The EMBO Journal Vol.17 No.24 pp.7382–7394, 1998

Leukemic transformation by the v-ErbA oncoprotein entails constitutive binding to and repression of an erythroid enhancer *in vivo*

Paolo Ciana[1,2], Georgia G.Braliou[1,3], Florence G.Demay[3], Marieke von Lindern[4,5], Domingo Barettino[1,6], Hartmut Beug[4] and Hendrik G.Stunnenberg[1,3,7]

[1]Gene Expression Program, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany, [3]Department of Molecular Biology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands and [4]Institute of Molecular Pathology, Dr. Bohrgasse 7 Vienna, Austria

[2]Present address: Institute of Pharmacological Sciences, University of Milan Via Balzaretti, 9 20133 Milan, Italy
[5]Present address: Institute of Hematology, Erasmus University, Box 1738, 3000 DR Rotterdam, The Netherlands
[6]Present address: IBMCP (CSIC-UPV) Camino de Vera s/n, 46022 Valencia, Spain

[7]Corresponding author
e-mail: stunnenb@sci.kun.nl

P.Ciana and G.G.Braliou contributed equally to this work

v-ErbA, a mutated thyroid hormone receptor alpha (TRα), is thought to contribute to avian erythroblastosis virus (AEV)-induced leukemic transformation by constitutively repressing transcription of target genes. However, the binding of v-ErbA or any unliganded nuclear receptor to a chromatin-embedded response element as well as the role of the N-CoR–SMRT–HDAC co-repressor complex in mediating repression remain hypothetical. Here we identify a v-ErbA-response element, VRE, in an intronic DNase I hypersensitive site (HS2) of the chicken erythroid *carbonic anhydrase II* (*CAII*) gene. *In vivo* footprinting shows that v-ErbA is constitutively bound to this HS2-VRE in transformed, undifferentiated erythroblasts along with other transcription factors like GATA-1. Transfection assays show that the repressed HS2 region can be turned into a potent enhancer in v-ErbA-expressing cells by mutation of the VRE. Differentiation of transformed cells alleviates v-ErbA binding concomitant with activation of *CAII* transcription. Co-expression of a gag–TRα fusion protein in AEV-transformed cells and addition of ligand derepresses *CAII* transcription. Treatment of transformed cells with the histone deacetylase inhibitor, trichostatin A, derepresses the endogenous, chromatin-embedded *CAII* gene, while a transfected HS2-enhancer construct remains repressed. Taken together, our data suggest that v-ErbA prevents *CAII* activation by 'neutralizing' *in cis* the activity of erythroid transcription factors.

Keywords: carbonic anhydrase II/repression/thyroid hormone/trichostatin A/v-ErbA

Introduction

Leukemic transformation of hematopoietic cells is manifested as an imbalance between proliferation and differentiation caused by the combined action of two or more co-operating oncogenes. Gradually, the concept has emerged that the decision of a hematopoietic cell to either self-renew (i.e. proliferate but not differentiate) or to undergo terminal differentiation is determined by the cooperative action of receptor tyrosine kinases and nuclear hormone receptors. In humans, 80% of acute promyelocytic leukemia (APL) patients bear translocations juxtaposing the *RAR*α gene locus to either the *PLZF* or *PML* genes. The resultant fusion proteins (Borrow *et al.*, 1990; De Thé *et al.,* 1990; Alcalay *et al.*, 1991; Chen *et al.*, 1993) block differentiation of hematopoietic progenitors (Grignani *et al.*, 1993; Rousselot *et al.*, 1994). Murine bone marrow cells expressing a dominant negative RARα lacking its ligand-dependent activation function, AF-2, are blocked at the stage of lymphohematopoietic progenitors (Tsai *et al.*, 1994). In chickens, the avian erythroblastosis virus (AEV) induces fatal erythroleukemia (for reviews see Beug *et al.*, 1994; Gandrillon *et al.*, 1995). AEV expresses two co-operating oncogenes, *v-erbA* and *v-erbB*, that together tip the balance between proliferation and differentiation towards self renewal. v-ErbB is a mutated and truncated viral variant of the epidermal growth factor receptor (EGFR) that promotes cell growth. v-ErbA is a highly mutated variant of chicken thyroid hormone receptor alpha, cTRα (Sap *et al.*, 1986; Weinberger *et al.*, 1986) that arrests the differentiation of erythroblast progenitors by preventing the expression of differentiation stage-specific erythroid genes (Zenke *et al.*, 1990; Disela *et al.*, 1991). Because v-ErbA requires cooperation with kinases to arrest differentiation, AEV-transformed cells can be induced to differentiate in the presence of specific kinase inhibitors (Choi *et al.*, 1986; Zenke *et al.*, 1988). Although it has been postulated that phosphorylation of v-ErbA is crucial for its oncogenic capacity, little is known about the consequences of kinase activity on v-ErbA function.

The discovery that v-ErbA is a mutated TRα initiated an extensive comparative analysis of presumed TR functions that are absent in the v-ErbA oncoprotein. The oncogenic requirements do not include transcriptional activation functions, since v-ErbA is severely crippled with respect to T3 binding (Muñoz *et al.*, 1988; Zenke *et al.*, 1990), dimerization with RXR (Selmi and Samuels, 1991; Barettino *et al.*, 1993) and transactivation (Saatcioglu *et al.*, 1993; Barettino *et al.*, 1994). The first clue as to the activity of v-ErbA required for oncogenicity stems from the observations that v-ErbA antagonizes ligand-dependent activation by TR (Damm *et al.*, 1989; Sap *et al.*, 1989; Zenke *et al.*, 1990). The finding that overexpression of TR and addition of ligand can overcome the block of differentiation by v-ErbA lent support to this notion (Disela *et al.*, 1991). A v-ErbA variant, td359, which failed to block differentiation also failed to repress transcription in transient transfection assays (Damm *et al.*,

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1987; Damm and Evans, 1993). Recently, the mutation in td359 causing the transformation defect has been shown to diminish the affinity of v-ErbA for the co-repressor SMRT *in vitro* (Chen and Evans, 1995). These and other observations have led to the formulation of an occlusion–repression model for the action of v-ErbA at the molecular level: v-ErbA occludes TR and/or RAR from binding to their cognate sites (Damm *et al.*, 1989; Sap *et al.*, 1989) and represses transcription of target genes *in cis* (Damm and Evans, 1993). Repression is assumed to involve a co-repressor complex (Chen and Evans, 1995; Hörlein *et al.*, 1995; Heinzel *et al.*, 1997).

Transient transfection experiments revealed that the ability of v-ErbA to repress transcription is an active mechanism shared by other unliganded class II nuclear receptors (Baniahmad *et al.*, 1990, 1992; Damm and Evans, 1993). Recently, ample biochemical data suggest that in the absence of a cognate ligand, TR and RAR can associate with co-factors, termed N-CoR and SMRT, that have intrinsic transcriptional repression activities (Chen and Evans, 1995; Hörlein *et al.*, 1995). N-CoR and SMRT in turn appear to be part of a large complex(es) consisting of factors that display transcriptional repression activities, such as SIN3A, or that are thought to stabilize repressive nucleosomal structures such as the histone deacetylase, HDAC (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). Following treatment with the cognate ligand, the receptors undergo conformational changes leading to dissociation of the repressor complexes thus enabling their interaction with a different set of proteins that include SRC-1/TIF2 type proteins (Oñate *et al.*, 1995; Voegel *et al.*, 1996; Hong *et al.*, 1997; Torchia *et al.*, 1997) and CBP/p300 (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996). These factors have intrinsic transcriptional activation activity as well as histone acetylase activity (Yang *et al.*, 1996). A picture emerges in which nuclear receptors act as ligand-operated, molecular on–off switches.

This model is questioned by several observations. A mutation in the DNA-recognition helix (P-box) of v-ErbA both diminishes its overall affinity for DNA and alters its sequence specificity (Bonde and Privalsky, 1990; Wahlstrom *et al.*, 1992; Barettino *et al.*, 1993; Judelson and Privalsky, 1996). Reverting that DBD mutation increases the affinity of that receptor for the canonical half-site AGGTCA (Nelson *et al.*, 1994). Unexpectedly, a v-ErbA variant with restored wild-type DNA-binding properties does not function as a 'super-oncoprotein'; on the contrary, it is now fully impaired in its ability to transform erythroid cells (Sharif and Privalsky, 1991; Bauer *et al.*, 1997). Furthermore, a mutation in the dimerization interface has caused a loss of affinity for the presumed partner, RXR (Selmi and Samuels, 1991; Barettino *et al.*, 1994). Collectively, these results suggest that v-ErbA binds to a repertoire of *cis*-acting elements that is distinct from, or only partially overlapping with, natural thyroid hormone response elements (TREs) and retinoic acid response elements (RAREs). Alternatively, v-ErbA may be targeted to chromosomal loci via protein–protein interactions such as described for AP1-GR (Konig *et al.*, 1992; Reichardt *et al.*, 1998).

An ensuing search for erythroid target genes repressed by v-ErbA identified the erythrocyte anion transporter (*band 3*) and *carbonic anhydrase II* (*CAII*) (Zenke *et al.*, 1990). Repression of these genes by v-ErbA is important for the v-ErbA-induced leukemic phenotype, and accounts for the tolerance of AEV-transformed erythroblasts to wide variations in the pH or HCO_3^- ion concentration required for survival of the leukemic cells in peripheral blood. Re-expression of these genes in transformed erythroblasts revealed that the v-ErbA-induced tolerance to pH variation was abrogated; however, the v-ErbA-induced block of differentiation remained largely unaffected (Fuerstenberg *et al.*, 1990, 1992). Transient transfection experiments involving v-ErbA expression vectors and either the promoter region of the *CAII* and/or synthetic reporters have yielded ambiguous and sometimes conflicting results (Disela *et al.*, 1991; Hermann *et al.*, 1993; Rascle *et al.*, 1994; G.G.Braliou, D.Barettino and H.G.Stunnenberg, unpublished observations).

Another wrinkle to the model is that binding of an unliganded receptor to its cognate response element *in vivo* in a chromosomal context has not yet been demonstrated. Although *in vivo* footprinting clearly revealed binding site occupancy by a ligand-activated retinoid receptor, it failed to reveal receptor binding in the absence of ligand (Minucci *et al.*, 1994; Chen *et al.*, 1996). Injection into *Xenopus* oocytes of a *TR*β A gene minilocus which reconstitutes chromatin, permitted analysis of the TRβ binding site and its effect on the chromatin structure (Wong *et al.*, 1995, 1997). These data corroborate and extend the model of an unliganded receptor that acts *in cis* to repress transcription and to induce changes in the chromatin topology. More experiments on natural target genes within their chromosomal loci *in vivo* are required to elucidate whether and how a class II unliganded receptor represses transcription as well as to ascertain the physiological role of repression.

To unravel the mechanism of transcriptional repression by v-ErbA *in vivo*, we set out to identify the regions required for transcriptional regulation of the *CAII* gene during erythroid differentiation. We assessed whether v-ErbA acts directly or indirectly through (one of) these regulatory regions. We identified a novel VRE in an intronic enhancer and found that this VRE is occupied *in vivo* in undifferentiated cells, but not in differentiating, *CAII*-transcribing erythroid cells. We discovered that v-ErbA represses the activity of the intronic enhancer by 'neutralizing' the positive action of transcription factors such as GATA-1. We show that a liganded thyroid receptor variant, gag–cTRα, overcomes v-ErbA action and unleashes enhancer activity. Finally, we show that addition of the histone deacetylase inhibitor, trichostatin A, results in derepression of the endogenous *CAII* gene, whereas a transfected, repressed HS2-enhancer construct remains unaffected by this treatment.

Results

DNase I hypersensitivity site induction in the CAII locus during erythroid differentiation

To identify regulatory regions in the *CAII* locus we explored the chromatin status using DNase I hypersensitivity assays in primary chicken erythroid progenitors. In both immature primary erythroblasts and in terminally differentiating primary erythrocytes, prominent DNase I hypersensitive sites were detected ~5 kb upstream and ~8 kb downstream of the

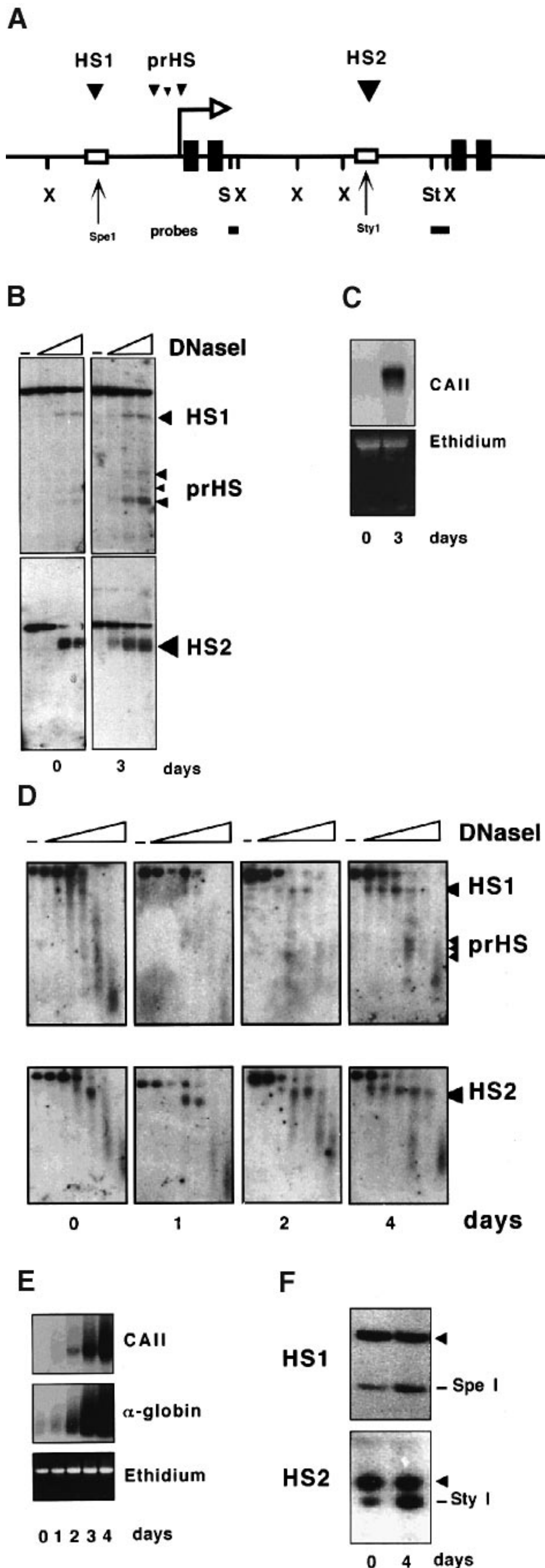

Fig. 1. Analysis of the chicken *CAII* locus during differentiation of primary erythroblasts and v-ErbA transformed HD3 cells. (**A**) Schematic diagram of the *CAII* genomic locus. Exons 1–4 (black boxes) and the relative positions of the *Xba*I (X), *Sac*I (S) and *Stu*I (St) sites, and riboprobes used for end-labeling are indicated. The DNase I hypersensitive regions designated HS1 and HS2 are indicated by open boxes, with the sizes of arrows indicative of the relative DNase I sensitivity. prHS indicates the expression-linked DNase I sensitive sites in the promoter region. (**B** and **C**) DNase I hypersensitive site mapping and corresponding Northern blot analysis using primary chicken erythroblasts before (day 0) and after induction of differentiation (day 3). (**D** and **E**) DNase I hypersensitive site mapping and corresponding Northern blot analysis using chicken erythroid HD3 cells before (day 0) and 1, 2 and 4 days after induction of differentiation. (**F**) Restriction enzyme accessibility assay of HS1 and HS2 using nuclei prepared from HD3 cells before (day 0) and 4 days after induction of differentiation. Arrows indicate fragments that were uncleaved *in vivo*, with *Spe*I and *Sty*I, respectively, marking the cleaved fragments.

transcription start site, designated HS1 and HS2, respectively (Figure 1A and B). Although the putative enhancers, HS1 and HS2, appear to be fully accessible in immature primary cells, *CAII* mRNA could not be detected on Northern blots (Figure 1C). Increased DNase I sensitivity was observed in the promoter region of *CAII*, designated prHS, only in differentiating, primary cells that actively transcribe the gene but not in non-CAII transcribing, proliferating erythroid progenitors (Figure 1B and C). We conclude that in proliferating, primary erythroid progenitors, the *CAII* locus is primed for expression.

In the AEV-transformed HD3 cell line, HS1 and HS2 were detectable only at relatively high DNase I concentrations as compared with primary erythroid progenitors (Figure 1D). Upon induction of differentiation, HS1 and HS2 became hypersensitive, concomitant with the appearance of *CAII* mRNA and of α-*globin* mRNA, an established differentiation marker (Figure 1D and E). The opening of chromatin at HS1 and HS2 in the course of differentiation in HD3 cells was confirmed and corroborated by restriction enzyme accessibility assays (Figure 1F). The extent of restriction enzyme cutting increased 2-fold in HS1 from 20% in proliferating erythroblasts to ~40% in terminally differentiating cells, and ~5-fold in HS2, from 11 to 54%. Taken together, these results identify HS1 and HS2 as the prime candidate regulatory regions involved in activation of *CAII* transcription and in conveying regulation by v-ErbA.

Localization of a v-ErbA response element

We used an unbiased immunoprecipitation approach to identify putative v-ErbA binding sites. A contiguous genomic fragment (17 kb) that includes the HS1, HS2 and *CAII* promoter regions (Figure 1A) was digested with frequently cutting restriction endonucleases, labeled and incubated with HD3 extracts. Protein–DNA complexes were precipitated with an anti-v-ErbA monoclonal antibody (1G10) coupled to paramagnetic beads. The predominant immunoprecipitated restriction fragments spanned the HS2 region (Figure 2A). *In vitro* DNase I footprinting using a fragment extending over HS2 and the v-ErbA-containing HD3 extracts yielded a distinct protection (bar) and enhanced DNase I cutting (arrow heads) (Figure 2B). Immuno-enrichment of HD3 extracts for v-ErbA prior to DNase I treatment diminished the enhanced DNase I cutting but did not affect the footprint (lanes 5 and 6). The comple-

mentary result was obtained using HD3 extracts immunodepleted of v-ErbA; the footprint was abolished but the enhanced DNase I cutting was unaffected (lanes 7 and 8).

Inspection of the sequence encompassing the footprinted region revealed a direct repeat consisting of one perfect and one imperfect half-site spaced by four nucleotides, designated VRE (Figure 2C). The arrangement of the half-sites is reminiscent of a conventional thyroid response element (DR4). Despite the mutation in the dimerization interface of v-ErbA that reduces the affinity of v-ErbA for RXR (Selmi and Samuels, 1991; Barettino *et al.*, 1993), a v-ErbA–RXR heterodimer complex with an oligonucleotide containing the VRE was revealed *in vitro* by bandshift and antibody-supershift assays (Figure 2D). Two sequences known to bind v-ErbA, a canonical DR4 sequence and the F2 sequence of the chicken lysozyme gene (Baniahmad *et al.*, 1990), competed for binding of v-ErbA–RXR, albeit less efficiently than the VRE probe itself. In contrast, the M1 oligonucleotide containing a mutation in the first half-site did not compete (Figure 2D). Thus, we have identified a novel v-ErbA binding site located in the second intron of the *CAII* gene and within the DNase I hypersensitive region HS2.

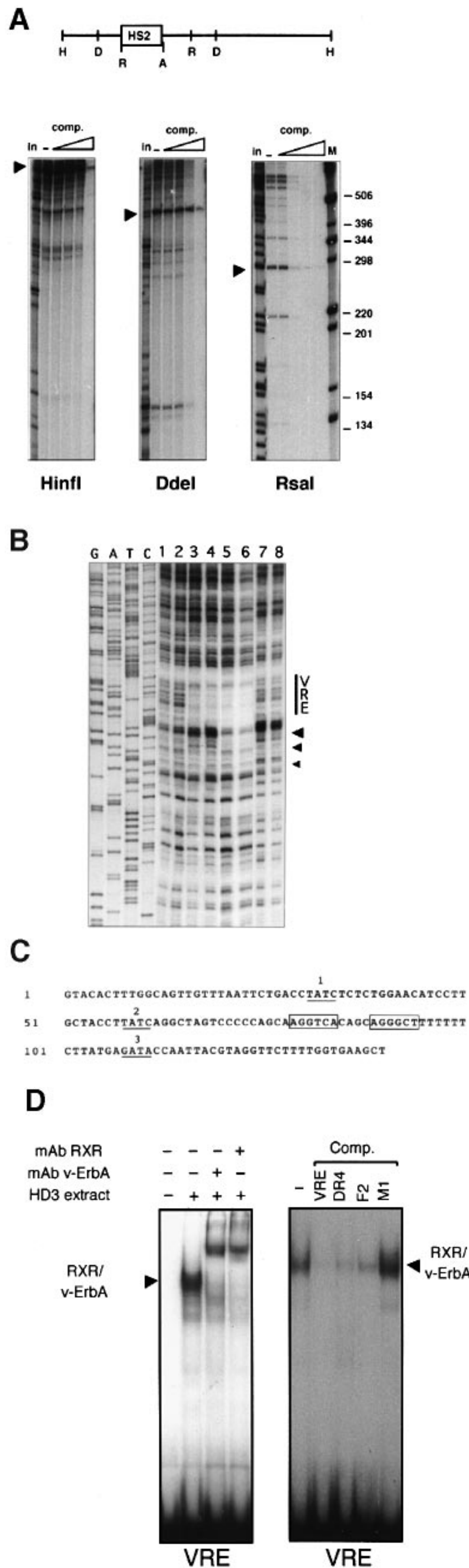

Fig. 2. Identification and characterization of a v-ErbA binding site in *CAII*. (**A**) Immunoprecipitation of v-ErbA–DNA complexes. ^{32}P-labeled DNA fragments generated by *Hin*fI (H), *Dde*I (D) and *Rsa*I (R) digestion of λ clone pCAIIX/N were incubated with HD3 extracts, with increasing concentrations of F2 competitor oligonucleotide. gag–v-ErbA–DNA complexes were immunoprecipitated with anti-gag 1G10 mAb bound to Dynal beads. Arrows indicate the position of the selected fragments. (In) input; (M) DNA marker and fragment size (bp). Also shown is a schematic presentation of the selected fragments with respect to the HS2 region. (**B**) *In vitro* DNase I footprinting on the *Rsa*I fragment (coding strand). Naked DNA (lanes 1–2), incubated with HD3 extract (lanes 3–4), with anti-gag 1G10 mAb-immunoenriched (lanes 5–6) or -immunodepleted HD3 extracts (lanes 7–8). The protected region is marked VRE; arrows point to subtle changes in the DNase I pattern that overlap a putative GATA-factor binding site; lanes labeled G, A, T and C are dideoxynucleotide sequencing reactions. (**C**) Nucleotide sequence of the *Rsa*I–*Alu*I DNA fragment spanning the HS2. The VRE is marked by arrows and three putative GATA-factor binding sites are underlined and numbered. (**D**) Gel-retardation assay of a ^{32}P-labeled synthetic oligonucleotide containing the VRE sequence using HD3 nuclear extract. The complex was super-shifted with mAb against v-ErbA (1G10) and RXR (4RX-1D12) or competed by cold VRE, TRE-DR4, F2 and M1-VRE oligonucleotides.

v-ErbA binds to the CAII-VRE in vivo

To determine whether v-ErbA binds to this putative HS2-VRE *in vivo*, dimethyl sulfate (DMS) and DNase I genomic footprinting experiments were performed in undifferentiated and differentiating HD3 cells, chicken embryo fibroblasts (CEF) and on naked genomic DNA. Comparison of the cleavage patterns revealed that two G-residues in the first, and one G-residue in the second half-site of the VRE were protected from DMS methylation in undifferentiated HD3 cells (Figure 3A, lane 2), but not in naked genomic DNA or CEF cells (Figure 3A, lanes 1 and 4). On the opposite strand, a single G-residue in the 3′ half-site was protected from DMS methylation and a DNase I protection was obtained within the VRE in undifferentiated HD3 cells (Figure 3B, lanes 2 and 6). In contrast, in differentiating HD3 cells that transcribe the *CAII* locus, DMS or DNase I protections of the VRE could not be detected (Figure 3, lanes 3 and 7). Western blot analysis (Figure 5E, lanes 3 and 4) shows that the absence of a footprint is not due to a reduction in the concentration of v-ErbA protein in differentiating as compared with fully transformed HD3 cells.

