In vivo repression of an erythroid-specific gene by distinct corepressor complexes

Luc E.G.Rietveld, Eric Caldenhoven and Hendrik G.Stunnenberg[1]

Department of Molecular Biology, NCMLS 191, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

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

L.E.G.Rietveld and E.Caldenhoven contributed equally to this work

To assess the mechanisms of repression of the erythroid-specific carbonic anhydrase II (*CAII*) locus we used chromatin immunoprecipitation and show that an NCoR–histone deacetylase (HDAC)3 complex is recruited by the nuclear receptor v-ErbA to the intronic HS2 enhancer turning it into a potent silencer. Furthermore we demonstrate that efficient *CAII* silencing requires binding of a MeCP2-targeted HDAC-containing corepressor complex to the hypermethylated CpG-island at the promoter. Activation of transcription by either AZAdC or thyroid hormone results in loss of one of the two corepressor complexes. Thyroid hormone further replaces the enhancer-bound NCoR–corepressor complex by the TRAP220 coactivator. Treatment with the HDAC inhibitor trichostatin A (TSA) causes activation of *CAII* transcription and histone H3 and H4 hyperacetylation at the enhancer, apparently without affecting binding of the two corepressor complexes. Unexpectedly, histone H3 and H4 at the fully repressed promoter are already hyperacetylated despite the close apposition of the MeCP2-targeted HDAC complex. Acetylation of histone H4, but not H3, at the promoter is moderately increased following TSA treatment. Our data suggest that the hyperacetylated but repressed *CAII* promoter is (partially) remodeled and primed for activation in v-ErbA-transformed cells.
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Introduction

Gene-specific regulation lies at the heart of cell differentiation in higher eukaryotes. Many genes in the human genome appear to be transiently active during differentiation and in small subsets of cells. This exquisite regulation is brought about by the selective assembly and disassembly of cell-type-specific transcription factor complexes, the enhanceosomes (reviewed in Merika and Thanos, 2001). Specificity is further determined by epigenetic events such as DNA methylation as well as by local remodeling of chromatin to permit or preclude binding of regulatory factors to nucleosomal DNA. In contrast to our biochemical understanding of the factors involved in gene activation and repression, *in vivo* data about gene regulation mediated by DNA-bound transcription factors and associated cofactor complexes remain scarce.

Nuclear hormone receptors provide a unique model to study the mechanisms of transcriptional activation as well as repression. Transcriptional activation by liganded nuclear hormone receptors is accomplished by recruitment of coactivator proteins, which contain intrinsic acetyltransferase activity. Coactivators that interact with nuclear receptors include p160-related factors and the acetyltransferases p300/CBP and PCAF (reviewed in Glass and Rosenfeld, 2000). A multiprotein complex, TRAP–Mediator (also referred to as ARC–DRIP–CRSP; reviewed by Malik and Roeder, 2000) has recently been shown to interact with liganded nuclear receptors (Fondell *et al.*, 1996; Näär *et al.*, 1999; Ranchez *et al.*, 1999). A single subunit, TRAP220, is thought to target the complex to the thyroid hormone receptor (TR) and other liganded nuclear hormone receptors through a LXXLL motif (Yuan *et al.*, 1998; Ito *et al.*, 1999). Chromatin immunoprecipitation (ChIP) experiments suggest that assembly of enhanceosome and coactivator complexes is ordered and dynamic (Agalioti *et al.*, 2000; Shang *et al.*, 2000).

Transcriptional repression by unliganded nuclear hormone receptors is thought to be brought about by recruitment of multi-subunit corepressor complexes containing intrinsic histone deacetylase (HDAC) activity. Most of our current knowledge on repression and corepressor proteins stems from *in vitro* binding studies. Two-hybrid analysis led to the identification of the nuclear receptor corepressor, NCoR (Horlein *et al.*, 1995), and its related family member, SMRT (Chen and Evans, 1995). The first *in vivo* proof for NCoR function in nuclear receptor action came from studies analyzing NCoR$^{-/-}$ mice which indicated that NCoR was required for active repression by nuclear receptors and other repressors (Jepsen *et al.*, 2000). The biochemical identification of multiple NCoR-containing complexes (Guenther *et al.*, 2000; Li *et al.*, 2000; Underhill *et al.*, 2000; Jones *et al.*, 2001) posed the question as to which of these complexes are involved in nuclear hormone receptor functioning *in vivo*.

Recent studies indicate that methylation is another important mechanism of gene silencing in cancer (Costello *et al.*, 2000). Although numerous studies suggested the involvement of methyl-binding proteins such as MeCP2 in gene silencing by methylation, direct *in vivo* evidence is currently lacking. The methyl-CpG binding protein MeCP2 is the founding member of a family of proteins that contain homologous methyl-CpG-binding domains (MBDs) (reviewed by Wade, 2001). The recent discovery that MBD family members reside in distinct complexes containing HDACs and other unique as well as common

subunits provided a link between DNA methylation, histone deacetylation and gene silencing.

Our current understanding of how corepressor and coactivator complexes associate with nuclear receptors *in vivo* on chromatin and exert their regulatory function is far from complete. To unravel these questions, we used the chicken erythroleukemia cell line, HD3, which is transformed by the avian erythroblastosis virus (AEV). AEV encodes two co-operating oncoproteins: v-ErbA, a mutated thyroid hormone receptor (Sap *et al.*, 1986) and v-ErbB, a constitutively active EGF–TGF-α receptor tyrosine kinase (Downward *et al.*, 1984). Notwithstanding extensive studies in the past decade, the molecular mechanisms by which v-ErbA deploys its oncogenic potential remain rather vague. It seems clear however that v-ErbA contributes to the leukemogenic phenotype by silencing erythroid-specific genes such as the *lysozyme* gene and the *carbonic anhydrase II* gene (*CAII*) (Zenke *et al.*, 1988; Baniahmad *et al.*, 1990; Pain *et al.*, 1990). We recently identified a v-ErbA responsive element (VRE) in the hypersensitive 2 (HS2) enhancer in intron 2 of the *CAII* gene. The VRE is adjacent to two GATA-1 sites (Ciana *et al.*, 1998; Braliou *et al.*, 2001). Mutation of the VRE unleashed the enhancer function resulting in a very marked increase in transcription (Braliou *et al.*, 2001; Rietveld *et al.*, 2001).

In this study, we used *in vitro* and *in vivo* approaches to assess the identity of the proteins associated with the *CAII* promoter and HS2 enhancer in its silent state. Using ChIP experiments, we show that v-ErbA and GATA-1 cohabitate the HS2 enhancer and that recruitment of a NCoR–HDAC3 corepressor complex by v-ErbA turns the erythroid enhancer into a silencer. Efficient silencing of the *CAII* gene further involves binding of a MeCP2-targeted HDAC-containing corepressor complex to the promoter. Dissociation of one of the corepressor complexes through the addition of either the methylation inhibitor AZAdC or thyroid hormone suffices to activate *CAII* transcription. Furthermore, we show that the HDAC inhibitor trichostatin A (TSA) activates *CAII* transcription and induces histone hyperacetylation at the HS2 enhancer without affecting corepressor binding. The repressed *CAII* promoter is hyperacetylated despite the presence of HDAC-containing complexes, suggesting that histone H3 and H4 at repressed promoter are not necessarily hypoacetylated.

Results

Association of a corepressor complex with v-ErbA *in vitro* and *in vivo*

Recently we have shown that the HS2 region within the *CAII* gene contains a binding site for v-ErbA as well as two functional GATA-1 binding sites (Figure 1A) (Ciana *et al.*, 1998; Braliou *et al.*, 2001). In this study, we set out to unravel the factors and mechanism involved in repression of the *CAII* gene by v-ErbA in HD3 cells. To identify corepressor components associated with v-ErbA in HD3 cells, immunoprecipitations were performed using an antibody directed against the gag moiety of v-ErbA (1G10). Western blot analysis of the precipitates revealed that the 270 kDa NCoR protein coprecipitated with v-ErbA (Figure 1B). The histone deacetylase HDAC3
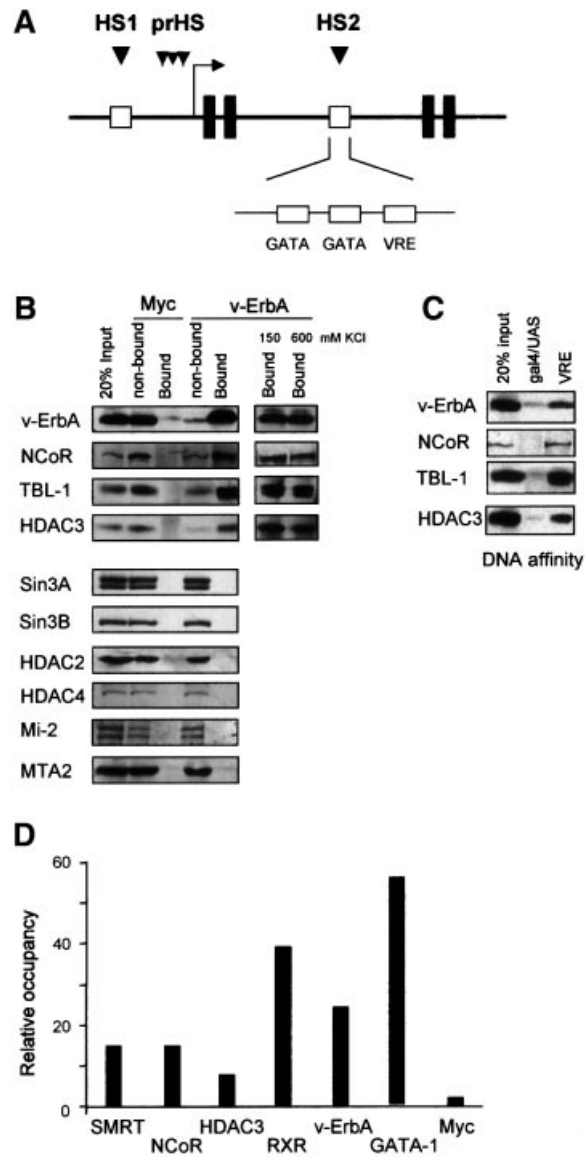

Fig. 1. v-ErbA recruits a specific corepressor complex. (**A**) Schematic depiction of the *CAII* genomic locus. Black boxes indicate exons 1–4. DNase I hypersensitive sites at the promoter (prHS) are indicated, as well as HS1 and HS2 (open boxes). At the HS2 region, protected regions in *in vivo* footprint experiments are indicated, these are two putative GATA-1 binding sites and a VRE. (**B**) Coupled co-immunoprecipitation–western blot experiment with v-ErbA antibody (1G10) showing association of the NCoR–HDAC3–TBL-1 proteins with v-ErbA but not with a control myc antibody. (**C**) DNA-affinity assay showing specific association of NCoR–TBL-1–HDAC3 with v-ErbA. DNA-affinity experiments were performed with specific oligomerized VRE and Gal4 oligonucleotides. (**D**) ChIP experiment performed with the indicated antibodies. The recovered DNA was quantified in real-time PCR with a primer set for the *CAII* HS2 region. The signal was compared with an unrelated control region, located 15 kb upstream of the HS2 and 7 kb upstream of the start site. This control signal was arbitrarily set at 1 and HS2 signal was calculated compared to this.

and the transducin β-like 1 protein (TBL-1) also efficiently co-immunoprecipitated with v-ErbA. The interaction between NCoR–HDAC3–TBL-1 complex and v-ErbA resisted stringent washing with buffers containing up to 600 mM KCl and 0.25% nonident P-40 (NP-40).

In contrast to a number of other studies (Heinzel *et al.*, 1997; Lee *et al.*, 2000; Underhill *et al.*, 2000) we found no

association of v-ErbA with Sin3A/B, HDAC2 or the class II histone deacetylase HDAC4 (Huang *et al.*, 2000; Kao *et al.*, 2000) (Figure 1B). Furthermore, association between v-ErbA and components of the NuRD complex such as the ATPase Mi-2 or the metastasis-associated protein MTA2 (Wade *et al.*, 1999; Zhang *et al.*, 1999) could not be detected (Figure 1B).

To obtain additional evidence for the association between v-ErbA and the NCoR–SMRT complex, a DNA affinity resin bearing oligomerized oligonucleotides containing the HS2–VRE was used. Figure 1C shows that VRE-bound v-ErbA associated with NCoR, HDAC3 and TBL-1, whereas these proteins are not bound to the unrelated Gal4 DNA binding site. Notably, components of the Sin3 and NuRD complexes could not be detected in these experiments (data not shown).

The outcome of our biochemical analysis in HD3 cells reveals that v-ErbA recruits a corepressor complex containing HDAC3. To show that this corepressor complex is targeted to the HS2 enhancer *in vivo*, we conducted ChIP experiments. Chromatin was purified from proliferating HD3 cells and ChIP assays were performed using the 1G10 antibody that is specific for the gag moiety of v-ErbA. This analysis revealed association of v-ErbA with the HS2 enhancer, but not with an unrelated control region, *in vivo* in HD3 cells (Figure 1D). An antibody directed against the heterodimeric partner RXR of v-ErbA efficiently precipitated the HS2 region indicating that RXR co-occupies the HS2 with v-ErbA. Antibodies against the GATA-1 transcription factor specifically precipitated the HS2 enhancer in HD3 cells that do not express *CAII*. The HS2 region was also efficiently precipitated with antibodies directed against NCoR, its close relative SMRT, and HDAC3 (Figure 1D), indicating the *in vivo* association of a v-ErbA–NCoR–HDAC3 complex with the HS2 region in HD3 cells. An unrelated anti-myc antibody did not specifically precipitate the HS2 regulatory region.

The ChIP experiments along with the previously published *in vivo* footprinting data (Ciana *et al.*, 1998) show that v-ErbA as well as GATA-1 are bound to the HS2 enhancer *in vivo* in proliferating HD3 cells that do not transcribe *CAII*. These data corroborate and extend the notion that in proliferating HD3 cells, v-ErbA nullifies the positive transcriptional activity of GATA factors (Ciana *et al.*, 1998; Braliou *et al.*, 2001). Our findings thus provide evidence for the *in vivo* association of an NCoR–corepressor complex to a presumed endogenous target gene by an unliganded nuclear hormone receptor.

MeCP2-instigated repression at the CAII promoter

Previous as well as unpublished experiments (Ciana *et al.*, 1998; data not shown) suggested that apart from repression instigated by v-ErbA, additional mechanism(s) involving HDACs contribute to *CAII* gene silencing. When carefully inspecting the *CAII* promoter region, we found that it is rather GC rich (82%) and that the CpG:GpC ratio in this region is 1.0, meeting the criteria for a CpG island (Gardiner-Garden and Frommer, 1987). Bisulfite sequencing revealed that the *CAII* promoter contains a high frequency (56 ± 8%) of methylated CpG dinucleotides in the region between –400 and –200 (data not shown). Analyzing different clones (*n* = 15) we found that the
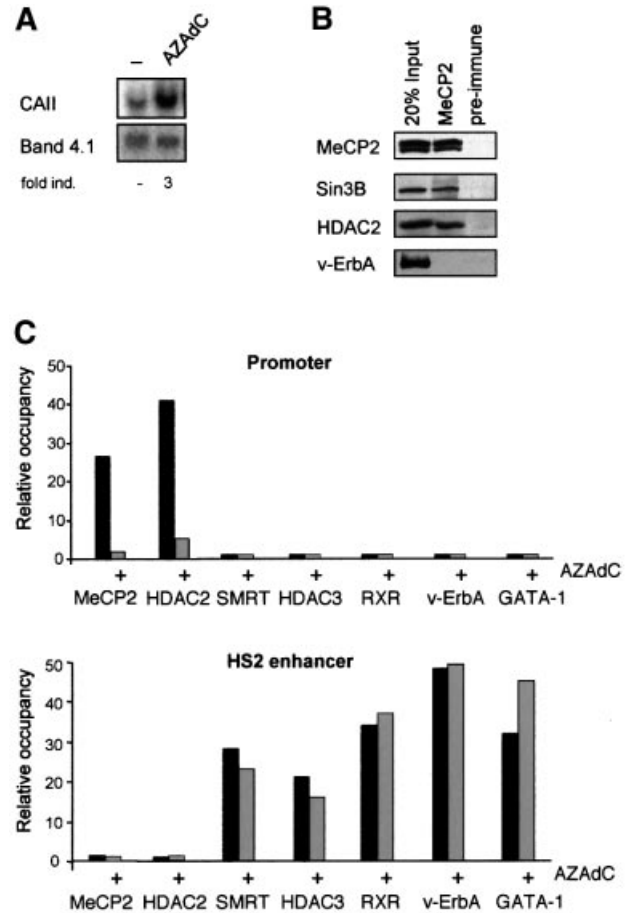

Fig. 2. The hypermethylated *CAII* promoter CpG island binds the MeCP2–corepressor complex *in vivo*. (**A**) Northern blot showing activation of *CAII* expression by treatment with the methylation inhibitor AZAdC (48 h, 500 nM) in HD3 cells. Band 4.1 serves as loading control. (**B**) Coupled co-immunoprecipitation–western blotting experiment with MeCP2 antibody showing association of the Sin3B–HDAC2 proteins with MeCP2, but not with preimmune serum. (**C**) ChIP experiment performed with indicated antibodies on chromatin from untreated HD3 cells and from cells treated for 48 h with AZAdC (500 nM). Recovered DNA was quantified using real-time PCR with control-, HS2- and promoter spanning primer sets. The background control region 7 kb upstream of the *CAII* promoter is used as control for which the PCR signal is arbitrarily set to 1 and signals were calculated compared to this.

methylation pattern was heterogeneous and disperse, and a consistent pattern of methylated CpG residues could not be observed. Direct involvement of methylation in *CAII* repression could be shown by treatment of HD3 cells with the methylation inhibitor AZAdC for 48 h, which efficiently activated *CAII* transcription (Figure 2A). Following treatment, the *CAII* promoter region was significantly demethylated (~50%) compared with untreated cells (data not shown).

Gene silencing by methylation reportedly involves binding of methyl-CpG binding proteins that recruit HDAC activity (Jones *et al.*, 1998; Nan *et al.*, 1998). Hence, the role of methyl-binding proteins in *CAII* repression was assessed. The methyl-binding protein MeCP2 appeared to be expressed at relatively high levels in HD3 cells. In co-immunoprecipitation experiments with MeCP2 antibody, HDAC2 and Sin3B proteins

copurified with the MeCP2 protein, indicating that MeCP2 recruited a Sin3–HDAC complex from HD3 cell extracts (Figure 2B). Furthermore, a Sin3B antibody efficiently co-immunoprecipitated MeCP2 from HD3 cells (data not shown).

ChIP assays were used to assess whether MeCP2 binds to the methylated promoter region *in vivo* and recruits a HDAC-containing complex to the CpG island in the *CAII* promoter. Antibodies against MeCP2 or HDAC2 specific-ally immunoprecipitated the *CAII* CpG island when using chromatin preparations derived from HD3 cells (Figure 2C). Treatment with AZAdC dislodged MeCP2 and HDAC2 from the promoter without significantly affecting binding of the factors bound to the HS2 enhancer (Figure 2C). Thus, the hypermethylated promoter recruits a MeCP2–HDAC2-containing corepressor complex that contributes to *CAII* repression in proliferating HD3 cells.

CAII activation by thyroid hormone treatment

The generally accepted 'on–off' model for repression and activation by nuclear receptors implies that ligand binding causes a conformational change in the ligand-binding domain resulting in dissociation of corepressor and subsequent association of coactivator proteins (reviewed in Glass and Rosenfeld, 2000). To visualize the 'on–off' switch, we turned to a HD3-derived cell line, HD3-V3, which expresses a ligand-responsive TR fused to the retroviral gag moiety (gag-c-ErbA) at levels equivalent to that of v-ErbA. Our previous studies had shown that addition of thyroid hormone (T3) triggers differentiation of these erythroblasts with a concomitant activation of *CAII* transcription (Disela *et al.*, 1991). HD3-V3 cells grown in hormone-depleted medium express low levels of *CAII* mRNA and addition of T3 boosts the levels of *CAII* mRNA notwithstanding the continued presence of the ligand-unresponsive, constitutive repressor v-ErbA in these cells (Ciana *et al.*, 1998; Figure 3A).

To identify factors recruited to the HS2 enhancer upon T3 activation, DNA-affinity resins were used to isolate interacting factors in nuclear extracts from HD3-V3 cells prepared before and after ligand treatment. