Dimerization Interfaces of v-ErbA Homodimers and Heterodimers with Retinoid X Receptor α*

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The oncprotein v-ErbA, a member of the zinc finger transcription factor superfamily, is a mutated version of thyroid hormone receptor α1 that is virtually incapable of binding T3. v-ErbA and other members of this family can bind as homodimers and heterodimers with retinoid X receptors to specific DNA sequences arranged as direct, inverted, or everted repeats. At least two regions in the C-terminal domain, the I box (10 and 11 helices in v-ErbA and thyroid hormone receptors) and the 20-amino acid region are involved in dimerization. However, it has not been entirely understood how these receptors dimerize on differently oriented core motifs and whether the domain(s) responsible for homodimerization and heterodimerization are identical. Therefore, deletions of the entire 20-amino acid region, the 10 helix, the 11 helix, and point mutations within these regions of v-ErbA were made by site-directed mutagenesis. The mutant proteins were tested for their ability to form v-ErbA homodimers and heterodimers with retinoid X receptor α on differently oriented core motifs by electrophoretic mobility shift assay. Transient transfections were performed to determine the dominant negative activity of the v-ErbA mutants. The data indicate that different dimerization interfaces are used for v-ErbA homodimerization and heterodimerization with retinoid X receptor α, and different dimerization interfaces are used on differently oriented core motifs. The data are of general interest because the information improves our understanding of the role of these dimerization interfaces in the mechanism of action not only of v-ErbA but also of other members of the superfamily.

The avian erythroblastosis virus (strain ES4) induces erythroleukemia and fibrosarcomas in birds (1, 2). This retrovirus possesses two oncogenes, v-ErbA and v-ErbB (1, 2). The retroviral oncprotein v-ErbA, a member of the zinc finger transcription factor superfamily, is a mutated form of thyroid hormone receptor α1 (TRα1) that is virtually incapable of binding T3 (3). v-ErbA is a dominant negative repressor in avian and mammalian cells; however, its mechanisms of action and molecular targets remain unknown (4, 5).

v-ErbA and TR belong to the ErbA superfamily of nuclear receptors. Other members of this family include glucocorticoid receptor, retinoic acid receptors, retinoid X receptors (RXRs), and the vitamin D receptor (6). These receptors interact with specific DNA sequences, called response elements, in the promoter region of target genes and thereby regulate diverse aspects of cellular development and homeostasis (6).

TR and v-ErbA can bind as homodimers or heterodimers to response elements arranged as everted, direct, and inverted repeats (7). The heterodimerization with RXR enhances the DNA binding of these receptors to their respective response elements (8–10). We recently described the DNA binding affinity of v-ErbA homodimers and v-ErbA-RXR heterodimers to differently oriented core motifs (11). We found that v-ErbA homodimers bind with the highest affinity to an imperfect everted repeat with a 5- or 6-bp spacer. Also, v-ErbA homodimers and v-ErbA-RXR heterodimers bind to direct repeats with different spacers (11).

At least two regions within the nuclear receptors are involved in the heterodimerization with RXR. One region is located within the DNA-binding domain (DBD). This region confers a weak dimerization interface; however, it dictates the spacing preference of direct repeat response elements (12–16).

In contrast to this weak dimerization interface of the DBD, a second region located in the C-terminal domain confers a strong dimerization interface (Fig. 1). This region, named the I box, encompasses helices 10 and 11 in TR and v-ErbA (17). Unlike the dimerization interface that forms between DBDs, the C-terminal region is capable of mediating dimerization in solution that would account for the high degree of cooperativity between dimeric partners on response elements. The highly conserved ninth heptad located within helix 11 has been shown to play a critical role in TR heterodimerization with RXR (18, 19). Another region located toward the N-terminal side of the ligand-binding domain is involved in dimerization. It consists of 20 amino acids that are highly conserved among the majority of nuclear receptors (20). Mutations in this region of TRβ1, vitamin D receptor, and retinoic acid receptor impair heterodimerization with RXR on direct and inverted repeat response elements (20, 21). However, little is known about the role of this region in homodimerization on direct and inverted repeats or in heterodimerization or homodimerization on everted repeats.

The crystal structures of the dimerization interfaces for several nuclear receptors have been published (22–25). However, in two of them, the studies were done with truncated DBDs in the presence of DNA (22, 23), whereas in others, the experiments were performed with the C-terminal region of the receptors in the absence of DNA (24, 25). Therefore, crystal struct-
amounts of 1,250 and 5,000 cpm for heterodimer binding. On each core motif, the between 2,500 and 10,000 cpm for homodimer binding and between DNA/sample. The amounts of \[ ^{3}H \]leucine v-ErbA proteins used were stranded DNAs were end-labeled with \[ ^{32}P \]-labeled DNA, and the protein(s) of interest. The double-mutations were performed in 35

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numbers of methionines in the specific proteins). After incubation for 30 min at 4 °C, the columns were washed with 60 ml of EMSA buffer. Their numbers indicate the amino acid positions in v-ErbA.