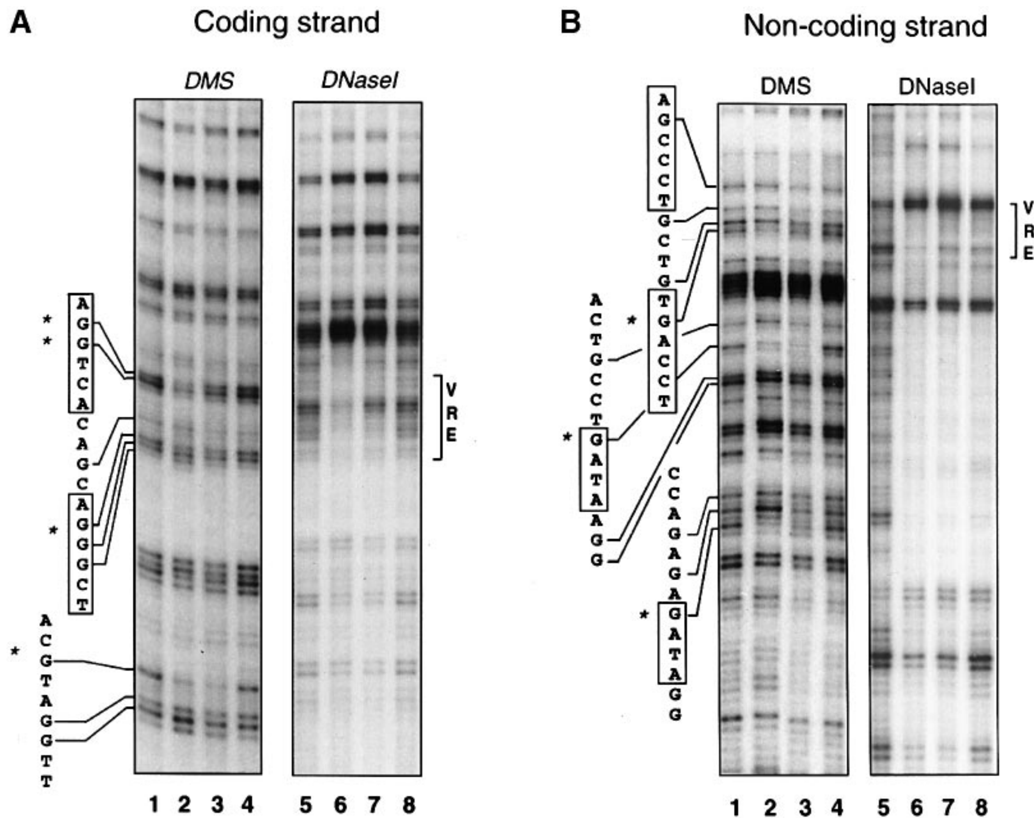

Fig. 3. DMS and DNase I *in vivo* footprinting. DMS (lanes 1–4) and DNase I (lanes 5–8) *in vivo* footprinting of (**A**) the coding strand, and (**B**), the non-coding strand, in HD3 cells before (lanes 2 and 6) and after (lanes 3 and 7) induction of differentiation, in chicken embryo fibroblasts (lanes 4 and 8) and on naked genomic DNA (lanes 1 and 5). The VRE and putative GATA-sites are boxed; protected G-residues are indicated by asterisks (*).

Intriguingly, additional G residues outside of the VRE were found to be protected from DMS methylation in erythroid cells regardless of their differentiation state. This protection indicates that the putative regulatory complex on HS2 may at least be partially assembled in undifferentiated cells prior to activation of *CAII* expression. Two of these DMS protections are within potential binding sites of members of the GATA family of transcription factors (Ko and Engel, 1993), located at nucleotides 31–35 (G1) and 58–61 (G2) (underlined in Figure 2C). Taken together, the occupancy of the HS2-VRE *in vivo* in undifferentiated erythroid progenitors correlates with the lack of transcription at the *CAII* promoter and lends support to the notion that v-ErbA represses *CAII* transcription through the putative HS2 enhancer.

HS2 is an enhancer

To test whether the HS2 indeed functions as an enhancer, the 137 bp *Rsa*I–*Alu*I fragment spanning the HS2 region was cloned in front of a tk promoter-CAT reporter and tested in HD3 cells (Figure 4A). Transcription originating from the tk promoter was minimally activated ~2-fold by HS2 (Figures 4B). However, a HS2 fragment carrying a mutation in the VRE that abolishes v-ErbA binding (Figure 2D and data not shown) and placed in front of the tk promoter (M1–HS2) boosted the level of transcription ~20- to 30-fold as compared with the level of transcription obtained with the wild-type HS2 enhancer (Figure 4B). The very potent activation of transcription from the HS2 enhancer obtained upon mutating the v-ErbA binding site can best be explained by the loss of v-ErbA repression. Placing an oligonucleotide comprising the VRE in front of tk repressed the level of transcription only ~2-fold whereas the F2-element from the chicken lysozyme gene (F2-tk) (Baniahmad *et al.*, 1990) conveyed 5- to 7-fold repression. A multimerized VRE placed in front of tk did not result in a significant potentiation of the repressive activity (data not shown); neither did an oligonucleotide M1 give significant enhanced activity. Furthermore, HS2 and M1–HS2 did not function as enhancers in the non-erythroid cell lines tested suggesting that the enhancer may be erythroid-specific (G.G.Braliou and H.G.Stunnenberg, unpublished observations). We conclude that HS2 comprises a genuine enhancer whose activity is repressed by the action of v-ErbA.

We next assessed the identity and biological significance of the putative GATA factor binding site as identified by *in vivo* DMS footprinting in immature as well as differentiating HD3 cells (Figure 3B). Bandshift assays revealed the presence of a protein in HD3 extracts that binds to an oligonucleotide spanning nt 24–44 (comprising the first putative GATA-factor binding site). This protein–DNA complex could be supershifted with a monoclonal antibody directed against GATA-1, but not by antibodies against GATA-2 and -3 (Figure 4C). To assess the biological significance of this GATA site *in vivo*, a mutation that abolished GATA binding in bandshift assays (data not shown) was introduced within the context of the HS2- and M1–HS2 fragments, yielding G1–HS2 and G1–M1–HS2 (Figure 4B). Mutation of the GATA-1 site reduced

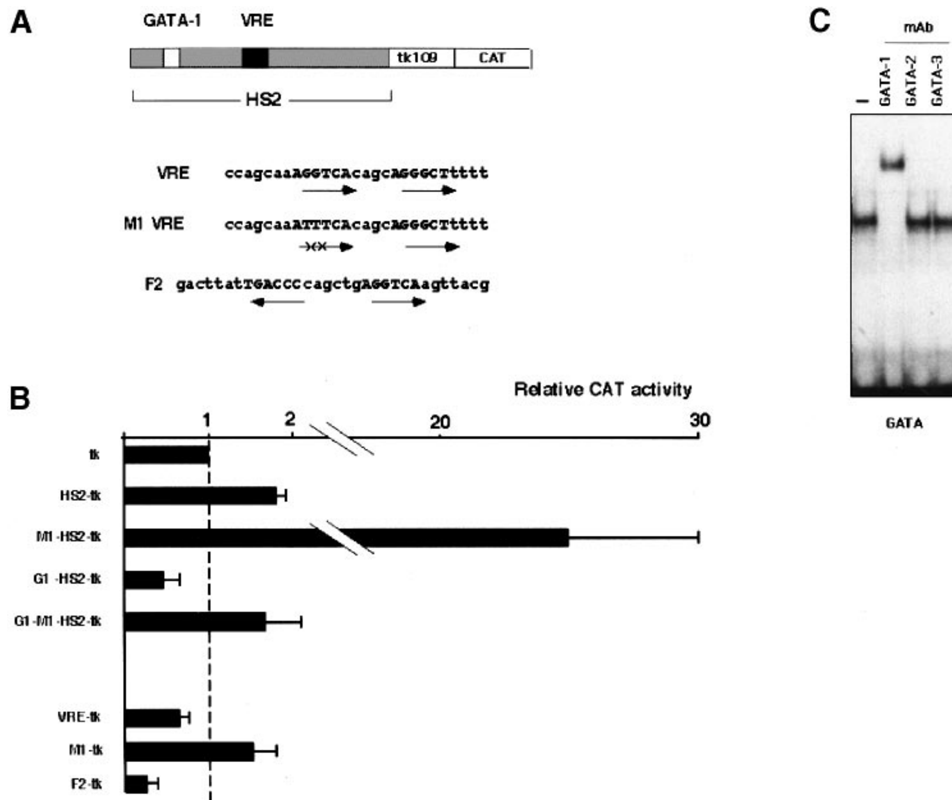

Fig. 4. Transcriptional repression in v-ErbA expressing HD3 cells is mediated by the VRE. (**A**) Schematic diagram of the tk reporter constructs containing either fragments of HSV or synthetic oligonucleotides. The nucleotide sequence of the VRE, M1-VRE and F2 oligonucleotides are shown, with arrows indicating the half-sites and the crosses indicating the mutated bases. In addition to the *Rsa*I–*Alu*I fragment spanning HS2, that fragment carrying mutations in the VRE (M1–HS2) or in a GATA site (G1–HS2) or in both sites (G1–M1–HS2) was tested. (**B**) Transient transfection assays of HD3 cells with the above tk reporter constructs. Transcription is expressed relative to that of the tk promoter alone. (**C**) Gel-retardation assay of a ^{32}P-labeled synthetic oligonucleotide containing the GATA factors binding site using HD3 nuclear extract. mAbs specific for GATA-1, GATA-2 and GATA-3 transcription factors were added.

the transcriptional activity of the HS2-enhancer from an ~2-fold activation obtained with HS2-tk to a 2-fold repression with G1–HS2-tk. Moreover, mutation of this GATA-1 site in the context of the M1-fragment (mutated v-ErbA binding site) caused a 15-fold reduction of the enhancer activity of HS2 as compared with M1–HS2-tk. This shows that the GATA site is critical for the activity of the HS2 enhancer.

