***v-erbA* overexpression is required to extinguish *c-erbA* function in erythroid cell differentiation and regulation of the *erbA* target gene CAII**

Christine Disela, Corinne Glineur,¹ Thomas Bugge,² Jan Sap,³ Gabi Stengl, Jerry Dodgson,⁴ Henk Stunnenberg,² Hartmut Beug, and Martin Zenke⁵

Institute for Molecular Pathology, A-1030, Wien, Austria; ¹ Institut National de la Santé et de la Recherche Médicale U186, Centre National de la Recherche Scientifique URA1160, Institut Pasteur, F-59019 Lille-Cedex, France; ²European Molecular Biology Laboratory, D-6900 Heidelberg, Germany; ³Department of Pharmacology, New York University Medical Center, New York 10016 USA; ⁴Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824-1101 USA

The *v-erbA* oncoprotein represents a retrovirus-transduced oncogenic version of the thyroid hormone (T3/T4) receptor *c-erbA* (type α). It contributes to virus-induced erythroleukemia by efficiently arresting differentiation of red cell progenitors and by suppressing transcription of erythrocyte-specific genes. Here, we show that *v-erbA* and *c-erbA* bind directly to sequences within the promoter of the erythrocyte-specific carbonic anhydrase II (CAII), a gene whose transcription is efficiently suppressed by *v-erbA*. This *erbA*-binding site confers thyroid hormone responsiveness to a heterologous promoter in transient expression experiments and is a target for efficient down-regulation of CAII transcription by the *v-erbA* oncoprotein. In stably transformed erythroblasts coexpressing the *v-erbA* oncoprotein and the *c-erbA*/T3 receptor at an approximately equimolar ratio, *c-erbA* activity is dominant over *v-erbA*. T3 efficiently induced erythroid differentiation in these cells, thus overcoming the *v-erbA*-mediated differentiation arrest. Likewise, T3 activated CAII transcription as well as transient expression of a T3-responsive reporter gene containing the CAII-specific *erbA*-binding site. The *c-erbA*-dependent activation of this CAII reporter construct could only be suppressed by very high amounts of *v-erbA*. Our results suggest that overexpression of *v-erbA* is required for its function as an oncoprotein.

[Key Words: *v-erbA* oncogene; *c-erbA*; thyroid hormone; thyroid hormone receptor; carbonic anhydrase; erythroid differentiation]

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The *v-erbA* oncoprotein represents a highly mutated version of the thyroid hormone (T3/T4) receptor *c-erbA* (type α ; Sap et al. 1986; Weinberger et al. 1986). However, owing to multiple point mutations and a small carboxy-terminal deletion, *v-erbA* neither binds nor responds to thyroid hormones nor does it *trans*-activate T3-responsive target genes. Yet it still binds to DNA in a sequence-specific fashion (Boucher et al. 1988; Munoz et al. 1988; Damm et al. 1989; Sap et al. 1989; Bonde and Privalsky 1990). In yeast, *v-erbA* seems to display a residual T3 response, rendering it a hormone-regulated transcriptional activator in this organism (Privalsky et al. 1990).

v-erbA was first identified as one of the two oncogenes of the avian erythroblastosis virus (AEV; Vennström et al. 1980; Debuire et al. 1984), a retrovirus causing sarco-

mas and an acute fatal erythroleukemia in virus-infected chickens (for review, see Graf and Beug 1978, 1983). Previous studies demonstrated that *v-erbA* contributes to erythroid cell transformation by efficiently blocking terminal differentiation of both normal and transformed erythroid precursor cells (Gandrillon et al. 1989; Schröder et al. 1990 and references therein). In addition, *v-erbA* abrogates the complex growth requirements of such transformed erythroblasts in culture (Beug et al. 1985; Kahn et al. 1986). These specific effects of *v-erbA* are at least partially due to its ability to interfere with transcription of three erythroid-specific genes whose expression is up-regulated during and most probably required for normal erythrocyte differentiation (Knight et al. 1988; Zenke et al. 1988, 1990). These *v-erbA*-regulated genes encode the erythrocyte-specific anion transporter band3, the erythroid-specific carbonic anhydrase II (CAII), and the enzyme aminolevulinic synthase

⁵Corresponding author.

(ALA-S, which catalyzes the first and rate-limiting step in heme biosynthesis). Two of these genes (band3, CAII) have been reintroduced into *v-erbA*-expressing leukemic erythroblasts, showing that repression of these two genes is responsible for the *v-erbA*-induced changes in the medium requirements of these cells (Fuerstenberg et al. 1990; I. Leitner et al. in prep.).

Recently, we have shown that *c-erbA*, when overexpressed in erythroid cells, efficiently regulates erythroid differentiation in a hormone-responsive fashion: Differentiation is blocked in the absence and induced in the presence of T3 (Zenke et al. 1990). In these cells transcription of the very same three genes (band3, CAII, ALA-S) whose expression is constitutively suppressed by the *v-erbA* oncoprotein, is modulated in a hormone-responsive fashion, that is, suppressed in the absence and activated in the presence of T3. These results have led to the notion that *v-erbA* represents a hormone-independent repressor of transcription that constitutively displays only one T3 receptor function (to repress transcription in the absence of hormone) but has lost the ability to respond to T3 and thus to activate transcription (Zenke et al. 1990). Evidence in favor of this idea has been presented by Damm et al. (1989) and Sap et al. (1989), who analyzed the ability of *c-erbA* and *v-erbA* to regulate transcription via an artificial thyroid hormone-responsive element (T3RE) and a T3RE present in the Moloney murine leukemia virus long terminal repeat (MoMLV LTR). They showed that the *v-erbA* oncoprotein constitutively repressed transcription of the respective T3-responsive reporter gene constructs in transient expression assays. In one study *v-erbA* was able to efficiently extinguish *c-erbA* receptor function, leading to the notion that *v-erbA* contributes to leukemogenesis by acting as a dominant repressor of gene expression (Damm et al. 1989). However, because none of the genes suppressed in leukemic erythroblasts has been identified as a direct target of *v-erbA* or *c-erbA*, this hypothesis could not be tested directly.

In this paper we have identified the erythroid-specific CAII gene as a direct target of *v-erbA* action. Both *v-erbA* and *c-erbA* bind specifically to the same sequence element within the promoter of the CAII gene. This *erbA*-binding site exhibits sequence motifs found in thyroid hormone-responsive elements and overlaps with potential binding sites for the *fos/jun/AP-1* complex and for the erythroid-specific transcription factor NF-E2. In transient expression assays this *erbA*-binding site confers thyroid hormone responsiveness to a heterologous promoter and is a target for efficient down-regulation of transcription by the *v-erbA* oncoprotein.

To directly test the dominant repressor hypothesis in leukemic cells and on naturally occurring target genes, we have stably introduced *c-erbA* into the *v-erbA*-expressing erythroblast cell line HD3 (Beug et al. 1982). Using this approach, we studied the effect of the coexpressed *v-erbA* and *c-erbA* proteins on (1) erythroid differentiation, (2) erythrocyte-specific gene expression, and (3) the activity of transiently transfected reporter gene constructs containing the CAII-specific *erbA*-bind-

ing site. We will show that *c-erbA* confers T3-dependent regulation of erythroid differentiation and transcriptional activation via the CAII T3RE when coexpressed with *v-erbA* at equimolar levels or in the presence of a moderate excess of *v-erbA*, indicating that under these conditions *c-erbA* is dominant over *v-erbA*. However, a large excess of *v-erbA* was able to extinguish the *c-erbA*-dependent activation of the CAII reporter gene. This suggests that in leukemic cells overexpression of *v-erbA* is required to efficiently suppress endogenous *c-erbA*/T3 receptor function, which appears to be an essential feature for *v-erbA* activity in erythroid cell transformation.