Although it has not been entirely understood how these receptors dimerize on these different elements, it is believed that the presence of a flexible hinge located between the DBD and the ligand-binding domain allows the DBD to rotate with respect to a common C-terminal interface (7, 17, 26). However, recent data on the role of the ninth heptad repeat in the dominant negative activity of TR variant 2 suggest that there are different dimerization interfaces as the half-site orientation changes (27, 28). Furthermore, it was unknown whether the domain(s) responsible for homodimerization and heterodimerization are identical.

To obtain a better understanding of the dimerization interfaces involved, we studied, by mutational analysis, the role of the I box and the 20-amino acid region of v-ErbA in the formation of v-ErbA homodimers and v-ErbA-RXR heterodimers on everted, direct, and inverted repeats.

The data described below indicate that different dimerization interfaces in the C-terminal domain are used in v-ErbA homodimers and v-ErbA-RXR heterodimers as well as within dimers on differently oriented core motifs.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The Promega Altered Sites kit was used to create point mutations in v-ErbA. For deletions, the polymerase chain reaction-based splice overlap extension technique was employed (29). Mutant products were sequenced to confirm the mutations and to exclude errors.

**Production and Purification of Proteins**—Wild type and mutant v-ErbAs (lacking gag sequences) and the mouse RXRα cDNAs (11, 30) were transcribed from pBluescript plasmids and then translated using the rabbit reticulocyte lysate system (Promega) in the presence of \[^{3}H \]leucine or \[^{35}S \]methionine (31). Trichloroacetic acid precipitable-protein counts per minute were determined. SDS-polyacrylamide gel electrophoresis and fluorography were performed to demonstrate that all proteins were of the appropriate size.

**Electrophoretic Mobility Shift Assays**—Protein-DNA binding reactions were performed in 35 \( \mu l \) of 20 mM HEPES, pH 7.8, 20% glycerol, 1.4 \( \mu g \) of poly(dIdC), 1 mM dithiothreitol, 50 mM KCl, 0.1% Nonidet P-40, \(^{32}P\)-labeled DNA, and the protein(s) of interest. The double-stranded DNAs were end-labeled with \[^{32}P\]ATP using T4 polynucleotide kinase. An EMSA was performed with 40,000 cpm of \[^{32}P\]-labeled DNA/sample. The amounts of \[^{3}H\]leucine v-ErbA proteins used were between 2,500 and 10,000 cpm for homodimer binding and between 1,250 and 5,000 cpm for heterodimer binding. On each core motif, the amounts of in vitro translated v-ErbA proteins used were equal as assessed by \[^{3}H\]leucine incorporation, taking into consideration the number of leucines in the specific proteins. For experiments involving heterodimerization, 2,500 cpm of RXRαs were employed. Reactions were incubated at room temperature for 45 min prior to electrophoresis. Electrophoresis was carried out on 0.25 \times \text{TBE} (22 mM Tris base, 22 mM boric acid, 0.5 mM EDTA) 6% polyacrylamide gels (29.1 acrylamide: bisacrylamide) at room temperature at 10% acetic acid, dried, and exposed to film with an intensifying screen for 6–24 h at −70 °C.

The DNA-protein complexes were quantified using a Molecular Dynamics PhosphorImager. Experiments were performed at least twice using two different batches of rabbit reticulocyte lysates.

The sequences of the oligonucleotides used in EMSA are shown below (the hexameric half-sites are underlined): M1, GATCCGGGGCATGTAATGAAATATTGGTACCGGATCT; ER5, GATCCGGGGCATGTAATGGGGTACCTAGTGGTACCGGATCT; ER6, GATCCGGGGCATGTAATGGGGTACCTAGTGGTACCGGATCT; DR4, GATCCGGGGCATGTAATGGGGTACCTAGTGGTACCGGATCT; IR0, GATCCGGGGCATGTAATGGGGTACCTAGTGGTACCGGATCT; IR6, GATCCGGGGCATGTAATGGGGTACCTAGTGGTACCGGATCT; and IR9, GATCCGGGGCATGTAATGGGGTACCTAGTGGTACCGGATCT.

**Results**

**Expression of v-ErbA Mutants**—v-ErbA mutants were confirmed by Western blot of nuclear cell extracts (Amersham Pharmacia Biotech ECL kit) with v-ErbA antibody (gift from M. Privalsky).

