the response of the DR-4 reporter to RA was strongly repressed in AEV-transformed erythroid cells (Fig. 6A, middle panel). A similar direct repeat of the AGGTCA half-site, but with a heptanucleotide spacer (DR-5), showed detectable, if very modest, RA responsiveness in AEV-transformed erythroid cells; this response element is also bound by both v-*erbA* and RARs (Fig. 4) but appears to have somewhat higher relative affinity for RARs than does the DR-4 repeat (2a, 37). These observations taken as a whole yielded a strong correlation between the ability of v-*erbA* protein to bind to an RRE in vitro and repression of RA-mediated gene expression in the erythroid cells.

Overexpression of RARs can partially overcome the presumptive v-*erbA*-mediated block to transcription in erythroleukemic cells. We next tested whether the apparent block to RAR function in AEV-transformed erythroid cells could be overcome by high levels of expression of an ectopically introduced RAR. We therefore included a pSG5-RAR-α expression vector in our next series of transient transfections in addition to the reporter gene construct (Fig. 6B). Introduction of high levels of exogenous RAR-α into these cells significantly overcame the block to reporter activation for the IR-RRE, DR-4, and DR-5 reporters, resulting in RA-induced reporter gene expression approaching (for IR-RRE) or exceeding (for DR-5) 50% of the levels seen with the (v-*erbA*-independent) β-RRE construct (Fig. 5). The response in the presence of exogenous RAR-α was specific for RA; thyroid hormone, glucocorticoids, and estrogens were inactive (Fig. 6B). These experiments confirm that the IR-RRE, DR-4, and DR-5 elements are capable of functioning in an RA-specific fashion in the erythroleukemic cell background but that a titratable inhibitor, presumably the v-*erbA* protein, must be removed before RAR-mediated activation can occur.

Recently a novel class of RARs, the RXRs, have been identified. Also members of the nuclear hormone receptor family, the RXRs appear to mediate cell responses to the 9-*cis* isomer of RA, although all-*trans* RA at high concentrations can mediate an RXR response through metabolic conversion to the 9-*cis* form (21). RXRs appear to play additional crucial roles by forming heterodimers with other members of the nuclear hormone receptor family, including the c-*erbA* protein and the RARs (39). We therefore repeated our experiments with use of an RXR-γ expression vector. As shown in Fig. 6C, exogenous expression of RXR-γ could partially induce reporter gene expression from an IR-RRE but had no effect on the DR-4 or DR-5-*tk*-CAT reporter.

Finally, we wished to determine whether an endogenous cellular target of v-*erbA* repression, the CAII gene (25), was induced by the introduction of ectopic RAR-α receptors. AEV-transformed erythroid cells were again transfected by the pSG5-RAR-α expression construct and treated with RA as before, and expression of the chromosomal CAII gene was analyzed by a Northern procedure. No CAII expression was detected by Northern analysis in either transfected or untreated cells, in the presence or absence of RA (data not shown). Similar results for untransfected cells were obtained with use of medium designed to support erythroid cell