Taken together, mutation of the v-ErbA binding site in the context of the HS2 enhancer resulted in a marked increase in the activity of the enhancer, as would be expected to occur upon inactivation of a repressor binding site. Moreover, transcription from the G1–HS2-tk reporter was lower than that obtained by the HS2-tk alone (Figure 4B), i.e. the balance between activation by GATA-1 and other (erythroid) factors and repression by v-ErbA is shifted towards repression. Intriguingly, the v-ErbA binding site does not appear to convey strong repression on its own outside of the HS2 context because the level of transcription from the heterologous tk- or any other tested promoter can only be reduced 2- to 3-fold (Figure 4B; data not shown). However v-ErbA very efficiently represses *in cis* the activity of the HS2-enhancer thus 'neutralizing' the transcriptional activity of GATA-1 and presumably other transcription factors (G.G.Braliou and H.G.Stunnenberg, unpublished observations).

Liganded TRα activates transcription through the HS2 enhancer

We reported previously that HD3-V3 cells expressing a gag–cTRα fusion protein (V3) to levels similar to that of v-ErbA (Disela *et al.*, 1991; Figure 5E, lanes 1 and 2) can be induced to express erythroid-specific marker genes such as *CAII* upon addition of T3 (Disela *et al.*, 1991; Schroeder *et al.*, 1992) without inducing differentiation. These and other experiments suggested that TR can overcome v-ErbA repression by binding to *cis*-acting sequences that might include the *CAII*-VRE. We therefore pursued the possibility that addition of T3, which should convert the gag–cTRα repressor to an activator, would revert the v-ErbA block of *CAII* expression and might induce chromatin changes. In undifferentiated HD3-V3 cells, the HS2 site was detectable at relatively high DNase I concentrations and became more pronounced within 24 h of T3 addition comparable with the results obtained in HD3 cells (Figure 5A; data not shown). HS1 and prHS also became more apparent upon T3 induction concomitant with the appearance of *CAII* mRNA (Figure 5B and C). The overall effects of T3-activated gag–cTRα are modest with respect to chromatin alterations, probably due to the presence of the constitutive repressor v-ErbA. These data nevertheless suggest that gag–cTRα instigates chromatin changes upon ligand activation in line with the studies of Wong and colleagues of the autoregulated *Xenopus* TRβ

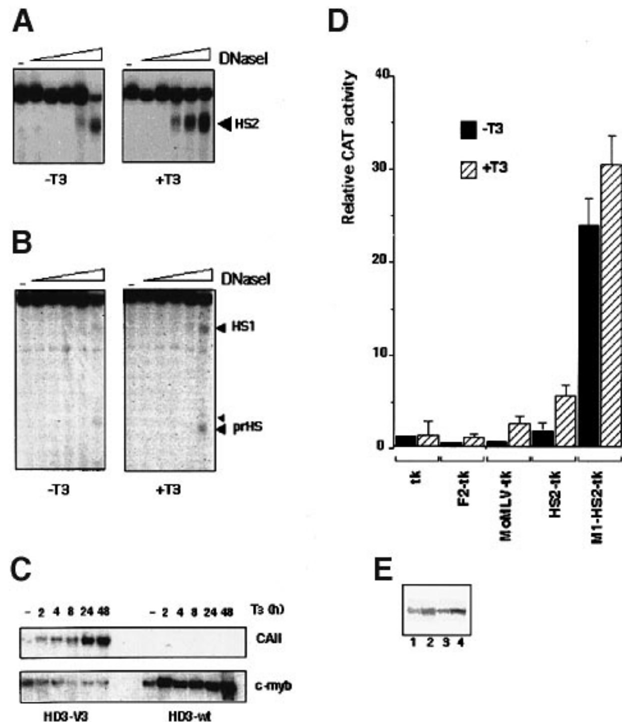

Fig. 5. Transcriptional activation and chromatin remodeling instigated by ligand-activated gag–cTRα. (**A** and **B**) DNase I hypersensitive site mapping in HD3 cells expressing gag–cTRα (named HD3-V3) before or after 24 h of T3 treatment. (**C**) Northern blot analysis of *CAII* and c-*myb* in the course of T3 induction in HD3-V3 and the parental HD3 cells. (**D**) Transient transfection analysis of MoMLV-TRE-, HS2-VRE- and M1–HS2-tk containing reporter constructs in HD3-V3 cells; before harvesting transfected cells were incubated 22 h in the absence (–) or presence (+) of T3. (**E**) Western blot analysis of gag–v-ErbA and gag–cTRα using anti-gag antibody 1G10. Lanes 1 and 2 are HD3-V3 cells 0 and 22 h after T3 treatment; lanes 3 and 4 are HD3 cells on day 0, and 4 days after induction of differentiation.

gene using an *in vivo* chromatin reconstitution system based on injection of single stranded plasmid DNA into *Xenopus* oocytes (Wong *et al.*, 1997).

Next, we tested the ability of the HS2-, M1–HS2-tk and of natural TREs from the Moloney murine leukemia virus (MoMLV) (Sap *et al.*, 1990) and from the lysozyme gene (F2) (Baniahmad *et al.*, 1990) placed in front of tk to mediate a T3 response in transient transfection assays in HD3-V3 cells. In the absence of ligand, the F2-tk as well as the MoMLV-tk appeared to be repressed (5- and 3-fold, respectively); addition of T3 boosted their level of transcription ~5- and 8-fold, respectively. For the HS2-tk reporter, addition of T3 resulted in a 4-fold activation, which is significantly lower than the maximal level of transcription obtained with the M1–HS2 construct that carries the VRE mutation. This reduced T3 responsiveness of the HS2-tk reporter is the sum of activation by liganded gag–cTRα and constitutive repression by v-ErbA, i.e. positive and negative factors competing for binding to the VRE.

Trichostatin A fails to induce HS2 activity on transfected plasmids, but induces transcription from the endogenous CAII gene

So far, we have demonstrated that the VRE bound by v-ErbA conveys strong repression of a transfected HS2-enhancer. Furthermore, ligand-activated gag-cTRα can partially relieve the repression of the *CAII* gene if expressed to equivalent levels as the constitutive repressor, v-ErbA (Disela *et al.*, 1991; Figure 5E). Taken together with our observations that the VRE is occupied *in vivo* in erythroid cells that do not transcribe the *CAII* gene and that v-ErbA binds to the VRE *in vitro*, we tentatively conclude that v-ErbA acts to repress the *CAII* gene. A plethora of biochemical, yeast two-hybrid and transient transfection assays suggest that an unliganded receptor represses transcription via the N-CoR–SIN3A–HDAC complex that possesses intrinsic histone deacetylase activity (Chen and Evans, 1995; Hörlein *et al.*, 1995; Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). Several recent observations have reinforced the notion that histone deacetylation plays an important role in repression. For example, trichostatin A (TSA) enhances the effects of RA on induction of differentiation of myeloid precursors (HL60, NB4 and U937 cells expressing PML–RAR and PLZF–RAR), and on activation of transiently transfected RARE reporters in these cells (Nagy *et al.*, 1997; Grignani *et al.*, 1998; Lin *et al.*, 1998). Inhibition of histone deacetylases can also relieve repression by unliganded TR–RXR bound on a TRE-containing template assembled into nucleosomes (Wong *et al.*, 1995, 1997).