**Co-transfections** included 1 \( \mu g \) of a human growth hormone (GH) expressing vector (pTKGH)/60-mm Petri dish. Mouse TRα1 and rat RXRβ1 were expressed from the vector pCDM (34); wild type and mutant v-ErbAs were expressed from the vector pRSV (36). The RSV-v-ErbA plasmid was modified from the original construct by deleting the gag sequences. Transfections included 100 ng of pCDMTRα1, which represents a nonsaturating dose of this expression plasmid, 1 \( \mu g \) of pCDMHRXβ1, and different amounts of pRSV-v-ErbAs (or vector). The amount of pRSV-v-ErbA used was 300 ng in experiments containing the inverted repeat ER5; 600 ng and 1.5 \( \mu g \) in experiments containing the direct repeat DR4; 1 and 3 \( \mu g \) in experiments involving the inverted repeat IR0. Vector pRSV was added to achieve a total of 3 \( \mu g \) of pRSV-based plasmid per transfection.

Expression of v-ErbA mutants was confirmed by Western blot of nuclear cell extracts (Amersham Pharmacia Biotech ECL kit) with v-ErbA antibody (gift from M. Privalsky).

**TRANSFECTED CELLS**—JEG-3 cells were grown in 90% Eagle's minimum essential medium plus 10% fetal bovine serum and were transfected using standard calcium phosphate methods (32). The oligonucleotides described above were used as potential v-ErbA response elements. These oligonucleotides were ligated as single inserts into pUTKAT3 at a BamHI site 5′ to the basal herpes simplex virus thymidine kinase promoter driving expression of CAT (35). Reporter plasmids were transfected at a dose of 4 \( \mu g \)-60-mm Petri dish. Mouse TRα1 and rat RXRβ1 were expressed from the vector pCDM (34); wild type and mutant v-ErbAs were expressed from the vector pTKGH (36). The RSV-v-ErbA plasmid was modified from the original construct by deleting the gag sequences. Transfections included 100 ng of pCDMTRα1, which represents a nonsaturating dose of this expression plasmid. 1 \( \mu g \) of pCDMRXβ1, and different amounts of pRSV-v-ErbAs (or vector). The amount of pRSV-v-ErbA was used was 300 ng in experiments containing the inverted repeat ER5; 600 ng and 1.5 \( \mu g \) in experiments containing the direct repeat DR4; 1 and 3 \( \mu g \) in experiments involving the inverted repeat IR0. Vector pRSV was added to achieve a total of 3 \( \mu g \) of pRSV-based plasmid per transfection.

Expression of v-ErbA mutants was confirmed by Western blot of nuclear cell extracts (Amersham Pharmacia Biotech ECL kit) with v-ErbA antibody (gift from M. Privalsky).

**RESULTS**

**Expression of v-ErbA Mutants**—v-ErbA mutants were confirmed by Western blot of nuclear cell extracts (Amersham Pharmacia Biotech ECL kit) with v-ErbA antibody (gift from M. Privalsky).
heptad was created (stop codon 352). In addition, point mutations were made within these regions in amino acids that are highly conserved throughout the superfamily of nuclear receptors. Most of these mutations were chosen to result in substantial alterations of charge or polarity. Mutations were also made outside the dimerization regions as controls (D254A, E258A, and L372R). These proteins were translated using the rabbit reticulocyte lysate system in the presence of [3H]leucine and analyzed by SDS-polyacrylamide gel electrophoresis. Unprogrammed reticulocyte lysate was used as a control (mock). Molecular mass (MW) markers (in daltons) are shown.

The 20-Amino Acid Region and Helices 10 and 11 Are All Involved in v-ErbA Homodimerization and Heterodimerization with RXR on Everted Repeats—Recently, we demonstrated that v-ErbA homodimers bind with the highest affinity to an imperfect everted repeat with a 5-bp spacer (ER5) (11). To determine the dimerization interfaces involved in this process, we intentionally used a dose of v-ErbA that gives minimal homodimer binding. As shown in Fig. 4A and Table II, deletion of the entire 20-amino acid region significantly decreased and disrupted v-ErbA homodimer and v-ErbA-RXR heterodimer formation, respectively, on this site. Among the point mutations tested within this region, P229R and D232A significantly interfered with homodimer formation. The mutant D232A but not P229R also decreased v-ErbA-RXR heterodimer formation on this core motif. The control mutants D254A and E258A did not affect dimerization.

Interestingly, the deletion of helix 10 did not affect v-ErbA homodimer formation. However, the same mutant disrupted heterodimer formation with RXR (Fig. 4B and Table II).

Deletions within helix 11, especially a deletion of the ninth heptad, affected homodimerization. Point mutations within the ninth heptad (L353R and L360R) significantly affected homodimerization as well. Heterodimerization with RXR was disrupted with deletions of the proximal portion of helix 11 (∆H11 A) and of the ninth heptad. However, a deletion within the I box distal to the ninth heptad (∆H 11 B) did not significantly affect heterodimerization on this site. The control mutant L372R outside the I box did not affect homodimerization or heterodimerization (Fig. 4C and Table II).

Neither the 20-Amino Acid Region nor Helices 10 and 11 Are Involved in v-ErbA Homodimerization on IR0—Higher amounts of v-ErbA were required on IR0 than on ER5. This is in agreement with our previous data, which indicated that v-ErbA homodimers bind with lower affinity to IR0 than to ER5 (11).