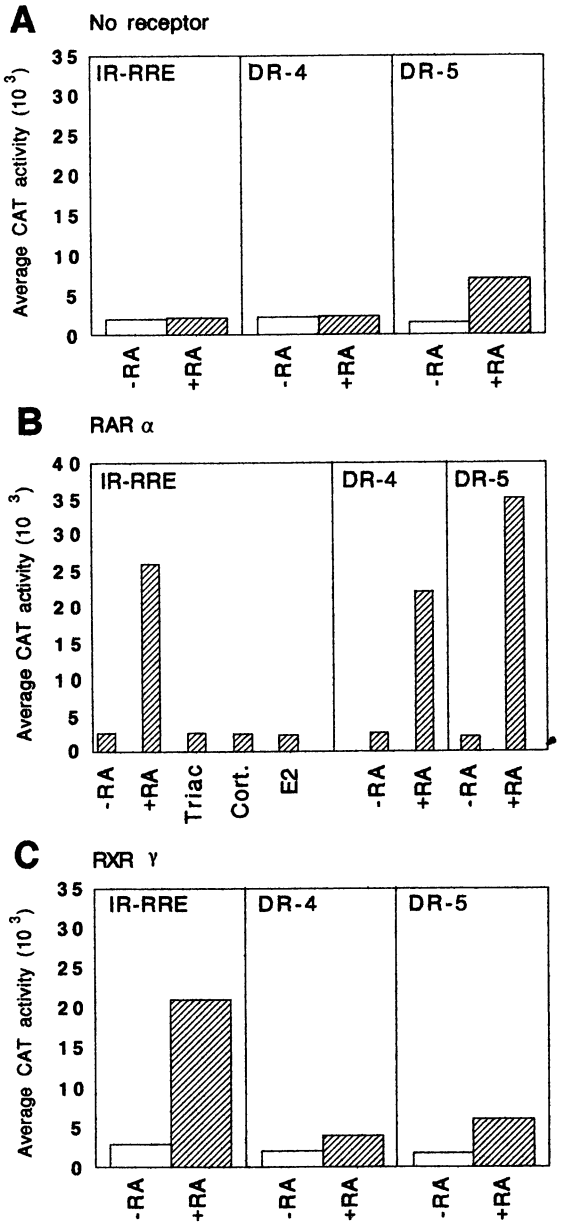

FIG. 6. Evidence that the RAR response in erythroleukemic cells is blocked on response elements recognized by the v-*erbA* protein and that overexpression of RAR-α can overcome this block. AEV-transformed erythroid cells were transfected with the *tk*-CAT reporters containing the IR-RRE, a DR-4 orientation of the AG GTCA half-site, or a DR-5 orientation of the AGGTCA half-site, as indicated. The cells were subsequently treated with the designated hormones and assayed for CAT activity. Averages of three independent experiments are presented. (A) No exogenous RAR gene was included in the transfections; (B) a pSG5-RAR-α expression plasmid was included in the transfection together with the reporters; (C) a pSG5-RXR-γ expression plasmid was included in the transfection together with the reporters. RA, all-*trans* RA; Cort., corticosterone; E2, estradiol.

differentiation (33). We were also interested in determining whether the exogenous RAR-α could overcome the v-*erbA* block to differentiation. The transfected cells were therefore also assayed for their differentiation properties by benzidine staining (to determine heme synthesis) and by microscopic examination of morphology. No enhancement of differentiation could be detected in RAR-α-transfected cells over that seen in control transfections (data not shown).

DISCUSSION

RARs are expressed in erythroleukemic cells. The v-*erbA* oncogene has been proposed to function in cancer cells as a dominant negative allele of the thyroid hormone receptor (3, 30). However, thyroid hormone receptors are only one member of a larger family of nuclear hormone receptors, and we have previously demonstrated that v-*erbA* can interfere with several members of this family, including RARs and estrogen receptors (34). Indeed, the ability of v-*erbA* protein mutants to participate in erythroid transformation was found to more closely correlate with their ability to repress RARs rather than thyroid hormone receptors, leading us to predict that the RA response might be an important target of v-*erbA* action in neoplasia. The experiments reported here served to confirm several crucial aspects of this initial hypothesis.

First and most critical to our original hypothesis, AEV-transformed erythroid cells express significant levels of RAR mRNAs (for the α and γ isoforms), and these mRNAs encode functional receptors which confer hormone responsiveness on a reporter element (β-RRE) immune to v-*erbA*-mediated repression. This abundant expression of RAR mRNAs is in marked contrast to the relatively low levels of thyroid hormone receptor mRNAs found in the same cells (data not shown), consistent with the idea that RARs may play a more substantive role in signal transduction in these cells. This conclusion is also supported by experiments demonstrating that the levels of thyroid hormone response in immature avian erythroid cells are very low in the absence of exogenously introduced thyroid hormone receptors (32).

A second prediction of our original hypothesis was also confirmed: although highly active on a v-*erb*A-immune response element (β-RRE), the RARs in AEV-transformed erythroid cells were relatively inactive when assayed on a series of reporters sensitive to v-*erbA* interference. We suggest that this inhibition of RAR action in AEV-transformed erythroid cells is due to the abundant v-*erbA* protein present in these cells, similar to the actions of v-*erbA* in transiently transfected HeLa cells or in MCF-7 cells. It has not been possible for us to propagate sufficient numbers of nontransformed erythroid cells to allow parallel studies to be performed in a v-*erbA*-minus background, and therefore we cannot at present completely eliminate the possibility that some factor in AEV-transformed cells other than v-*erbA* protein interferes with RAR function on the IR-RRE, DR-4, and DR-5 reporters. However, this hypothetical factor would have to display DNA sequence recognition properties precisely identical to those displayed by the v-*erbA* protein on the four distinct response elements (IR, β-RRE, DR-4, and DR-5) used in our studies.

v-*erbA* has effects on all three RAR isoforms. RARs play crucial roles in vertebrate morphogenesis and differentiation (5, 27). In vertebrates, at least three distinct loci (RAR-α, -β, and -γ) encode receptors for all-*trans* RA (2, 5, 7, 20). The different RAR isoforms are evolutionarily conserved and are expressed in distinct tissues and at distinct times during vertebrate development, probably reflecting divergent roles in the organism (5). Interestingly, both the RAR-β and RAR-γ forms appeared to confer a limited hormone-independent activation of the reporter in our transient transfections of HeLa cells, whereas the RAR-α form did not; perhaps this

differential hormone-independent activity reflects to some extent the differing roles of RAR-α, -β, and -γ in the normal organism.