Results

c-erbA regulates erythroid differentiation in the presence of *v-erbA*

Erythroblasts expressing the *v-erbA* oncoprotein are blocked completely in their ability to differentiate, whereas erythroblasts overexpressing *c-erbA* can be efficiently induced to differentiate into erythrocytes by T3 (Zenke et al. 1988, 1990; Pain et al. 1990; Schröder et al. 1990). This finding led to the notion that *v-erbA* exerts its oncogenic activity by interfering with a possible function of the endogenous *c-erbA* or of related receptors in erythroid cell differentiation (Schröder et al. 1991a,b).

To study this question we tried to express *c-erbA* at levels high enough to overcome the *v-erbA*-mediated block in differentiation. Consequently, recombinant retroviral vectors have been constructed that efficiently express the *c-erbA*/T3 receptor either as a gag/*c-erbA* fusion protein or as bona fide *c-erbA*, together with the neomycin-resistance gene as a selectable marker (Fig. 1A,B; Munoz et al. 1988; Zenke et al. 1990). To investigate if and to what extent the point mutations present in the *v-erbA* DNA-binding domain might affect T3 receptor function and its ability to compete with *v-erbA* for potential target sequences, a chimeric gag/*v-c-erbA* T3 receptor was constructed containing the *v-erbA*-specific DNA-binding domain joined to the hormone-binding domain of *c-erbA* (Fig. 1A; *v2-erbA* in Munoz et al. 1988; Zenke et al. 1990). These retroviral vectors have been introduced into cells of the *v-erbA*/t*serbB*-transformed erythroblast cell line HD3 (Beug et al. 1982), and neomycin-resistant colonies were selected in semisolid methocel medium (Schröder et al. 1990). Neomycin-resistant colonies obtained after 7–10 days were isolated and tested for their response to thyroid hormone. Such cells were therefore incubated at 42°C (to inactivate t*serbB*) and their ability to differentiate in response to T3 was evaluated both by changes in morphology and by a reduction in their growth rates (measured as inhibition of ³H-thymidine incorporation).

As shown in Figure 2 the majority of clones coexpressing *v-erbA* and *c-erbA* or gag/*c-erbA* showed a clear response to T3 as evidenced by a strong inhibition of [³H]thymidine incorporation. In clones coexpressing *v-erbA* and the chimeric gag/*v-c-erbA* protein, this inhibition in growth by T3 was less prominent. As ex-

Figure 1. Schematic representation of the *erbA* proteins, the *erbA*-neo retroviral vectors, and of the CAII promoter. (A) The structure of *c-erbA*, *v-erbA*, *gag/c-erbA*, and the chimeric *gag/v-c-erbA* is shown. The virus-derived *gag* sequences (Δ gag), DNA-binding, and hormone-binding domains, the *v-erbA*-specific point mutations (●), and the carboxy-terminal deletion present in *v-erbA* (Δ gag) are indicated. (B) Schematic representation of recombinant retroviral vectors containing Δ gag/erbA or *c-erbA*, together with the neomycin-resistance gene (neo and Δ gag/neo, respectively). (C) The CAII promoter is depicted from position -940 to +430. The nucleotide position of the mRNA start site +1 is indicated. The position of the -660 *erbA*-binding site (*erbA*; positions -672 to -655), the TATA sequence, and the cleavage sites for the restriction endonucleases *Ava*II (A) and *Bss*HII (B) are shown. The CAII upstream sequence is listed as accession number X15807 in the EMBL data library.

pected, untreated clones or clones transfected with a neo vector lacking *erbA* (Fig. 2; *v-erbA*) did not respond. The same picture emerged if these clones were analyzed for morphological changes in response to T3. As shown in Figure 3A erythroblast clones coexpressing *v-erbA* and *c-erbA* (either as *gag/c-erbA* or as bona fide *c-erbA*) behaved very similarly to erythroblast clones expressing *c-erbA* only (Zenke et al. 1990). They formed large colonies of essentially immature cells in the absence of T3 and small reddish colonies consisting of partially mature cells in the presence of T3 which, however, disintegrated with prolonged T3 treatment (Fig. 3A; data not shown). In these assays the *c-erbA* and *gag/c-erbA* T3 receptors were consistently more efficient in inducing differentiation than the chimeric *gag/v-c-erbA* receptor containing the *v-erbA*-specific DNA-binding domain. However, one

T3-responsive erythroblast clone coexpressing *v-erbA* and *gag/v-c-erbA* (clone 16, Fig. 2) could be isolated that showed a partial arrest of differentiation in the absence of hormone, whereas normal differentiation into mature erythrocytes was observed in the presence of T3 (Fig. 3B). As expected, T3 had no effect on erythroblasts expressing only *v-erbA* (Fig. 3B). Taken together, these results demonstrate that the normal *c-erbA* protein and variants thereof can overcome the *v-erbA*-mediated block of differentiation and cause a T3-dependent modulation of the differentiated phenotype if coexpressed in *v-erbA*-containing cells.

To determine the relative amounts of the *c-erbA* and *v-erbA* proteins coexpressed in such cells, the same erythroblast clones as tested above (see Fig. 3) were labeled with [³⁵S]methionine and subjected to immunoprecipitation analysis by using anti-*erbA*-specific antisera (Beug and Hayman 1984; Glineur et al. 1990). Quantification of the *c-erbA*- and *v-erbA*-specific bands in the autoradiograph shown in Figure 4A (and data not shown) revealed that the transduced *c-erbA* was expressed at equimolar to threefold lower levels than the *v-erbA* oncoprotein. Because the *gag/c-erbA* and *gag/v-c-erbA* proteins could not be distinguished in size from *v-erbA*, only a rough estimate of their amounts could be made from the 1.7- to 2.1-fold increase in intensity of the superimposed *gag/c-erbA*- (or *gag/v-c-erbA*-) and *v-erbA*-specific bands as compared to the intensity of the *v-erbA*-specific bands in several control clones (Fig. 4A and data not shown).

Finally, we investigated whether coexpression of *c-erbA* in *v-erbA* erythroblasts would allow T3-dependent activation of, for example, CAII, a gene whose transcriptional activity is efficiently suppressed by *v-erbA* (Zenke et al. 1990). Figure 4B shows that in cells expressing similar levels of *c*- and *v-erbA* proteins, *c-erbA* clearly dominated *v-erbA* activity and induced CAII transcription in response to T3. Band3 and ALA-S, whose transcription is also repressed by *v-erbA* (Zenke et al. 1988), were also induced by T3 in such cells (Fig. 4B and data not shown).

CAII transcription is directly regulated by hormone-responsive *erbA* proteins

The results described above led to the unexpected conclusion that *c-erbA* and variants thereof were able to overcome the *v-erbA*-mediated arrest of erythroid differentiation and erythrocyte gene transcription. To answer the question whether or not this effect was the result of a direct competition of the *erbA* proteins for similar regulatory sequences in the promoters of the affected genes, efforts have been made to investigate whether any of these genes might be directly regulated by *v-erbA* or *c-erbA*.

To test whether or not CAII, band3, or ALA-S would qualify as potential direct targets for *erbA* action, two assays were performed. First, we checked whether in cells stably expressing hormone-responsive *erbA* proteins transcription of any of these genes would be rapidly induced by T3 within a few hours. Second, we tested

Figure 2. Analysis of erythroblast clones coexpressing *v-erbA* together with *c-erbA*, *gag/c-erbA*, or *gag/v-c-erbA* for their differentiation response to T3. The extent of growth inhibition [^3H]thymidine incorporation in the presence of T3 divided by the respective value measured in the absence of T3 (Zenke et al. 1990) in response to T3 treatment is shown for individual HD3 erythroblast clones coexpressing *v-erbA* and *c-erbA*, *gag/c-erbA*, or *gag/v-c-erbA* as indicated. HD3 clones transfected with the neo-retroviral vector alone and thus expressing *v-erbA* only were used as a control (*v-erbA*).

whether the T3-induced up-regulation of these genes would also be observed in the presence of the protein biosynthesis inhibitor cycloheximide, because direct transcriptional activation by *erbA* should occur in the absence of protein synthesis. As shown in Figure 5A, CAII mRNA was rapidly induced, reaching maximal levels after 2–5 hr of T3 treatment, irrespective of the presence or absence of an active *tserbB* oncogene (37°C and 42°C, respectively; data not shown). T3 induction of band3 mRNA followed somewhat slower kinetics, achieving maximal levels after 5–10 hr. No such T3-dependent increase of β -globin mRNA was observed, whereas *c-myb* mRNA levels decreased (probably as a result of the induction of differentiation in these cells).