As shown in Fig. 5 and Table III, deletions and point mutations within the 20-amino acid region and helices 10 and 11 did not affect the homodimer formation of v-ErbA on the inverted repeat IR0. In fact, deletions of helices 10 and 11, and the mutant carrying a stop codon prior to the ninth heptad (stop codon 352) significantly increased homodimer formation on this core motif.

In contrast, the heterodimerization of v-ErbA with RXR was disrupted with deletions of either the 20-amino acid region, helix 10, or helix 11. Also, point mutations within these regions, particularly D232A, Y338L, L353R, and L360R, significantly decreased the formation of heterodimers. The control mutants, D254A, E258A, and L372R, did not affect homodimerization or heterodimerization with RXR on IR0.

It was important to consider the independent occupancy of two half-sites by two protein molecules rather than protein-protein interactions on DNA because none of the v-ErbA mutants tested affect the formation of v-ErbA homodimers on the inverted repeat IR0. However, this possibility was very unlikely, because in dose-response curves, homodimer is the pre-
dominant complex observed bound to DNA (there is minimal evidence of a monomeric intermediate). Nevertheless, “spac- ing” mutants of the inverted repeat with a 6-bp spacer (IR6) and with a 9-bp spacer (IR9) were created. As seen in Fig. 6, v-ErbA binding was not observed on IR6 or IR9, confirming that the v-ErbA-DNA complex seen with IR0 was due to homodimer formation rather than to the independent occupancy of two half-sites by two molecules.

v-ErbA Dimerization in the Absence of DNA—Because the ability of v-ErbA mutants to homodimerize and heterodimerize with RXR differed on the differently oriented core motifs, we wished to determine whether such homodimers and heterodimers can form in the absence of DNA. To accomplish this, MBP-RXR and MBP-v-ErbA fusion proteins were produced in E. coli and absorbed to amylose affinity columns (MBP was adsorbed as a control). After extensive washing, reticulocyte lysate-translated,35S-radiolabeled wild type v-ErbA, D20AA, DH10, DH11A, DH11B, or the D9 heptad was applied to the columns. After further washing, the bound radiolabeled proteins were eluted, and the radioactivity was determined. As shown in Fig. 7, v-ErbA-RXR heterodimers were easily detected under these conditions (48% of input cpm). Proteins harboring deletions of either the 20-amino acid region, helix 10, or within helix 11, heterodimerize with RXR very poorly. Interestingly, in contrast to its inability to heterodimerize with RXR in solution, DH11B was able to heterodimerize with RXR on the direct repeat DR4 (Fig. 4C).

Also, v-ErbA homodimers were clearly detected under these conditions (31% of input cpm). The v-ErbA mutants described above dimerized with MBP-v-ErbA poorly, with DH11A the least affected (31% of the level seen for the wild type). Thus, homodimerization in the absence of DNA most closely resem-

### Table I

Relative binding of v-ErbA mutants as homodimers and heterodimers with RXRa to everted repeats (ERS)

| v-ErbA mutant | Relative binding of v-ErbA homodimer<sup>a</sup> | Relative binding of v-ErbA-RXR heterodimer<sup>a</sup> |
|---------------|---------------------------------|---------------------------------|
| A219D         | 0.51                            | 0.06                            |
| N221I         | 1.01                            | 1.05                            |
| D229R         | 0.66                            | 0.75                            |
| D232A         | 0.21                            | 0.23                            |
| Δ20AA         | 0.17                            | <0.05                           |
| D254A         | 0.97                            | 0.96                            |
| E258A         | 0.97                            | 0.97                            |
| Q328L         | 0.76                            | 0.57                            |
| Y338L         | 0.34                            | 0.18                            |
| ΔH10          | 0.32                            | 0.06                            |
| L353R         | 0.11                            | <0.05                           |
| L360R         | 0.18                            | <0.05                           |
| L372R         | 0.92                            | 0.92                            |
| ΔH11A         | 0.37                            | <0.05                           |
| ΔH11B         | 0.28                            | 0.07                            |
| Δ9 heptad     | 0.13                            | <0.05                           |

<sup>a</sup> Binding of v-ErbA mutants as homodimers or as heterodimers with RXRa were determined relative to wild type v-ErbA, which was arbitrarily designated as 1.00. Results are the averages of two to three experiments. Binding was determined by PhosphorImager analysis.