The v-*erbA* protein interfered with reporter gene activation by all three RAR isoforms in transient transfections of HeLa cells. This finding both extends our initial observations on endogenous RARs in MCF-7 cells (in which the precise nature of the RARs mediating the response could not be defined) and indicates that the v-*erbA* effects are relatively broad and not limited to a single RAR species. In addition, we have recently been able to demonstrate that the v-*erbA* protein can interfere with the actions of the RXR class of 9-*cis* retinoic acid receptors (data not shown). This effect probably accounts for the relatively low level of RXR function observed in our AEV-transformed erythroid cells, despite the presence of detectable levels of RXR mRNA in these cells (data not shown), and for the ability of ectopically introduced RXR to partially overcome this block to RXR activity (Fig. 6C).

A subset of RREs are targets of v-*erbA* repression in cancer cells. Although v-*erbA*-mediated repression is not limited to a particular RAR isoform, it is restricted by the nature of the DNA response element. The v-*erbA* protein is a strong repressor of RAR action on a subset of response elements consisting of either an inverted repeat of an AGGTCA half-site (IR-RRE) or a direct repeat of the same consensus hexamer with a four- or (to a slightly lesser degree) five-nucleotide spacer. However, v-*erbA* is unable to interfere with RARs on a β-RRE element (a direct repeat of related half-sites: GGTTCACCGAAAGTTCA). Gel retardation analyses confirm that the v-*erbA* protein binds to the IR-RRE, DR-4, and DR-5 oligonucleotides and not to the β-RRE element. This finding appears to be consistent with our concept of v-*erbA* interference being mediated at the level of DNA half-site recognition and requiring binding to the response element for repression. The potential targets of v-*erbA* repression are therefore likely to include a wide variety of RREs containing the AGGTCA consensus half-site; however, another distinct set of RREs containing other half-site sequences (such as β-RRE) will be immune to the effects of v-*erbA*.

Overexpression of an ecotopic RAR-α in erythroleukemic cells was able to partially or fully overcome the block to expression of a variety of exogenously introduced RRE reporter genes, yet we were unable to observe enhanced expression of an endogenous gene (the CAII gene) or to detect an induced differentiation of these cells in response to RA. This result may simply reflect an insensitivity of the transfection technique, as only a relatively small percentage of the cell population is expected to acquire and express the exogenous receptor after electroporation. This hypothesis appears to be supported by our Northern analysis of the RAR-α-transfected erythroid cell population, which did not reveal detectably greater expression of RAR-α in the bulk population over those levels seen with nontransfected cells (Fig. 2, lanes 4 and 5). We therefore believe that only a small percentage of the erythroid cells acquire the RAR-α and reporter genes in these transfections and that expression in only these few cells, although detectable by use of the cotransfected CAT reporter, is inadequate to alter the CAII expression or differentiation properties of the bulk cell culture. Alternatively, it has been reported that the v-*erbB* oncogene, which is also encoded on the AEV genome, plays a cooperative role in blocking differentiation in these cells and may therefore prevent differentiation and CAII expression despite the abrogation of v-*erbA* action by RAR-α overexpression (31). Finally, we cannot rule out the possibility that our AEV-transformed cell line has undergone unknown secondary changes that may have stabilized its immature phenotype independent of v-*erbA* expression, thereby conferring a resistance to RAR-induced differentiation.

When this study was nearing completion, we became aware of independent results from another research group that support several aspects of the work cited here. Schroeder et al. (31, 32) have observed accelerated differentiation of immature erythroid progenitors when these cells are treated with RA or a combination of RA and T3, and this differentiation is blocked by AEV infection (notably, joint expression of v-*erbA* and v-*erbB* was more effective in this block to differentiation than was expression of v-*erbA* alone). The details of this hormonally regulated differentiation pathway remain unclear at this time, but the RA-mediated differentiation was paralleled by an RA induction of CAII expression, suggesting an action at the level of transcription. These findings further support the notion that v-*erbA*, perhaps in combination with v-*erbB*, acts in neoplastic cells by interfering with RAR action, thereby potentially disrupting a network of hormonally regulated genes responsible for normal erythroid cell differentiation. Effects of v-*erbA* on other hormone receptors may play additional contributory roles.

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S.S. and M.S. contributed equally to the work leading to this report.

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