In addition, no T3-dependent activation of CAII and band3 transcription was observed in *v-erbA*-containing cells where, as expected, CAII and band3 mRNA levels were efficiently suppressed (Fig. 5A). Using run-on analysis, we have demonstrated previously that the modulation of CAII and band3 mRNA levels by *erbA* proteins is the result of an efficient regulation of CAII and band3 gene transcription (Zenke et al. 1988, 1990). Furthermore, the induction of CAII transcription by T3 followed the same pattern irrespective of whether bona fide *c-erbA*, *gag/c-erbA*, or the chimeric *gag/v-c-erbA* T3 receptor had been used (data not shown), suggesting that neither the *gag* sequences fused 5' to the *erbA* proteins nor the *v-erbA*-specific point mutations contained in the

Figure 3. T3-dependent induction of differentiation of erythroblast clones coexpressing *v-erbA* and *c-erbA*, *gag/c-erbA*, or *gag/v-c-erbA*. (A) The erythroblast clone coexpressing *v-erbA* and *gag/c-erbA* (clone 24 in Fig. 2) was seeded into semisolid methocel medium in both the absence and presence of T3 (–T3 and +T3, respectively; see Materials and methods). Photographs of typical colonies obtained after 3–4 days at 42°C are shown (colonies). In parallel, mass cultures of the same clone were incubated at 42°C for 3 days in the absence and presence of T3. Cytospin preparations of these cells stained with neutral benzidin and histological dyes (Zenke et al. 1990) and photographed under blue light to specially reveal the hemoglobin content are shown (hemoglobin). (B) Erythroblast clones coexpressing *v-erbA* and *c-erbA* or *gag/v-c-erbA* (clones 12 and 16, respectively; see Fig. 2) were induced to differentiate by T3 in liquid culture as described in A. An HD3 clone expressing *v-erbA* only was used as a control (*v-erbA*). Cytospin preparations of these cells as described in A are shown.

Figure 4. Immunoprecipitation analysis of *erbA* proteins and T3-dependent regulation of CAII and band3 transcription in cells coexpressing *v-erbA* and *c-erbA*. (A) Erythroblast clones coexpressing *v-erbA* and *c-erbA* (clones 7 and 12 in Fig. 2; lanes 1 and 2, respectively), or *gag/c-erbA* (clones 2 and 24 in Fig. 2; lanes 3 and 4, respectively) or expressing *v-erbA* only (clones 6, 12, and 15 in Fig. 2; lanes 5, 6 and 7, respectively) were labeled with [³⁵S]methionine and subjected to immunoprecipitation analysis as described in Materials and methods. The positions of p46^{c-erbA}, p75^{v-erbA} and p75^{gag/c-erbA} are indicated. (B) Erythroblast clones (see A) expressing *v-erbA* and *c-erbA* (lanes 1–4) or *v-erbA*

only (lanes 5,6) were treated with T3 for 16 hr or left untreated as indicated. Total RNA prepared from these cells was analyzed by Northern blot hybridization for CAII- and band3-specific transcripts. The 28S rRNA was stained with methylene blue (Khandjian 1986) to show equal RNA loading in all lanes.

DNA-binding domain of the chimeric *gag/v-/c-erbA* receptor grossly affect the ability of these proteins to induce CAII transcription.

To investigate whether the activation of CAII and band3 transcription would also occur in the absence of protein biosynthesis, the same cells as described above were treated with T3 in both the absence and presence of cycloheximide. As shown in Figure 5B CAII mRNA levels were elevated rapidly by T3, irrespective of the absence or presence of cycloheximide, indicating that de novo protein synthesis is not required for the T3-specific induction of CAII mRNA in these cells [similar results were obtained by Pain et al. (1990) when this manuscript was in preparation]. In contrast, following addition of T3, band3-specific transcripts accumulated less rapidly in the presence than in the absence of cycloheximide, suggesting that a cycloheximide-sensitive step is involved in the T3-dependent transcription of this gene. As expected, β -globin mRNA levels were only marginally af-

fected by T3. Essentially the same result was obtained when the experiment was performed at 42°C when the second retroviral oncogene (*tserbB*) contained in these cells was inactivated by temperature shift (data not shown).

In conclusion, both the rapid kinetics of CAII mRNA induction by T3 and its insensitivity to cycloheximide treatment strongly suggest that the CAII gene might be subject to direct transcriptional control by hormone-activated *erbA* proteins.

v-erbA and *c-erbA* bind to upstream sequences of the CAII gene

To directly assess the ability of the *erbA* proteins to bind to specific sequences in the CAII gene, a combined DNA-binding/immunoprecipitation protocol was employed (McKay 1981). Therefore, *v-erbA*, *c-erbA*, and *gag/v-/c-erbA* were expressed in recombinant vaccinia

Figure 5. The induction of CAII mRNA by T3 is rapid and insensitive to cycloheximide treatment. (A) Kinetics of CAII mRNA induction by T3. Erythroblasts expressing *gag/v-/c-erbA* (*v2-erbA* in Zenke et al. 1990) were shifted to 42°C for 18 hr (to inactivate *tserbB*) and treated with 100 nM T3 for various periods of time as indicated. As a control, *v-erbA*-transformed erythroblasts (Zenke et al. 1988; 1990) were treated similarly. RNA was prepared and subjected to slot blot hybridization and analyzed for CAII-, band3-, β -globin-, and *c-myb*-specific transcripts as described in Materials and methods. (B) Effect of cycloheximide treatment (CX; 20 μ M/ml) on the induction of CAII mRNA by T3. The same *gag/v-/c-erbA*-expressing erythroblasts as used in A were treated for 4 and 8 hr with T3, cycloheximide, or both or were left untreated. Cycloheximide was added 30 min prior to the addition of T3. RNA was prepared and analyzed by slot blot hybridization with CAII-, band3-, and β -globin-specific probes (see Materials and methods).

virus vectors in HeLa cells (Stunnenberg et al. 1988), and extracts were prepared and incubated with a mixture of 32 P-labeled DNA fragments derived from an *Ava*II digestion of the CAII gene (Fig. 1C). The DNA-*erbA* protein complexes formed in this assay were immunoprecipitated by an anti-*erbA*-specific antiserum. Preimmune serum was used as a control. The DNA fragments precipitated were then resolved by gel electrophoresis. Figure 6 shows that of the five fragments precipitated, two were specifically precipitated by the anti-*erbA* antibody only (0.56 and 0.8 kb). The three additional fragments were also precipitated by the control serum. In addition, *c-erbA*-containing extracts and extracts containing *v-erbA* or the chimeric *gag/v-c-erbA* protein (Fig. 1A) yielded a very similar pattern of bands, although the 0.56-kb band was found to be slightly stronger when *c-erbA*-containing extracts were used. No specific precipitation was observed with cell extracts from wild-type vaccinia virus-infected HeLa cells. We also noted that nonspecific competitor DNA interfered more efficiently with *erbA* binding to the 0.56-kb than to the 0.8-kb band, suggesting stronger binding to the larger fragment (data not shown). Finally, a synthetic oligonucleotide containing a T3RE (see below) competed efficiently for *erbA*-specific binding and immunoprecipitation of the 0.56- and 0.8-kb fragments, whereas an oligonucleotide containing an unrelated sequence did not compete (data not shown).