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**Fig. 3. EMSA analysis of DNA binding of wild type and mutant v-ErbA proteins to the DNA element ER5 in the absence and in the presence of RXRa.**

A, in vitro translated wild type 3H-labeled v-ErbA, v-ErbA proteins containing mutations within the 20 AA region, the control mutant outside the 20-amino acid region (D254A), or unprogrammed reticulocyte lysate (mock) were incubated with a 32P-labeled DNA probe ER5 in the absence or in the presence of in vitro translated wild type 3H-labeled RXRa. The protein-DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. B, experiments were performed with wild type or mutant v-ErbA proteins containing mutations within helix 10 in the absence or in the presence of RXRa. C, experiments were performed with wild type, mutant v-ErbA proteins containing mutations within helix 11, or the control mutant outside the 1 box (L372R) in the absence or in the presence of RXRa. VM, VD, and VR indicate v-ErbA monomer, v-ErbA homodimer, and v-ErbA-RXR heterodimer-DNA complexes, respectively. The asterisk indicates a faint band seen with RXR alone. The amount of in vitro translated v-ErbA used was 2,500 or 1,250 cpm for homodimer or heterodimer formation with RXR, respectively. Equal amounts of mutant v-ErbAs were used, as assessed by [3H]leucine incorporation, taking into account the number of leucines in the specific proteins. For experiments involving heterodimerization, 2,500 cpm of RXRa were employed.
Dominant Negative Activity of v-ErbA Mutants on ER5—The functional consequences of the mutations in the C-terminal region of v-ErbA was investigated. We determined the ability of the mutant proteins to direct v-ErbA repression of T3-dependent CAT expression mediated by TRα1 in JEG-3 cells. As depicted in Table IV, wild type v-ErbA supports potent suppression of T3 induction on the response element ER5. The control mutants, D254A and L372R, are able to suppress T3 mediated CAT activity similar to the wild type. However, the difference in the effect on dimerization between the wild type v-ErbA and the mutants was dramatic. Specifically, suppression of T3-mediated CAT activity was about 5–6-fold greater for the wild type v-ErbA than for the mutants that significantly affect homodimerization and heterodimerization with RXR (A219D, D232A, D20AA, D9 heptad).

v-ErbA proteins containing the point mutations P229R, and Y338L have a less deleterious effect, suppressing T3-mediated CAT activity to 38 ± 1.7 and to 55 ± 2.3%, respectively, of that observed in the absence of v-ErbA. Within helix 11, the mutant DH11A suppressed T3-mediated CAT activity to 49 ± 4.1% of that observed in the absence of v-ErbA. This indicates that the proximal region of helix 11 has a less important role in the dominant negative activity of v-ErbA than the ninth heptad or the distal portion of this helix. In addition, mutations within the 20-amino acid region, helices 10 or 11 affect the repressor activity of v-ErbA in the absence of T3 on this response element (Table IV).

v-ErbA Homodimers and v-ErbA-RXR Heterodimers Are Both Involved in the Dominant Negative Activity of v-ErbA on DR4; However, RXR Is Required to Achieve Maximal Repressor Activity on This Response Element—We have recently shown that cotransfected RXR enhances the dominant negative activity of v-ErbA on the response element arranged as a direct repeat DR4.

### Table II

| v-ErbA mutant | Relative binding of v-ErbA homodimer* | Relative binding of v-ErbA-RXRα heterodimer* |
|---------------|--------------------------------------|---------------------------------------------|
| A219D         | 0.80                                 | 0.34                                        |
| N221I         | 1.03                                 | 1.01                                        |
| P229R         | 0.48                                 | 0.91                                        |
| D232A         | 0.27                                 | 0.34                                        |
| Δ20AA         | 0.24                                 | <0.05                                       |
| D254A         | 1.07                                 | 1.07                                        |
| E258A         | 1.28                                 | 1.1                                         |
| Q328L         | 0.88                                 | 0.87                                        |
| Y338L         | 0.72                                 | 0.48                                        |
| ΔH10          | 0.97                                 | 0.06                                        |
| L353R         | 0.29                                 | 0.14                                        |
| L360R         | 0.33                                 | 0.28                                        |
| L372R         | 0.91                                 | 1.24                                        |
| ΔRXR11        | 0.37                                 | 0.10                                        |
| ΔH11A         | 0.53                                 | 0.81                                        |
| Δ9 heptad     | 0.20                                 | <0.05                                       |

* Binding of v-ErbA mutants as homodimers or as heterodimers with RXRα were determined relative to wild type v-ErbA, which was arbitrarily designated as 1.00. Results are the averages of two to three experiments. Binding was determined by PhosphorImager analysis.
with a 4-bp spacer (DR4) (11). This effect was not observed when RXR was cotransfected with v-ErbA on a response element arranged as an everted repeat (ER5) or inverted repeat (IR0) (11).

Experiments were conducted to test the role of the 20-amino acid region, helix 10, and helix 11 in the dominant negative activity of v-ErbA on DR4 in the absence and presence of cotransfected RXR. Because some v-ErbA mutants selectively affect heterodimerization (Δ H 10) and others do not (Δ H 11 B), these studies may provide a better understanding of the role of v-ErbA homodimers and v-ErbA-RXR heterodimers in the repressor activity of v-ErbA on this core motif.