We therefore examined whether histone deacetylases play a role in the repression of *CAII* transcription in HD3-V3 and HD3 cells (Figure 6A; data not shown). In HD3-V3 cells, addition of T3 resulted in a 3-fold activation of the HS2-tk reporter, while addition of TSA did not affect the level of transcription from this promoter. Unexpectedly, addition of both T3 and TSA reproducibly resulted in a reduction of transcription rather than an additional increase, as compared with that obtained with T3 alone. T3 or TSA treatment activated transcription from the F2-tk reporter and the combination of T3 plus TSA resulted in an additional 2-fold enhancement. Whether the additive effect of T3 plus TSA is relevant remains to be determined, since T3 plus TSA also caused a 2- to 3-fold activation of the parental tk-reporter. Transfection of a 3×(RAREβ2)-tk reporter and addition of RA plus TSA resulted in a very strong synergistic activation of transcription (G.G.Braliou and H.G.Stunnenberg, unpublished observations). These data imply that although the HD3-V3 cells can respond to TSA and T3 or RA treatment as described for other cell lines, transcription from the HS2-tk reporter was not similarly affected.

Finally, we performed in parallel Northern blot analysis of the transfected and TSA and/or T3 treated cells to test whether the endogenous *CAII* gene was activated upon these treatments (Figure 6B). Surprisingly, the TSA treatment alone resulted in significant activation of transcription from the endogenous *CAII* locus; in addition, TSA further boosted the strong activation given by T3 alone. In contrast, the level of transcription from the endogenous *MYB* gene, a marker of undifferentiated erythroid cells, was weakly reduced upon T3 treatment (see also Figure 5C) and markedly down-regulated by TSA treatment. A combined TSA plus T3 treatment enhanced this down-regulation as obtained with TSA alone. The mRNA levels from a constitutive gene, *band 4.1*, were not markedly affected by T3 and/or TSA. Thus, the endogenous *CAII* gene was sensitive to TSA as well as to T3 treatment whereas the transfected HS2 enhancer only responded to ligand, but

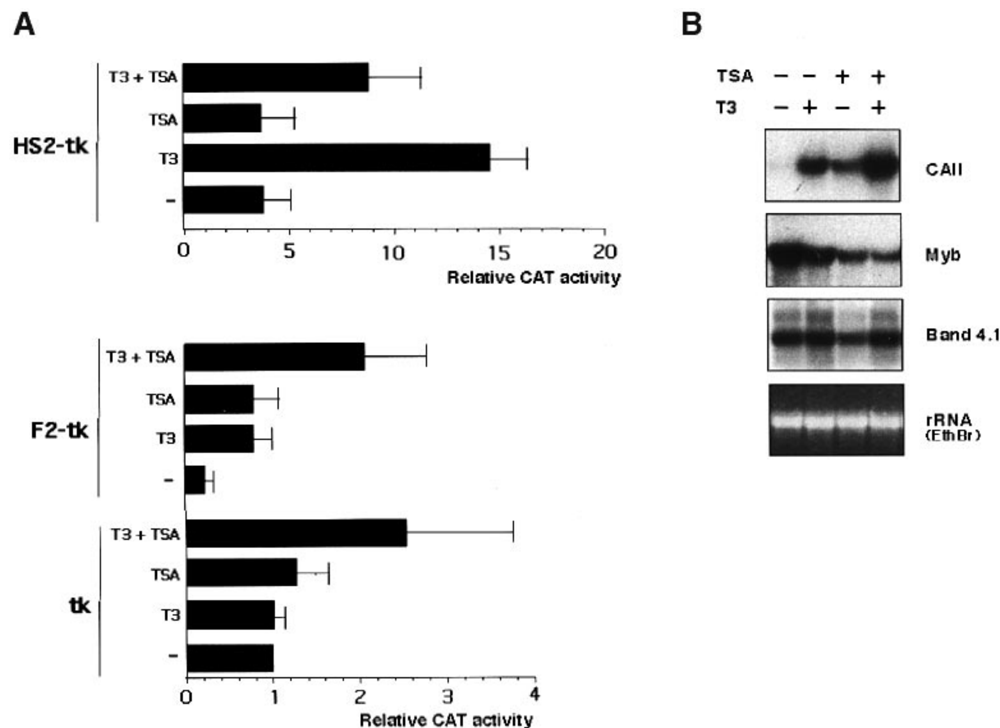

Fig. 6. Effect of TSA and T3 treatment of HD3-V3 cells. (**A**) Transient transfection assays with the indicated constructs, with CAT activities expressed relative to the tk reporter alone. (**B**) Northern blot analysis of endogenous genes in the treated cells. Cells were treated with the indicated reagents for 22 h before harvesting.

not to TSA treatment. Similar results were obtained with the HD3 cells (data not shown).

Discussion

v-ErbA acts in cis to repress CAII transcription

To date, the best-documented biological phenomenon which correlates with repression by a nuclear receptor is the block of differentiation of chicken erythroid progenitors mediated by the *v-ErbA* and *v-ErbB* oncogenes (Zenke *et al.*, 1988, 1990; reviewed in Beug *et al.*, 1996). v-ErbA is postulated to contribute to erythroleukemia by repression of erythroid-specific target genes such as *CAII* (Zenke *et al.*, 1990; Disela *et al.*, 1991; Bauer *et al.*, 1997). It has remained unclear whether this presumptive negative function of v-ErbA is mediated *in cis* through a v-ErbA response element or via protein–protein interactions with other transcription factors. We and others initially identified and characterized a TRE in the promoter region of *CAII* (Disela *et al.*, 1991; Rascle *et al.*, 1994). Notwithstanding extensive analysis, we could not demonstrate unambiguously that this element functioned as a genuine v-ErbA *cis*-acting element.

In this study, we have assessed the chromatin state and the regulation of *CAII* expression in primary erythroid progenitors and in AEV-transformed erythroid cell lines during the course of differentiation. Using *in vivo* DNase I mapping, we have identified two hypersensitive regions, one positioned ~5 kb upstream of the transcription start site and one ~8 kb downstream, in the second intron, termed HS1 and HS2, respectively. Transient transfection experiments revealed that the HS2 hypersensitive region functions as a genuine enhancer that governs *CAII* expression. Several lines of evidence suggest that v-ErbA binding to the HS2-VRE causes repression of transcription. First, immunoprecipitation assays identified a high affinity v-ErbA binding site, VRE, that is located in the HS2. Secondly, bandshift assays as well as *in vitro* footprinting showed that v-ErbA specifically binds to this VRE. Thirdly, a mutation causing loss of v-ErbA binding to the VRE *in vitro* resulted in a marked derepression of the HS2 enhancer activity *in vivo*. Fourthly, in transient transfection experiments, ligand activation of a gag–cTRα fusion protein partially reverted the repression of transcription from the HS2 enhancer which was dependent on the presence of the VRE. Finally, DMS and DNase I *in vivo* footprinting revealed that the VRE was protected in undifferentiated cells, whereas protection was lost in differentiating cells in which the v-ErbA oncoprotein is inactive.

Besides v-ErbA, other (erythroid) factors are bound to the HS2 enhancer in undifferentiated cells, indicating that an enhancer complex is at least partially assembled on the HS2 before the onset of *CAII* transcription. One of the bound factors was identified as the erythroid-specific GATA-1 factor. In undifferentiated cells, that is in the presence of an active v-ErbA oncoprotein, the pre-assembled enhancer complex does not instigate productive transcription. In differentiating cells, v-ErbA binding to the VRE cannot be detected and *CAII* is transcribed, suggesting that v-ErbA prevents the activity of the enhancer complex by 'neutralizing' the activity of transcription factors such as GATA-1. In line with this hypothesis, transient transfection assays in undifferentiated HD3 cells revealed that mutation of the VRE unleashed potent enhancer activity. Therefore, transcription factors capable of driving the HS2 enhancer are present in an active state in undifferentiated cells.