The DNA fragments specifically immunoprecipitated as DNA-*erbA* protein complexes in this assay map to the promoter region of the CAII gene (position -939 to +426; Fig. 1C), strongly suggesting a direct and specific interaction of the *erbA* proteins with CAII promoter sequences. Digestion of the CAII gene with another restriction endonuclease (*Bss*III; Fig. 1C) results in a different

Figure 6. Immunoprecipitation of DNA-*erbA* protein complexes with anti-*erbA*-specific antibodies (McKay assay; McKay 1981). 32 P-Terminally labeled *Ava*II fragments spanning the CAII promoter (see Fig. 1C) were incubated with HeLa cell extracts containing vaccinia virus-expressed *c-erbA*, *v-erbA*, and *gag/v-c-erbA* proteins or with extracts from wild-type vaccinia virus-infected HeLa cells (no *erbA*). Following immunoprecipitation with an anti-*erbA*-specific antibody (lanes 1,3,5,7) fragments were resolved in 2% agarose (lanes 1–4) or 5% polyacrylamide gels (lanes 5–8). Preimmune serum was used as a control (lanes 2,4,6,8). The two uppermost bands and a small 0.2-kb band represent plasmid-derived fragments. Protein binding and/or immunoprecipitation of these fragments apparently is non-specific, as these bands are also precipitated, albeit less efficiently, by preimmune serum.

pattern of fragments, yet if employed in the DNA binding/immunoprecipitation assay, yielded *erbA*-specific precipitation of two DNA fragments, which also span the CAII promoter region (positions -682 to +206; data not shown).

To delineate more precisely the *erbA*-binding site(s) within the CAII promoter, an *in vitro* DNase I protection assay was employed. Because *v-erbA* appeared to bind more strongly to the 0.8-kb *Ava*II fragment (Fig. 6), we focused (in the following text) on the analysis of this fragment (position -938 to -133; Fig. 1C). Therefore, DNA fragments were terminally labeled with 32 P and incubated with extracts containing vaccinia virus-expressed *v-erbA* and *c-erbA* proteins. Following DNase I digestion, fragments were resolved by gel electrophoresis as described (Wildeman et al. 1986). As shown in Figure 7, A and B, both *v-erbA* and *c-erbA* effectively protected sequences between position -655 and -672 on either strand (lanes d and c; referred to as the -660 *erbA*-binding site in the following text; see also Fig. 10A, below). No protection was seen without extract or when extracts from wild-type vaccinia virus-infected HeLa cells were used (Fig. 7A,B, lanes a,b). In addition, we observed that extracts prepared from *v-erbA*-expressing chicken erythroblasts generate a footprint at an identical position, whereas no such protection was observed with extracts from no *erbA*-expressing erythroblasts (data not shown). As depicted in Figure 10 and discussed below, the protected sequences contain a cluster of nucleotides (position -655 to -665) that exhibit dyad symmetry and a striking homology to thyroid hormone-responsive elements occurring in other genes. Additional protected sequences have been observed both farther upstream and proximal to the mRNA initiation site, the nature of which is currently under investigation.

To demonstrate unambiguously that the observed protection of the -660 *erbA*-binding site was the result of specific binding of *erbA* proteins to these sequences, an oligonucleotide competition experiment was performed. Figure 7C shows that an oligonucleotide comprising a palindromic T3 receptor consensus-binding site ($T3RE_{pal}$, AGGTCATGACCT; Glass et al. 1988; see also Figure 10B, below) efficiently competed for *erbA*-specific binding and lifted off the footprint (lane c) as did an oligonucleotide containing the homologous CAII-specific sequence (lane f). Oligonucleotides containing unrelated sequences or a glucocorticoid regulatory element (GRE)-like sequence did not compete (lanes d and e, respectively).

Specific binding of the *v-erbA* and *c-erbA* proteins to the -660 *erbA*-binding site was also demonstrated by electrophoretic mobility-shift assay. However, the poly[d(I-C)] titration experiment shown in Figure 8 demonstrates that *v-erbA* binds to this site only at low poly[d(I-C)] concentrations and therefore less efficiently than *c-erbA*. A similar observation was made by Sap et al. (1989), who investigated *v*- and *c-erbA*-specific binding to a T3RE present in the MoMLV LTR. To determine whether this low-affinity binding was the result of the *gag* sequences present in *v-erbA*, a mutant *v-erbA* devoid of *gag* was used as a control. As shown in Figure 8 (lanes

Figure 7. Sequence-specific binding of *v-erbA* and *c-erbA* proteins to the CAII promoter. (A,B) DNase I protection analysis of a CAII promoter region with vaccinia virus-expressed *v-erbA* and *c-erbA* proteins. ³²P-Terminally labeled DNA fragments were incubated with extracts containing vaccinia virus-expressed *c-* and *v-erbA* proteins (3 μ l extract, lanes c and d, respectively), with 3 μ l extract prepared from wild-type vaccinia virus-infected HeLa cells (lane b) or without extract (lane a). Following DNase I digestion, fragments were subjected to polyacrylamide gel electrophoresis (see Materials and methods) and exposed to film for 24–36 hr. The analysis for the sense (A) and anti-sense strand (B; radiolabeled at position –583 and –755, respectively) is shown. (C) Specific oligonucleotides compete for *erbA*-specific binding. DNase I protection analysis was performed as described in A for *c-erbA* in the absence of competitor oligonucleotides (lane b) or in the presence of 100 fmols of specific or nonspecific oligonucleotides (lanes c–f). As a specific oligonucleotide a synthetic palindromic T3RE [lane c; 5'-GGATCTCAGGTCATGACCTGA-3' (Glass et al. 1988)] and an oligonucleotide comprising the CAII-specific sequences that correspond to the protected region (lane f; 5'-GATCTAAGGTGAGTGAACGA-3'; position –667 to –654) were used. As nonspecific oligonucleotides, a permuted version of the CAII-specific oligonucleotide used in lane f was employed (lane d; 5'-GATCTCAGAA-CAGTGTCTGA-3') and a GRE-like oligonucleotide (lane e; 5'-GATCTGTGGTGATTCAACTA-3').

cleotides (T3RE and T3RE*, respectively) as indicated (lanes c–f). As a specific oligonucleotide a synthetic palindromic T3RE [lane c; 5'-GGATCTCAGGTCATGACCTGA-3' (Glass et al. 1988)] and an oligonucleotide comprising the CAII-specific sequences that correspond to the protected region (lane f; 5'-GATCTAAGGTGAGTGAACGA-3'; position –667 to –654) were used. As nonspecific oligonucleotides, a permuted version of the CAII-specific oligonucleotide used in lane f was employed (lane d; 5'-GATCTCAGAA-CAGTGTCTGA-3') and a GRE-like oligonucleotide (lane e; 5'-GATCTGTGGTGATTCAACTA-3').

11,12), this *v-erbA* mutant binds more efficiently to the –660 *erbA*-binding site than bona fide *v-erbA*. These results contrast our in vivo data on *c-erbA*, which show that the bona fide *c-erbA*/T3 receptor and gag-fused *c-erbA* did not exhibit any significant difference in biological activity (Fig. 3; Zenke et al. 1990). To demonstrate the specificity of *erbA* binding, oligonucleotide competition experiments were performed. As expected, an oligonucleotide containing the palindromic T3 receptor consensus-binding site T3RE_{pal} competed efficiently both for *v-* and *c-erbA*-specific binding to the –660 *erbA*-binding site, whereas an oligonucleotide containing a GRE did not compete. Band-shift experiments in which *v-erbA* and *c-erbA* simultaneously compete for binding to a limiting amount of oligonucleotide (to specifically reveal their different affinities to the –660 *erbA*-binding site, see below) have so far not been successful probably due their low affinity to this site (data not shown).