As shown in Fig. 8, the cotransfection of wild type v-ErbA expressing vector at 600 ng and 1.5 μg suppressed T3-mediated CAT activity to 37 ± 3.7 and 22 ± 1.2% of that observed in the absence of v-ErbA, respectively. When 600 ng of mutant v-ErbA expression vector was cotransfected, the v-ErbA mutants elicit a nil to modest repression of T3 induction on DR4. Specifically, the CAT activities were:

- D20AA, 100 ± 7%;
- ΔH10, 93 ± 5%;
- ΔH11A, 110 ± 3%;
- ΔH11B, 99 ± 1.6%;
- and Δ9 heptad, 80 ± 7.8%, of that observed in the absence of v-ErbA. At 1.5 μg, their repressor activities were enhanced, but remained weaker than that of the wild type. Specifically, the CAT activities on this response element were:

- D20AA, 59 ± 6%;
- ΔH10, 66 ± 4%;
- ΔH11A, 65 ± 10%;
- ΔH11B, 59 ± 6%;
- and Δ9 heptad, 46 ± 8%, of that observed in the absence of v-ErbA. Interestingly, ΔH10, a v-ErbA mutant able to homodimerize but not to heterodimerize with RXR, suppressed T3 induction modestly. This finding suggests that v-ErbA homodimers can, albeit weakly, repress...
v-ErbA, a maximal suppression of T3 induction was observed in inverted repeats. 32P-Labeled inverted repeats with no spacer (IR0), ErbAs, cotransfected RXR absence of RXR T3-mediated CAT activity (92 6

6 bp spacer (IR6), or 9-bp spacer (IR9) between half-sites were incubated with in vitro translated wild type 3H-labeled v-ErbA and analyzed by EMSA. VD indicates v-ErbA homodimer-DNA complexes.

transcription on direct repeats. When RXRβ was cotransfected with 600 ng of wild type v-ErbA, a maximal suppression of T3 induction was observed (6 ± 0.6% of that observed in the absence of v-ErbA). In contrast, with the exception of ΔH11B, RXRβ did not enhance significantly the repressor activity of the v-ErbA mutants. Specifically, the cotransfection of RXRβ with 600 ng of the v-ErbA mutants Δ20AA, ΔH10, ΔH11A, and Δ9 heptad, suppressed T3-mediated CAT activity to 91 ± 3.7, 79 ± 2.8, 108 ± 5.9, and 81 ± 3.7% of that observed in the absence of v-ErbA, respectively. However, the mutant ΔH11B was not only able to heterodimerize with RXR on DR4 by EMSA (Fig. 4C), but also its repressor activity was significantly enhanced when RXRβ was cotransfected. Specifically, ΔH11B was without effect (99 ± 2%) in the absence of cotransfected RXRβ and suppressed T3-mediated CAT activity to 39 ± 7% in the presence of cotransfected RXRβ. In the absence of wild type or mutant v-ErbAs, cotransfected RXRβ did not have a significant effect on T3-mediated CAT activity (92 ± 5% of that observed in the absence of RXRβ).

Taken together, the above data indicate that v-ErbA-RXR heterodimers rather than v-ErbA homodimers are critical for the dominant negative activity of v-ErbA on direct repeats. Moreover, the results obtained by EMSA and transfection analyses with ΔH11B suggest that sequences within helix 11 distal to the ninth heptad of v-ErbA do not play an important role in the interaction with RXR on direct repeats with a 4-bp spacer.

The Repressor Activity of v-ErbA on IR0 Is Most Likely Mediated by v-ErbA Homodimers—Experiments were conducted to examine the role of the 20-amino acid region, helix 10, and helix 11 in the dominant negative activity of v-ErbA on the inverted repeat IR0. For this purpose, 1 μg of wild type or mutant v-ErbAs was transfected. As shown in Fig. 9, wild type v-ErbA suppressed T3-mediated CAT activity to 72 ± 5% of that observed in the absence of v-ErbA, confirming that v-ErbA is a weak repressor on this response element. v-ErbA mutants containing deletions of either the 20-amino acid region, helix 10, helix 11A, helix 11B, or the ninth heptad suppressed T3-mediated CAT activity to 61 ± 6, 64 ± 7, 77 ± 2, 99 ± 4, and 76 ± 3%, respectively. In addition, the mutant harboring a stop codon at position 352 suppressed T3-mediated CAT activity to 57 ± 1.4% of that observed in the absence of the v-ErbA mutant. Thus, except for ΔH11B, the mutants were as

| v-ErbA mutant | Relative binding of v-ErbA homodimer* | Relative binding of v-ErbA-RXRα heterodimer* |
|---------------|--------------------------------------|--------------------------------------------|
| A219D         | 1.66                                 | 0.18                                       |
| N221I         | 1.14                                 | 0.98                                       |
| P229R         | 0.92                                 | 0.69                                       |
| D232A         | 1.28                                 | 0.26                                       |
| Δ20AA         | 0.93                                 | 0.07                                       |
| D254A         | 1.11                                 | 1.03                                       |
| E258A         | 1.20                                 | 0.96                                       |
| Q328L         | 1.12                                 | 0.54                                       |
| Y338L         | 2.24                                 | 0.16                                       |
| ΔH10          | 2.44                                 | <0.05                                      |
| L353R         | 1.13                                 | 0.06                                       |
| L360R         | 1.57                                 | 0.07                                       |
| L372R         | 0.95                                 | 0.94                                       |
| ΔH11A         | 1.75                                 | 0.12                                       |
| ΔH11B         | 2.16                                 | 0.11                                       |
| Δ9 heptad     | 1.36                                 | 0.09                                       |
| Stop codon 352| 1.87                                 | <0.05                                      |