The question arises how v-ErbA prevents the activity of a pre-assembled enhancer complex. The widely accepted molecular switch model for nuclear receptor action suggests that the unliganded receptor tethers the co-repressor complex containing N-CoR–SMRT, SIN3A/B and the histone deacetylase, HDAC (Chen and Evans, 1995; Hörlein *et al.*, 1995; Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). An extension of this model predicts that the v-ErbA–co-repressor complex either participates in or instigates the local organization of the chromatin into a repressive state. Actually, initial studies suggested that thyroid hormone action was mediated by a receptor that stably associates with chromatin independent of the presence or absence of ligand (Perlman *et al.*, 1982). This concept was corroborated and extended by the recent data from Wolffe and co-workers showing that unliganded TR may indeed assist or even be instrumental in setting up a repressive chromatin state in *Xenopus* oocytes (Wong *et al.*, 1995). Our observation that the DNase I hypersensitivity of the HS2 as well as of the HS1 region is markedly reduced in fully-transformed, v-ErbA-expressing HD3 progenitors as compared with primary erythroid progenitors is consistent with a role of v-ErbA in setting up or stabilizing repressive chromatin. Our TSA experiments strongly suggest that histone deacetylases play a role in repression of the chromatin-embedded *CAII* gene. Similarly, Wong and co-workers showed that histone acetylation/deacetylation plays a role in repression and activation by wild-type TR in *Xenopus* oocytes (Wong *et al*., 1997). It seems likely that the histone deacetylase-containing N-CoR–SMRT–SIN3A–HDAC complex is targeted to the *CAII* locus by v-ErbA; however, formal proof is lacking at this stage. Recruitment of the HDAC activity to the locus is, however, unlikely to be the only step leading to repression. In fact, our transient transfection assays performed in the presence of TSA and/or T3 suggest that v-ErbA can repress the HS2-enhancer activity independent of histone deacetylase activity. Hence, v-ErbA binding to its site in the HS2 enhancer may also act directly or via the N-CoR–SMRT or SIN3A components of the co-repressor complex on (erythroid) transcription factors bound to the *CAII* enhancer to 'neutralize' their transcriptional activity. The observed non-responsiveness from the HS2 enhancer to TSA or TSA plus T3 treatments is particularly striking in light of the results with the F2-tk reporter. Furthermore, a strong synergism between RA and TSA has been observed in NB4 cells (Lin *et al*., 1998), in U937 cells stably transfected with PML–RAR and PLZF–RAR (Grignani *et al*., 1998), in P19 EC cells (Minucci *et al*., 1997) or HD3 cells (G.G.Braliou and H.G.Stunnenberg, unpublished observation). In all these cases, however, artificial reporter configurations were tested. One interpretation of our results is that HDACs do not contribute to repression of the HS2 enhancer by v-ErbA. Alternatively, acetylation/deacetylation of histones or other (basal) transcription factors may be effective only if the HS2 enhancer contains a positioned nucleosome. We favor the latter explanation which is in agreement with the frequent observations that nucleosomal assembly of transfected plasmids is anomalous, for example with the MMTV promoter (Archer *et al*., 1992). Our findings and those of Wong and colleagues underscore the importance of the topology of the chromatin-embedded TRE to support TR binding and transcriptional regulation (Wong *et al.*, 1997 and this study).

Intriguingly, induction of HD3 differentiation lead to a loss of v-ErbA binding to the HS2-VRE, which did not result from a markedly decreased concentration of v-ErbA in differentiating versus undifferentiated HD3 cells (Figure 5E). One possible explanation could be that loss of v-ErbA binding to the VRE was due to changes in concentrations of auxiliary factors or in the phosphorylation status of v-ErbA. Phosphorylation of v-ErbA at serine residues 16 and 17 was previously shown to be critical for its oncogenic activity (Glineur *et al.*, 1990). Also, nuclear hormone receptors need to cooperate with receptor tyrosine kinases to block differentiation of multipotent hematopoietic cells (Bauer *et al.*, 1997). For example, v-ErbA-expressing primary erythroblasts can only be triggered into differentiation upon omission of stem cell factor (SCF), whereas AEV-transformed HD3 cells can be triggered into differentiation only upon inactivation of the tyrosine kinase oncogene with specific inhibitors and upon addition of erythropoietin and insulin (Choi *et al.*, 1986; Zenke *et al.*, 1988). It therefore seems plausible that this interruption of tyrosine kinase signaling may have affected phosphorylation of v-ErbA or associated (co)factors.

CAII-HS2 enhancer is activated in response to T3

Our previous studies have shown that TRα is likely to take part in erythrocyte differentiation as well as in *CAII* activation (Disela *et al.*, 1991; Schroeder *et al.*, 1992; Gandrillon *et al.*, 1994). We now show that the HS2 becomes fully open only upon T3 treatment and that increased DNase I sensitivity can be observed in the promoter region. This ability of liganded TR to remodel the chromatin structure is consistent with biochemical experiments, describing the physical interaction among nuclear receptors and protein complexes that possess an intrinsic histone acetyltransferase activity (Yang *et al.*, 1996). Thus, liganded TR indeed appears to counteract the repressive action of v-ErbA by destabilizing the repressive chromatin configuration and setting up active chromatin, thereby explaining the observation that the HS1 and HS2 hypersensitive sites of *CAII*, which are only poorly developed in v-ErbA expressing cells, are very prominent in HD3-V3 cells.

Our transient transfection data with HD3-V3 cells, show that the *CAII*-HS2 enhancer can mediate T3-dependent transactivation. The data corroborate and extend the notion that v-ErbA occludes TRα from binding to the HS2-VRE, because in the presence of T3 the enhancement of transcription by the HS2 is significantly lower compared with the M1–HS2, i.e. in the absence of a v-ErbA binding site. *In vitro* DNA binding studies indicate that the v-ErbA–RXR heterodimer has a relatively high affinity for the HS2-VRE as compared with a canonical DR4 or F2 element (Figure 2C). The VRE deviates from the consensus DR4 in the sequence of the 3′ half-site (-AGGGCT-). Intriguingly, v-ErbA presumably contacts the 3′ half-site and the G-residue at that fourth position was shown to be preferred by a DNA-binding domain containing the Gly→Ser mutation present in the P-box of v-ErbA (Nelson *et al*., 1994).

Is repression in cis by unliganded receptors a general phenomenon?

Our data clearly show that v-ErbA binds to a response element embedded in chromatin and represses transcription of CAII *in cis*. It is tempting to speculate that, in the absence of their respective ligands, other wild-type class II receptors function in a manner similar to that observed for v-ErbA. Up to now, only a few biological phenomena have been described that may be attributed to repression. The *Xenopus TRβA* gene is repressed by unliganded TRβ through a TRE-DR4 (Wong *et al*., 1995). Unliganded TR and v-ErbA repress transcription of the chicken lysozyme gene *in vivo* through the TRE-F2 element (Baniahmad *et al*., 1990). The 3′ *hoxb-1* gene is reportedly regulated by an enhancer which contains two activating RAREs and one repressing RARE (Studer *et al*., 1994; reviewed in Marshall *et al*., 1996). In the latter two cases, however, it has not yet been demonstrated that the unliganded receptor indeed binds to its target site *in vivo*.

In contrast to the occupancy of the HS2-VRE we observed in erythroid progenitors, *in vivo* footprint assays did not reveal occupancy of the RARE present in the *RARβ2* promoter in the absence of ligand, although a clear protection was seen upon RA treatment (Minucci *et al*., 1994; Chen *et al*., 1996; Bhattacharyya *et al*., 1997). This result is surprising because the *RARβ2* promoter displays DNase I hypersensitivity in undifferentiated P19 embryonal carcinoma cells (Bhattacharyya *et al*., 1997; our unpublished observations) before the onset of transcription. It is not inconceivable that the binding of an endogenous RAR receptor may not as readily be demonstrable by *in vivo* footprinting as with the highly expressed oncogenic v-ErbA receptor.

The role of unliganded receptors in hematopoietic disorders

Although v-ErbA may not be the prototypic unliganded receptor, the phenomenon of repression linked to hematopoietic disorders is a recurring theme. The hybrid proteins PML–RAR and PLZF–RAR, the causative agents of APL (Hofmann, 1992), have recently been shown to block differentiation at a promyelocytic stage by acting as transcriptional repressors. Intriguingly, APL cells carrying the PLZF–RAR fusion have lost their response to RA treatment and do not differentiate; this correlates with the ability of PLZF to interact, independently from the RAR hinge region, with co-repressors such as N-CoR or SMRT (Grignani *et al*., 1998; Lin *et al*., 1998). Overexpression of a dominant negative variant of RARα lacking activation functions in lymphohematopoietic progenitors reveals the ability of this truncated protein to block differentiation of these cells; the repressor activity of RAR seems to contribute at least in part to this phenotype (Tsai *et al*., 1994). Thus, class II nuclear receptors in the repressive ‘off’ mode may play an important role, both in hematopoiesis and in other biological processes. Unraveling the mechanisms of gene silencing is likely to provide novel insight into the multifaceted activities of class II nuclear receptors either as transcriptional repressors or as activators, in normal and in disregulated differentiation.

Materials and methods

Cell culture

Two derivatives of the AEV-transformed cell line HD, namely HD3-EpoR and HD3-V3, expressing, respectively, the murine erythropoietin receptor or a gag–chicken TRα fusion, were used. Primary erythroblasts and these cell lines were grown in CFU-E medium (Dolznig *et al*., 1995); the medium for primary erythroblasts was supplemented with SCF to promote proliferation (Mellitzer *et al*., 1996). Differentiation was induced in differentiation medium (Dolznig *et al*., 1995). In HD3-EpoR cells, 5 μM of the tyrosine kinase inhibitor PD 153035 (Fry *et al*., 1994) was added to inhibit signaling from the v-ErbB oncoprotein. Before T3 treatment, HD3-V3 cells were grown for 48 h in medium containing stripped serum; 150 nM T3 was added to the medium where indicated. CEF were grown as described (Fuerstenberg *et al*., 1992).

DNase I hypersensitivity assay

In vivo DNase I hypersensitivity assays were performed essentially as described (Stewart *et al*., 1991). Briefly, cells were washed twice with phosphate-buffered saline (PBS) and incubated for 4 min at room temperature in a buffer containing 0.2% NP-40 and increasing concentrations of freshly prepared DNase I; HD3-EpoR cells with 1, 2, 4, 8, 16 and 32 and primary erythroblasts with 1, 2, 4 U per 10^6 cells, respectively. The reactions were stopped by the addition of 20 mM EDTA and 20 μg/ml RNaseA (final concentrations). Cells were lysed by addition of 1% SDS, 50 mM Tris–HCl pH 8 and 200 μg/ml Proteinase K and incubation overnight at 37°C; DNA was then purified by phenol extraction and ethanol precipitation. Genomic DNA (20 μg) was digested with *Xba*I and hybridized with the ^{32}P-labeled riboprobes, *Sac*I–*Xba*I and *Stu*I–*Xba*I, as indicated schematically in Figure 1A.

Enzyme accessibility assay

Nuclei were prepared from differentiating and undifferentiated HD3-EpoR cells, resuspended in the appropriate restriction enzyme buffer and incubated for 1 h with 200 U of *Spe*I or *Sty*I for HS1 and HS2 analyses, respectively. DNA was extracted and analyzed as described in the above paragraph.

Northern blot

Total RNA was extracted using the guanidium–CsCl method; *CAII*, c-*myb*, *band4.1* and α-*globin* mRNA level were detected by Northern blot analysis as previously described (Zenke *et al*., 1990).

Co-immunoprecipitation of v-ErbA–DNA complexes

The λ clone pCAIIX/N containing a 17 kb fragment of the *CAII* gene (cloning will be described elsewhere) was digested with *Hin*fI, *Rsa*I and *Dde*I, respectively, and labeled. DNA (5 pmol) was incubated with HD3 extracts in 20 mM HEPES pH 7.9, 100 mM NaCl, 5 mM $MgCl_2$, 15% (v/v) glycerol, 0.1% Triton-X 100, 0.3 mg/ml poly(dI–dC) and 2 mM dithiothreitol in the presence of increasing amounts (0.1, 1, 10 and 100 ng) of a unlabeled competitor oligonucleotide TRE-F2 containing the v-ErbA binding site from the chicken lysozyme gene (Baniahmad *et al*., 1990). v-ErbA–DNA complexes were immunoprecipitated using anti-gag 1G10 mAb and goat-anti mouse IgG-coated paramagnetic beads (Dynal). Precipitated fragments were analyzed in a 6% sequencing gel.

Oligonucleotides used for gel-retardation assays and for cloning in pBLCAT2 vector

Coding strand:
VRE: 5′-TCGACCCAGCAAGGTCACAGCAGGGCTTTTTTTC-3′;
M1-VRE: 5′-TCGACCCAGCAATTTCACAGCAGGGCTTTTTTTC-3′,
F2: 5′-TCGACTTATTGACCCCAGCTGAGGTCAAGTTACC-3′
GATA: 5′-TCGACTCTGACCTATCTCTCTGGAAC-3′
Non-coding strand:
VRE: 5′-TCGAGAAAAAAAGCCCTGCTGTGACCTTGCTGGG-3′;
M1-VRE: 5′-TCGAGAAAAAAAGCCCTGCTGTGAAATTGCTGGG-3′,
F2: 5′-TCGAGGTAACTTGACCTCAGCTGGGGTCAATAAG-3′
GATA: 5′-TCGAGTTCCAGAGAGATAGGTCAGAG-3′
Oligonucleotide used for mutagenesis:
mutagenesis-VRE 5′-CCCTGCTGTGAAATTGCTG-3′;
mutagenesis-GATA 5′-CTGACCTAAATCTCTGGAAC-3′.

Gel-retardation assay

Labeled VRE oligonucleotide (20 pmol) was incubated with HD3 protein extract for 15 min on ice in the same binding buffer as used for the co-immunoprecipitation assays; in antibody-supershifting experiments, anti-gag 1G10 mAb, anti-RXR mAb or anti-GATA-1, -GATA-2, -GATA-3 were added and incubated on ice for an additional 15 min; competition was performed by adding a 100-fold molar excess of unlabeled VRE, TRE, M1-VRE or F2 oligonucleotides. Reactions were loaded on pre-cooled 0.5×TBE, 5% acrylamide gels.

Transient transfection assays

HD3, HD3-EpoR and HD3-V3 cells were transfected using the DEAE–dextran transfection procedure as previously described (Choi and Engel, 1988). In a typical experiment 10^7 cells were transfected with 5 μg of reporter construct together with 1 μg of EF1α-Luc as an internal control, and harvested after 48 h. CAT and luciferase activity were measured as described previously (Barettino *et al.*, 1993). Additions of 150 nM T3 and/or 100 nM TSA were made to HD3-V3 transfected cells grown in culture medium containing stripped serum for the last 22 h.

Cloning

An *Rsa*I–*Alu*I fragment of 137 bp spanning HS2 was inserted into the *Hin*dII–*Bam*HI site of pBLCAT2 vector, yielding pHS2-tk. The HS2 mutants M1–HS2-tk, G1–HS2-tk and G1–M1–HS2-tk were generated by oligonucleotide-directed site-specific mutagenesis using the oligonucleotides, M1-VRE and G1-GATA, respectively. pVRE-tk, pM1-VRE-tk and F2-tk, were constructed by cloning the corresponding oligonucleotides containing cohesive *Sal*I and *Xho*I termini into the *Xho*I site of pBLCAT2.

In vitro DNase I solid-phase footprinting

In vitro DNase I solid-phase footprinting was performed as described (Sandaltzopoulos and Becker, 1994). A 291 bp *Rsa*I fragment spanning the HS2 was labeled and incubated with HD3 extracts or with HD3 extracts either immunoenriched or immunodepleted for v-ErbA using mAb 1G10.

In vivo DMS and DNase I footprinting

Cells resuspended in 1 ml media were treated with 2 μl DMS for 2 min at room temperature, washed twice with cold PBS and resuspended in nuclei buffer (NB: 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris–HCl pH 8.2, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM EGTA, 2 mM EDTA, 0.5% NP-40). After incubation at 4°C for 5 min, nuclei were pelleted, washed twice with NB without sucrose and NP-40; DNAs were then extracted as described above for the DNase I hypersensitivity assays. DMS-treated DNA samples were treated with piperidine (1 M) for 45 min at 96°C, chloroform-extracted and precipitated. DNA samples prepared for DNase I hypersensitive site mapping were also used for ligand-mediated PCR (LM–PCR) mediated DNase I *in vivo* footprinting using a biotinylated oligonucleotide approach as described (Quivy and Becker, 1993) and 1.2 μg of genomic DNA. The following gene-specific primers sets were used: coding strand: 5′-AGATGTGAACCTGAATGA-3′, 5′-CCAGTCTGTGCCAAGTAGT-TC-3′, 5′-GCTGAGTTGAAATCACTG-3′; non-coding strand: 5′-TGA-CAAGCAGGAGAGTAA-3′, 5′-GAGTAAGAACAGGACGCAA-3′, 5′-AGCGGATGATGTAGAGAT-3′; the PCR cycling was: 1 min denaturation at 96°C, 2 min annealing at 50°C and 3 min elongation at 72°C using Expand Long Template PCR system (Boehringer Mannheim). Labeled fragments were separated on 6% sequencing gels.

Acknowledgements

We thank Francis Stewart, Joan Betz and members of the Stunnenberg laboratory for continued discussions and critical reading of the manuscript, and thank Willem Klaassen for assistance. We thank P.Chambon and D.Engel for kindly providing the RXR and GATA antibodies, respectively, and thank J.P.Quivy for help with the LM–PCR procedure. P.C. was supported by an EU Institutional Fellowship; F.G.D. was supported by an EU TMR Fellowship.

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Received April 16, 1998; revised October 7, 1998; accepted October 21, 1998