The –660 *erbA*-binding site functions as a T3RE

We next investigated whether the –660 *erbA*-binding site identified in the CAII promoter was biologically active and would operate in vivo as a T3RE. Therefore, synthetic oligonucleotides comprising this CAII *erbA*-binding site as a monomer or trimer were inserted upstream of a minimal thymidine kinase promoter in luciferase-encoding reporter plasmids (pTL15 and pTL29, respectively; Fig. 9A). The ability of these *erbA*-binding

sites to stimulate transcription in response to T3 in transient *trans*-activation experiments was analyzed by transfection of such reporter plasmids into the *c-erbA*-expressing erythroblast clones described above, hence, in the cell type where the endogenous CAII gene is normally active and induced by T3. In all experiments a constant amount of pRSV- β -gal (Gorman et al. 1982) was cotransfected to correct for variations in the transfection efficiency. Following transfection, cells were incubated for 24 hr, treated with T3 for an additional 24 hr, or left untreated and assayed for luciferase and β -galactosidase activity. Figure 9A shows that the –660 CAII *erbA*-binding site does indeed function as a T3RE in transient transfection experiments stimulating luciferase activity by a factor of 3.4 after addition of T3 (pTL15) and, therefore, to a similar extent as the T3RE present in the rat growth hormone promoter (Glass et al. 1988; Brent et al. 1989 and references therein). Trimerization of this element further increased the T3-dependent induction of luciferase reporter activity (sixfold, pTL29). However, this might be due largely to a hormone-dependent relief of repression rather than a true stimulation of transcription because the luciferase activity achieved in the presence of T3 is similar to that observed for pT109 luc and pTL19 containing no or a mutant T3RE, respectively. The specificity of *c-erbA* for the CAII T3RE in T3-dependent *trans*-activation is shown by the fact that a reporter gene containing the retinoic acid response element (RARE) of the β -retinoic acid receptor promoter (pTL19) was not subject to T3-depen-

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Figure 8. Electrophoretic mobility-shift assay demonstrates binding of *v-erbA* and *c-erbA* to the -660 *erbA*-binding site. 32 P-terminally labeled double-stranded oligonucleotides comprising the -660 *erbA*-binding site (positions -677 to -645 ; Fig. 10) were incubated with extracts containing vaccinia virus-expressed *c-* and *v-erbA* proteins (lanes 2,4,6,8–13) or with an extract prepared from wild-type vaccinia virus-infected HeLa cells (lanes 1,3,5,7) as indicated, and subjected to 6% native polyacrylamide gels [electrophoresis time (lanes 1–6,7–13), 3 and 2 hr, respectively]. The *v-erbA* mutant used in lanes 11–13 has the virus-derived gag sequences up to the gag-specific *Xho*I site deleted (T. Bugge and H. Stunnenberg, unpubl.). The poly[d(I-C)] concentrations used in the experiment are indicated. In the competition assay a 50-fold molar excess of unlabeled T3RE_{pal} (see above, lanes 10,13) or GRE_{pal} (5'-GATC-CATCGTAGCTAGAACAGACTGTTCTCGA-3'; lanes 9,12) oligonucleotides were used.

dent *trans*-activation by *erbA* (Fig. 9A), although the same element readily functioned in retinoic acid receptor-driven *trans*-activation (data not shown).

Similar results have been obtained by cotransfection of the pTL15 or pTL29 reporter plasmids with a *c-erbA*-encoding expression vector into chicken embryo fibroblasts or HD3 cells (data not shown). However, in all experiments the T3-dependent *trans*-activation of the -660 CAII T3RE was not as strong as the one achieved with the trimerized palindromic T3RE consensus sequence used as a positive control (T3RE_{pal} in recombinant pTL13; Fig. 9A).

Trans-activation of the CAII T3RE by *c-erbA* and *gag/v-c-erbA* is differentially suppressed by *v-erbA*

We then investigated whether the *v-erbA* oncoprotein could be expressed at levels high enough to compete efficiently with *c-erbA* for binding to the -660 CAII *erbA*-binding site in the luciferase-encoding reporter plasmids and thus affect the T3-dependent *trans*-activation measured for these constructs in transient expression exper-

iments. Therefore, stably transformed erythroblasts expressing a constant amount of *c-erbA*/T3 receptor protein (expressed either as a *gag/c-erbA* fusion protein or as bona fide *c-erbA*; see above) were transfected with an increasing amount of *v-erbA*-encoding expression plasmid, along with the pTL29 reporter recombinant containing the -660 *erbA*-binding site of the CAII gene. To test whether the *v-erbA*-specific point mutations in the DNA-binding domain would alter the binding characteristics of *erbA* to the CAII T3RE, the T3-responsive erythroblast clone 16 expressing the chimeric *gag/v-c-erbA* T3 receptor was treated similarly.

Figure 9B shows that in cells expressing the chimeric *gag/v-c-erbA* receptor increasing amounts of cotransfected *v-erbA* expression plasmid efficiently suppressed the T3-dependent *trans*-activation of the reporter plasmid containing the CAII T3RE. This efficient suppression was not the result of nonspecific "squenching" of ubiquitous transcription factors, because expression of the pRSV- β -gal internal control plasmid remained virtually unaffected (data not shown). Thus, *v-erbA* can compete efficiently with a T3-responsive *erbA* protein containing the *v-erbA*-specific DNA-binding domain (*gag/v-c-erbA*). In contrast, cells expressing *c-erbA* had to be transfected with a 10-fold higher amount of *v-erbA* expression plasmid to blunt the T3 response of the CAII reporter (Fig. 9B). This demonstrates that a larger excess of *v-erbA* is required to extinguish the T3-dependent *trans*-activation of the CAII T3RE by *erbA* proteins that contain the DNA-binding domain of the wild-type T3 receptor.

In a similar type of competition experiment the synthetic response element T3RE_{pal} (Glass et al. 1988) was used instead of the naturally occurring CAII T3RE. In this instance, the same high amount of *v-erbA* expression was required for efficient competition of both *c-erbA* and the chimeric *gag/v-c-erbA* containing the normal and mutated T3 receptor DNA-binding domain, respectively (Fig. 9C). Therefore, it appears that the different affinity of *v-erbA* and *c-erbA* for specific DNA sequences (see Discussion) is only revealed when the naturally occurring -660 *erbA*-binding site has been used, underscoring the importance of using *erbA* target sequences as they occur in nature instead of artificial consensus sequences for such studies.

In conclusion, our data strongly suggest that overexpression of the *v-erbA* oncoprotein is essential both for its ability to repress differentiation and erythrocyte-specific gene expression and for its ability to suppress *c-erbA* function at a naturally occurring *erbA*-binding element, the T3RE of the CAII gene.

Discussion

The CAII gene is a direct target for transcriptional regulation by *erbA*

Previous studies have led to the proposal that *v-erbA* acts as a transcriptional repressor of erythrocyte-specific genes involved in erythroid differentiation and thus con-

containing the CAII T3RE. T3-responsive reporter plasmids (2 μg) containing either the trimerized CAII T3RE (pTL29; B) or a trimerized palindromic T3RE (pTL13; C) were cotransfected with increasing amounts of v-erbA-encoding expression plasmids into erythroblasts coexpressing v-erbA and gag/c-erbA or gag/v-erbA (● and ■, clones 24 and 16, respectively; see Fig. 2). Following T3 treatment as described in Materials and methods the induction of luciferase activity in response to T3 is shown after correcting for variations in the transfection efficiencies according to the β-galactosidase activity as described in A. The amounts of v-erbA expression plasmids did not grossly affect the expression of the reporter gene by unspecific squelching, as the β-galactosidase activity measured remained virtually unaffected. For 15 μg of v-erbA expression plasmid transfected, a marginal effect on β-galactosidase internal control activity was observed, however, it was the same in both the presence and absence of T3.