* Binding of v-ErbA mutants as homodimers or as heterodimers with RXRα were determined relative to wild type v-ErbA, which was arbitrarily designated as 1.00. Results are the averages of two to three experiments. Binding was determined by PhosphorImager analysis.

![EMSA analysis of v-ErbA binding to variably spaced inverted repeats](image1)

![Dimerization of v-ErbAs in the absence of DNA](image2)
The C-terminal domain (ligand-binding domain) of the nuclear receptors contains a strong dimerization interface named the I box (17). In addition, there is evidence that the 20-amino acid region located toward the N-terminal end of the ligand-binding domain is also involved in dimerization (20, 21).

Previous studies have suggested that there is a common C-terminal dimerization interface used on all classes of binding sites. Specifically, these studies have indicated that whereas the C-terminal domain is physically constrained as a consequence of dimerization, the DBD in contrast retains spatial flexibility (7, 17, 26). This flexibility would allow the DBD to rotate to bind to everted, direct, and inverted repeats. Although these important studies have improved our understanding of dimerization, the question of whether or not a common C-terminal domain interface exists has not been answered for the following reasons: 1) the data were obtained from DNA binding assays in which large deletions and chimeras within the C-terminal domain were created and 2) the crystal structures of the C-terminal domain of nuclear receptors were done in solution and not in the presence of the DBD bound to differently oriented core motifs (24, 25).

Therefore, we performed a detailed mutational analysis within the 20-amino acid region and helices 10 and 11 of v-ErbA to determine whether different dimerization interfaces in the C-terminal region are used for v-ErbA homodimerization and heterodimerization with RXR and whether different dimerization interfaces are used on core motifs with different orientations. Furthermore, the creation of v-ErbA mutants that disrupt heterodimerization with RXR without affecting homodimerization and vice versa helped clarify the role of RXR in the dominant negative activity of v-ErbA.

Our results indicate that different dimerization interfaces are used for v-ErbA homodimers and for v-ErbA-RXR heterodimers and also that different dimerization interfaces are used on core motifs with different orientations. Specifically, for v-ErbA homodimers, helices 10 and 11 and the 20-amino acid region are all important when bound to everted repeats. For v-ErbA homodimers bound to direct repeats, helix 11 and the 20-amino acid region are involved in this process, whereas helix 10 does not play an important role. When bound to inverted repeats, neither the 20-amino acid region, helix 10, nor helix 11 are involved in v-ErbA homodimerization. Furthermore, v-ErbA mutants containing deletions of helices 10 and 11 or a stop codon prior to the ninth heptad display an enhanced binding to inverted repeats as compared with the wild type v-ErbA. These results indicate that sequences within the C-terminal domain of v-ErbA interfere with v-ErbA homodimerization on inverted repeats, probably by a steric mechanism. In contrast, chicken ovalbumin upstream promoter transcription factor (COUP-TF) homodimers require the I box for binding to
a core motif arranged as an inverted repeat (17).

The above data indicate that steric interactions between two v-ErbA molecules are not identical on everted, direct, or inverted repeats. We have found that everted repeats with a 5- or 6-bp spacer display a higher affinity binding for v-ErbA homodimers than direct or inverted repeats (11). The differences in affinity of these homodimers for the corresponding response elements may be explained by differences in the dimerization interfaces of v-ErbA homodimers. Two possibilities may be considered in the process of DNA binding by v-ErbA homodimers. In the first model, v-ErbA homodimers are formed in solution and subsequently bind to the response element with high affinity. In the second model, a v-ErbA monomer binds first to DNA, and then a second v-ErbA molecule binds, and the overall complex is stabilized by protein-protein as well as protein-DNA interactions.

Our data support the first model in that v-ErbA can indeed homodimerize in solution. The pattern of homodimerization in solution by the v-ErbA mutants resembles more closely that seen on the high affinity binding sites, the everted repeats. However, the dimerization interfaces involved in solution differed from those involved when bound to direct or inverted repeats, probably imposed by the orientation of these core motifs. This in turn could explain the lower affinity for v-ErbA homodimer binding for these sites as compared with everted repeats. In addition, the inhibitory effect of the C-terminal region of v-ErbA would explain the weak binding of v-ErbA homodimers to inverted repeats.

It is unknown which region(s) are involved in v-ErbA homodimerization on inverted repeats. It is likely that this is mediated by the dimerization interface of the DNA-binding domain or by specific sequences in the C-terminal domain. In addition, v-ErbA dimerization could be regulated by sequences in the N-terminal domain as described with TRα (37).

Regarding v-ErbA-RXR heterodimers, the 20-amino acid region and helices 10 and 11 of v-ErbA are all involved in the dimerization with RXR. However, sequences within helix 11 distal to the ninth heptad of v-ErbA are critical for heterodimerization with RXR in solution and when bound on response elements arranged as inverted and everted repeats but not on direct repeats, as shown with the mutant ΔH11B. This mutant is not only able to heterodimerize with RXR on DR4, but its repressor activity is also enhanced when RXR is cotransfected in a system containing a direct repeat as a response element. Thus, these data indicate that the nature of DNA-binding sites play an active role in the formation of dimerization interfaces of v-ErbA with RXR.

Overall, there is a good correlation between the DNA binding affinity of v-ErbA, the repressor action of v-ErbA on inverted repeats, and the transfection data, although some discrepancies are noted. For example, the mutant A219D has similar binding characteristics as the mutant ΔH11A for homodimer and heterodimer formation with RXR on everted repeats. However, the latter is a more potent repressor in transfection assays. A likely explanation could involve the nuclear corepressors. Recently, it has been shown that helices 5 and 6 in v-ErbA (named SSD-2 subdomain) are involved directly in protein-protein interactions with nuclear corepressors (NCoR and SMRT), thus playing an important role in the silencing activity of v-ErbA (38). Therefore, because the 20-amino acid region is included in this subdomain, it is probable that some mutations within this region could affect the interaction with the corepressors.

Although RXRs can enhance the DNA binding of v-ErbA, the significance of RXR in the dominant negative activity of v-ErbA remains to be elucidated. Based on the data obtained with the v-ErbA mutants, it is difficult to precisely determine the role of RXR in v-ErbA action on everted repeats, because the mutants that affect dimerization interfere with both homodimerization and heterodimerization. Nevertheless, important information can be obtained from these data. Specifically, ΔH11A, a v-ErbA mutant that partially retains its ability to homodimerize but is unable to heterodimerize with RXR, is capable of repressing T3-mediated action. Furthermore, we have shown that cotransfected RXR did not enhance the dominant negative activity of wild type v-ErbA on everted repeats (11). Thus, our data suggest that v-ErbA homodimers rather than v-ErbA-RXR heterodimers play an important role in v-ErbA action on response elements arranged as everted repeats. However, we cannot rule out the possibility that heterodimer formation between v-ErbA and endogenous RXR(s) accounts for some of the repressor activity on this core motif.

On direct repeats, the transfection data with the v-ErbA mutants show that v-ErbA homodimers and v-ErbA-RXR heterodimers are both involved in the dominant negative activity of v-ErbA. However, heterodimerization with RXR is required to achieve maximal repressor activity on this response element.

The v-ErbA mutants were as potent as the wild type in directing repression of T3-mediated action on inverted repeats. These mutants were capable of binding as homodimers but not as heterodimers with RXR on this response element. Furthermore, we have shown previously that cotransfected RXR does not affect the dominant negative activity of v-ErbA on this response element (11). Taken together, these results would indicate that the repressor activity of v-ErbA on inverted repeats is most likely mediated by v-ErbA homodimers rather than heterodimers with RXR. In conclusion, the data indicate that different dimerization interfaces are used for v-ErbA homodimerization and heterodimerization with RXRα, and different dimerization interfaces are used on differently oriented core motifs.

Data obtained from knock-out mice devoid of all known thyroid hormone receptors have shown a mild overall phenotype obtained with the TRα/"/TRβ− mice compared with those with hypothyroidism (39). The difference in phenotype could be explained by the ability of TRs to bind T3 response elements and repress transcription in the absence of T3. This would suggest physiological consequences for such T3-independent action of TR. Thus, a better understanding of the dimerization and repressor action of v-ErbA may provide insight into the fundamental mechanism underlying physiologic hormone regulation of TR as well as into that of other members of the nuclear receptor superfamily.

Also, investigation of v-ErbA may lead to important findings that can be applied to other clinical situations involving dominant negative transcription factors, such as the thyroid hormone resistance syndrome. This syndrome is an autosomal dominant condition with a variety of phenotypes caused by mutations in TRβ. The mutant receptor appears to function as a dominant negative form inducing the disease by interfering with the action of the normal receptor counterpart. These mutations disrupt ligand (T3) binding, affect interaction with corepressors, or disrupt dimerization (40–42). Because v-ErbA and TR are highly related, the data described above could predict the effect of mutations in the carboxy terminal dimerization interface of TR on homodimerization or heterodimerization with RXR on differently oriented core motifs and therefore could help explain differences in phenotype in this syndrome.

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