tributes to leukemogenesis by efficiently arresting terminal differentiation of erythroid precursor cells (Zenk et al. 1988, 1990; Damm et al. 1989; Sap et al. 1989; Gandrillon et al. 1989). Here, we provide the first direct evidence in favor of this hypothesis. We demonstrate that the v-erbA oncoprotein, as well as the normal thyroid hormone receptor c-erbA, binds directly to a sequence element within the promoter of the erythrocyte-specific CAII gene. This element displays high homology to known T3REs (Glass et al. 1988; Banihmad et al. 1990; Glass and Holloway 1990; see also Fig. 10B), confers T3-dependent transcriptional regulation to an unrelated promoter in transient expression assays, and is a target sequence for efficient down-regulation of CAII transcription by the v-erbA oncoprotein. It also exhibits some limited homology to RARE and the vitamin D3-responsive element (VDRE) of the human osteocalcin gene promoter (Schüle et al. 1990; data not shown). Preliminary evidence indeed suggests that the CAII gene is regulated by retinoic acid in normal bone marrow progenitors (C. Schröder and H. Beug, unpubl.). Other studies have demonstrated that CAII is also regulated by T3, vitamin D3, and androgen (Harkonen and Väänänen

1988; Hillstrom-Shapiro et al. 1989; Billecocq et al. 1990; Pain et al. 1990). However, no cis-acting hormone-response element has been identified thus far in the regulatory region of the CAII gene. Given the high degree of homology between the T3, retinoic acid, and vitamin D3 receptors and their respective response elements, it will be very important to determine whether regulation of CAII transcription by hormones other than T3 occurs through the very same DNA element (i.e., the -660 erbA-binding site), implying that its regulation by retinoic acid and vitamin D3 would also be affected by v-erbA. However, at present, we cannot exclude the possibility that sequences other than the -660 erbA-binding site contribute to CAII regulation by erbA, although sequence elements homologous to the -660 erbA-binding site have not been detected thus far in the CAII promoter sequence available.

Further inspection of the -660 erbA-binding site and its surrounding sequences reveals a potential AP-1-binding site overlapping with the T3RE (Fig. 10B; position -657, TcACTCA, read from the opposite strand), a situation very reminiscent of a common response element of the fos/jun/AP-1 complex and the retinoic acid and

Figure 9. The -660 erbA-binding site functions as a T3RE. (A) T3-dependent expression of transiently transfected reporter plasmids containing the CAII erbA-binding site in front of a minimal TK promoter either as a monomer or trimer (pTL15 and pTL29, respectively) were analyzed for their T3 response in transient cotransfection experiments in c-erbA-expressing HD3 erythroblasts (clone 12; Figs. 2 and 3) as described in Materials and methods. As controls, the reporter plasmid pT109Luc containing the TK promoter only and pTL13 containing a trimerized palindromic T3RE (Glass et al. 1988) were used. pTL19 contains the RARE of the retinoic acid receptor (type β) promoter replacing the erbA-binding site in pTL15. The luciferase activity measured in response to T3 treatment was normalized according to the β-galactosidase activity of the cotransfected pRSV-β-gal recombinant (Gorman et al. 1982) to correct for variations in transfection efficiencies. Mean values plus standard deviations from three to seven independent transfection experiments are shown. (B,C) v-erbA competes for T3-dependent expression of transiently transfected reporter plasmids

Figure 10. Anatomy of the -660 *erbA*-binding site in the CAII upstream sequence. (A) Sequences protected by the *v-erbA* and *c-erbA* proteins in the DNase I protection assay. The solid bars show the region of protection on the sense (upper) and antisense (lower) strands. The open boxes indicate the distance between the last protected cleavage site and the first unprotected cleavage site. The solid triangle indicates the position of a weak hypersensitive site seen on the antisense strand (see Fig. 7B). (B) Sequence alignment of the -660 *erbA*-binding site (T_3RE_{CAII}) with the T3RE consensus sequence (T_3RE_{pal} ; Glass et al. 1988) and the NF-E2-binding sites of the human β -globin gene (Ney et al. 1990; Talbot and Grosfeld 1991). Direct and indirect repeats are indicated. Potential AP-1-binding sites are boxed. Sequence divergence from the -660 *erbA*-binding site (T_3RE_{CAII}) is indicated by lowercase letters.

vitamin D3 receptors found in the osteocalcin gene (Schüle et al. 1990 and references therein). We also noticed a second, less conserved potential AP-1 site (position -648 , TG.CTCA, read from the opposite strand). Both putative AP-1-binding sites could possibly cooperate with each other because they are spaced by one helical turn (11 nucleotides), thereby allowing the *fos/jun*/AP-1 complex to bind to both sites on the same face of the DNA helix. That these AP-1 sites might be functional is suggested by our observation that in transient transfection assays the activity of reporter plasmids containing the CAII T3RE plus flanking sequences (which include both potential AP-1 sites) is severely impaired (data not shown), indicating a possible interference of the *fos/jun*/AP-1 complex with the activity of the *c-erbA*/T3 receptor.

Finally, the -660 *erbA*-binding site exhibits a distinct sequence homology to the binding site of the erythroid-specific transcription factor NF-E2 identified in the human β -globin locus (Ney et al. 1990; Talbot and Grosfeld 1991 and references therein). Despite the large evolutionary distance (chicken vs. human) this homology extends >23 nucleotides, being most pronounced at the presumptive contact sites of the NF-E2 protein (Fig. 10B). Whether this element is functionally important for CAII promoter activity is not known at present. It appears conceivable, however, that *v-erbA* binding to the -660 *erbA*-binding site precludes NF-E2 from acting at this site and thereby suppresses CAII gene activity in leukemic cells. In addition, the potential AP-1 and NF-E2 sites overlapping the -660 *erbA*-binding site may well explain our present difficulties in demonstrating a strong T3-dependent regulation in transient expression assays

of the entire CAII promoter region, comprising up to 1.2 kb of upstream sequence. Even when tested together with the β -globin enhancer fused to the 3' end of the luciferase reporter gene, only a weak *trans*-activation by T3 (2.5-fold) is seen (data not shown).

We have observed previously that in erythroblasts containing retrovirus-transduced *c-erbA*, transcription of the endogenous CAII gene is suppressed in the absence of T3 and is activated, in the presence of T3, to levels found in erythroblasts lacking exogenous *erbA* proteins, suggesting that in these cells *c-erbA* shows a T3-dependent relief of repression rather than a true stimulation of CAII transcription (Zenke et al. 1990). This situation is largely reproduced in transient expression experiments by the CAII T3RE present in the reporter plasmid pTL29. It is therefore tempting to speculate that in *c-erbA* erythroblasts activation of the receptor by T3 relieves the transcriptional arrest seen in the absence of T3, thereby allowing NF-E2, for example (see above), to activate the promoter. Further experiments are clearly required to substantiate such a model.

Does v-erbA act as a dominant repressor of erythrocyte genes?

The identification of CAII as a direct target of *erbA* action and of a *cis*-acting DNA element through which the *erbA* proteins affect CAII promoter activity has allowed us to investigate for the first time how *v-erbA* might interact with the normal T3 receptor *c-erbA* in a cell type where the *v-erbA* oncoprotein shows its pathologic effects. We show that *c-erbA* dominates *v-erbA* activity, mediating T3-dependent differentiation and erythrocyte-specific gene transcription when expressed simultaneously with *v-erbA* in erythroid cells at approximately equimolar levels. Likewise, in such cells *c-erbA* can modulate the activity of transiently expressed reporter plasmids containing the -660 *erbA*-binding site of the CAII gene. However, a large excess of *v-erbA* was able to extinguish the *c-erbA*-dependent activation of the CAII reporter gene. From these data we conclude that *v-erbA* does not act as a dominant repressor of transcription on naturally occurring erythrocyte genes at an equimolar ratio but that overexpression of *v-erbA* is required for its repressor function and thus for its function as an oncoprotein.

These results are in contrast with the observations of Damm et al. (1989), where *v-erbA* was found to efficiently suppress *c-erbA* function in cotransfection experiments by using a synthetic T3RE. However, because the rat T3 receptor (type α) was combined with avian *v-erbA* in these studies, the use of a synthetic T3RE, as well as the species difference, might well explain this divergence of results. This idea is strengthened by the fact that our results are in line with those of Sap et al. (1989), who showed that *v-erbA* is only a weak competitor of *c-erbA* for interaction with a T3RE present in the Mo-MLV LTR. In addition, our findings strongly underscore the necessity of using naturally occurring *erbA* target sequences in such studies.

We also demonstrate that the chimeric gag/v-/c-erbA T3 receptor (containing the v-erbA-specific DNA-binding domain) was less efficient than c-erbA in overriding v-erbA function. Thus, only a minority of clones coexpressing gag/v-/c-erbA with v-erbA were distinctly T3 responsive for differentiation induction (Fig. 2). However, because the exact relative amounts of the erbA proteins expressed in these cells could not be determined (see Results) it is not known whether the reduced biological activity of gag/v-/c-erbA in some of the clones correlates with its lower expression. Furthermore, ~10-fold less v-erbA expression was required to blunt the T3-dependent regulation of a CAII T3RE reporter construct by gag/v-/c-erbA as compared to c-erbA or gag/c-erbA. These results are consistent with the observation that the v-erbA oncoprotein and the chimeric T3 receptors containing the v-erbA-specific DNA-binding domain exhibit a reduced affinity for sequence-specific DNA binding in vitro (Sap et al. 1989). Recently, further studies have shown that a specific point mutation present in the v-erbA DNA-binding domain (Gly → Ser at amino acid position 61) affects both sequence-specific DNA binding and its activity in erythroid cell transformation (Bonde et al. 1991). In addition, the very same point mutation reduced the transcriptional efficiency of erbA-estrogen receptor chimeras in transient trans-activation studies (de Verneuil and Metzger 1990). This point mutation corresponds to the position of one of the discriminating amino acids that are thought to interact with a specific base of the responsive element (Mader et al. 1989; Umesono and Evans 1989; Forman and Samuels 1990).

These findings suggest that the specific point mutation(s) present in the DNA-binding domain of v-erbA impair its ability to act as a dominant repressor of c-erbA, perhaps through its altered binding affinity to specific target sequences. However, this might not be true for other genes regulated by v-erbA. It is even conceivable that v-erbA may interact more efficiently with control elements of related receptors and/or other transcription factors, thus enabling it to act on genes that are normally not subject to regulation by thyroid hormone. Our observation that gag/c-erbA and gag/v-/c-erbA have the same effect on CAII transcription but slightly different effects on erythroid cell differentiation might point to such genes whose expression is differentially affected by these erbA proteins as a result of the differences in their DNA-binding domain. In line with this we also note that erythroblasts expressing the chimeric gag/v-/c-erbA protein grow significantly faster than those expressing c-erbA or gag/c-erbA (C. Disela and M. Zenke, unpubl.). Thus, other properties of v-erbA than its function as a repressor of late erythrocyte-specific genes might contribute to its activity as an oncoprotein.

Does repression of CAII by v-erbA contribute to the leukemic phenotype?

Our data demonstrating that the CAII gene is subject to direct regulation by hormone-responsive erbA proteins and is repressed by the v-erbA oncoprotein raise the

question of whether suppression of CAII expression can account, at least in part, for the phenotypic changes induced by v-erbA in transformed erythroblasts. CAII deficiency in man leads to rather pleiotropic effects such as impaired growth, kidney function, bone resorption, and brain development (Sly et al. 1985). However, in red cells of CAII-deficient individuals the CAI isoenzyme appears to compensate perfectly for the absence of CAII and maintains the carbonic anhydrase activity required for red cell function. Interestingly, chicken red cells only express the CAII isoenzyme and completely lack CAI (Tashian 1989), probably as a result of the loss of erythroid-specific promoter elements (R. Tashian, pers. comm.). Thus, suppression of CAII expression by v-erbA in transformed chicken erythroblasts renders them completely devoid of carbonic anhydrase activity.

We have recently addressed the questions if and how suppression of CAII and band3 by v-erbA might contribute to the leukemic phenotype by reexpressing these genes in v-erbA-containing erythroblasts with suitable retroviral vectors (Fuerstenberg et al. 1990; I. Leitner et al. in prep.). Our results show that reexpression of either CAII or band3 in leukemic erythroblasts abrogates the altered growth medium requirements induced by v-erbA (Beug et al. 1985; Kahn et al. 1986), causing a phenotype similar to the one seen for erythroblasts lacking v-erbA. In addition, reexpression of CAII partially relieved the v-erbA-induced differentiation arrest in these cells, leading to slow, incomplete maturation of such cells (I. Leitner et al. in prep.). In contrast, no effects of band3 reexpression on the v-erbA-induced differentiation arrest was observed. Thus, direct repression of CAII gene transcription by v-erbA seems to be responsible for part, but not all of, the phenotypic changes induced by this oncogene in leukemic cells.

Materials and methods

Construction of recombinant retroviral vectors

The recombinant retroviral vectors gag/c-erbA-neo and gag/v-/c-erbA-neo (Fig. 1B) were generated by replacing v-erbA in pCRA-neo (Schröder et al. 1990) by gag/c-erbA and gag/v-/c-erbA, respectively (v3-erbA and v2-erbA in Munoz et al. 1988). The gag/neo-c-erbA vector (pNEO-CEA+) contains the chicken c-erbA cDNA of pKCR2-CEA (Sap et al. 1989) as an EcoRI fragment in pSFCV-LE (Fuerstenberg et al. 1990). To achieve efficient c-erbA protein expression, the c-erbA ATG was modified to meet the requirements for a perfect Kozak consensus sequence (Sap et al. 1990).

Cells and cell culture

The origin and culture conditions of the v-erbA-expressing cell line HD3 (transformed by v-erbA + tserbB of ts34 AEV) have been described previously (Beug et al. 1982). Erythroblast clones coexpressing v-erbA and c-erbA were generated by transfecting HD3 cells with the recombinant retroviral vectors described above (see Fig. 1A,B), together with helper virus (RAV-1) DNA (6 µg of retroviral vector DNA plus 1 µg of helper virus DNA/10⁷ cells) by using the DEAE-dextran procedure (Choi et al. 1988). Cells were allowed to recover from transfection by cultivation

in CFU-E medium (Radke et al. 1982) for 2 days and then seeded in CFU-E methocel containing 3 mg/ml of G418 to select for neo-resistant colonies (Schröder et al. 1990). After 7–10 days, colonies were isolated, expanded in CFU-E medium containing T3-depleted fetal calf serum and avian transferrin (150 µg/ml instead of chicken serum) and screened for T3 responsiveness as described for *c-erbA*-expressing clones in Zenke et al. (1990). The methods for induction of erythroid differentiation of such clones in methocel and liquid medium have been described previously (Glineur et al. 1990; Zenke et al. 1990).

Preparation of extracts from HeLa cells containing vaccinia virus-expressed erbA proteins

Extracts were prepared from HeLa cells infected with either wild-type or recombinant vaccinia viruses expressing *c-erbA*, *v-erbA*, or *gag/v-c-erbA* as described previously (de Magistris and Stunnenberg 1988; Sap et al. 1989, 1990). Twenty-four hours after infection cells were harvested, cell extracts were prepared according to Wildeman et al. (1984) and used for *in vitro* DNase I protection assays. For use in electrophoretic mobility-shift assays, whole-cell extracts were prepared by lysis of cells in 50 mM Tris-HCl (pH 7.5), 420 mM NaCl, 5 mM MgCl₂, and 0.5% (vol/vol) Triton X-100. Clarified lysates were snap-frozen in liquid nitrogen and stored at –70°C. The levels of the various *erbA* proteins present in these extracts were comparable as judged by Western blotting analysis (data not shown).

Detection of erbA proteins by immunoprecipitation

Cells were labeled with [³⁵S]methionine, and extracts were immunoprecipitated with anti-*erbA*-specific antibodies as described previously (Goldberg et al. 1988; Glineur et al. 1990).

RNA extraction and Northern blot analysis

RNA was prepared and subjected to blot hybridization as described previously (Zenke et al. 1990).

DNA binding/immunoprecipitation assay

The recombinant plasmid pBHcaXIII-3.1, containing 1.3 kb of chicken CAII upstream sequence, the first and second exon, and part of the second intron (Yoshihara et al. 1987; J. Dodgson, unpubl.), was digested with *Ava*II or *Bss*HII (see Fig. 1C) and terminally labeled with [³²P]dCTP and *Escherichia coli* DNA polymerase (Klenow fragment). Approximately 2 fmoles of terminally labeled fragments were incubated for 30 min at 30°C in 1× binding buffer [(10 mM HEPES (pH 7.8), 40 mM KCl, 2 mM MgCl₂, 1 mM DTT, 5% (vol/vol) glycerol] containing 0.1 mg/ml of BSA and 0.1 mg/ml of poly[d(I-C)] in a total volume of 26–28 µl with 0.2–2 µl cell extracts containing vaccinia virus-expressed *erbA* proteins (see above). The DNA–protein complexes were precipitated with anti-*erbA*-specific antisera [3 µl of antibody (Ab)21 per reaction (Goldberg et al. 1988; Glineur et al. 1990)] 30 min at 4°C, followed by addition of 20 µl of 10% *Staphylococcus aureus* protein A (in 1× binding buffer) and incubation at 4°C for 30 min. After centrifugation, the pellet was washed three times with 1× binding buffer, dissolved in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.2% SDS, 10 µg/µl of *E. coli* tRNA, and 20 µg/µl of proteinase K and incubated for 1 hr at 55°C. Following phenol–chloroform extraction and ethanol precipitation, fragments were resolved in 2% agarose or 5% polyacrylamide gels, dried, and exposed to film for 6–16 hr.

In vitro DNase I protection assay

The *Pst*I–*Aha*II fragment of the CAII upstream sequences (positions –925 to –583) was subcloned into pBluescriptKS+ (Stratagene) between *Pst*I and *Sma*I (pC102). In addition, a CAII promoter fragment (positions –755 to –600) was amplified by PCR thereby generating new *Hind*III and *Xho*I sites at position –755 and –600, respectively, which were used for subcloning into pBluescript KS+ (pC103). For footprinting analysis recombinants pC102 and pC103 were cut with *Xba*I or *Xho*I, respectively, terminally labeled with [³²P]dCTP and *E. coli* DNA polymerase (Klenow fragment), recut with a second enzyme (*Xho*I or *Xba*I, respectively), and purified from preparative gels by standard techniques (Maniatis et al. 1989).

In a typical DNase I protection assay, reaction mixes containing 20 mM HEPES (pH 7.8), 40 mM KCl, 3 mM MgCl₂, 17% (vol/vol) glycerol, 25 mg/ml of poly[d(I-C)], various amounts of nuclear extracts containing the vaccinia virus-expressed *erbA* proteins (0–10 µl) and 2 fmoles of end-labeled DNA were incubated in a total volume of 25 µl for 10 min at 20°C. Following DNase I digestion (90 sec at 20°C), reactions were terminated by addition of 200 µl of 140 mM NaCl, 50 mM NaAc (pH 6.5), 0.3% SDS, and 200 µg/µl of *E. coli* tRNA. After phenol–chloroform extraction and ethanol precipitation the samples were resolved in 8% sequencing-type gels alongside G + A tracks of the same DNA (Maxam and Gilbert 1980). In competition assays oligonucleotides in a 50-fold molar excess were added to the reactions before addition of the end-labeled fragments.

Electrophoretic mobility-shift assay

Two microliters of whole-cell extract (~4 µg of protein) containing vaccinia virus-expressed *erbA* proteins was preincubated on ice in 13 µl of 20 mM HEPES (pH 7.8), 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, and 15% (vol/vol) glycerol containing 0.5–2.0 µg of poly[d(I-C)]. Oligonucleotides (~20 fmoles) were terminally labeled with [³²P]dCTP and *E. coli* DNA polymerase (Klenow fragment), added to the reaction and the incubation continued for another 15 min. The samples were then loaded onto 6% native polyacrylamide gels (0.25× TBE), and the complexes were separated by electrophoresis at 200 V for 2 hr. Subsequently, the gel was dried and subjected to autoradiography. For competition assays a 50-fold excess of unlabeled competitor oligonucleotide was added, together with the labeled oligonucleotide.

Transient expression assays

The DNA segment containing the –660 *erbA*-binding site (position –712 to –655) was ligated as a synthetic oligonucleotide between *Xho*I and *Hind*III into the luciferase-encoding expression plasmid pTluc109 (Nordeen 1988) upstream of the minimal thymidine kinase (TK) promoter, thereby generating recombinant pTL15. Various permutations of this CAII promoter-specific fragment have been synthesized as synthetic oligonucleotides and inserted into pTluc109 at identical positions to the wild-type sequence (see Fig. 9A). DNA sequencing was used to confirm the nucleotide sequence of the synthetic DNA after cloning. Recombinants pTL13 and pTL29 are derivatives of pTL15 containing a trimerized palindromic T3RE (Glass et al. 1988) or a trimerized version of the –660 CAII *erbA*-binding site (position –666 to –655) between *Xho*I and *Hind*III of pTluc109. In pTL19 CAII-specific sequences (position –666 to –649) have been replaced by the RARE of the retinoic acid receptor (type β) promoter (position –54 to –36; deThe et al. 1990). The plasmid DNA used for transfection was prepared

by the Triton lysis procedure [Maniatis et al. 1989], followed by CsCl/ethidium bromide equilibrium density gradient centrifugation, destaining with 1-butanol and extensive dialysis against 20 mM Tris-HCl (pH 7.5), 1 mM EDTA.

In a typical transfection experiment 2 μ g of luciferase-encoding reporter plasmid DNA (pTL series) and a constant amount of pRSV- β -gal DNA [2 μ g (Gorman et al. 1982) to be able to correct for variations in the transfection efficiencies] were used. In cotransfection experiments 0.5 μ g of c-erbA expression plasmid pKCR2-CEA [Sap et al. 1989] or various amounts of the v-erbA expression plasmid pKCR2-VEA [Sap et al. 1989] was added. pBluescript plasmid DNA was used to achieve a final amount of 20 μ g of DNA and 10 μ g of DNA for calcium phosphate and DEAE-dextran transfection, respectively.

Semiconfluent primary chicken embryo fibroblasts (CEFs) were transfected by using the calcium phosphate/DNA coprecipitation method as described [Graham and Van der Eb 1973]. Sixteen to twenty hours before transfection 2×10^6 CEFs were plated on Greiner tissue culture dishes (10 cm diameter) in culture medium containing T3-depleted FCS. The calcium phosphate precipitate was left in contact with the cells for 20–24 hr, followed by a complete medium change. Cells were then treated with 100 nM T3 for 20–24 hr and harvested by means of a rubber policeman. Cell extracts were prepared by three cycles of freeze/thawing. Aliquots of such extracts were analyzed for their protein content, β -galactosidase activity [Herbomel et al. 1984], and luciferase activity [(deWet et al. 1987) using the bioluminescence counter, Clinilumat, Berthold, Wildbach, Germany].

Nonadherent cells were transfected by using the DEAE-dextran procedure [Choi and Engel 1988] or transfection infection [Zenke et al. 1990] and grown for 24 hr in CFU-E medium containing T3-depleted FCS and chicken serum. Cells were then treated with 100 nM T3 for 20–24 hr and harvested by centrifugation. For preparation of cell extracts and analysis of protein content, β -galactosidase and luciferase activity, see above.

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Note added in proof

As indicated by the direct repeat shown in Figure 10B the –660 CAII-specific erbA-binding site fits the AGGTCA/4-nucleotide spacer/AGGTCA direct repeat consensus sequence proposed recently for T3REs by Umesono et al. 1991. *Cell* 65: 1255–1266.

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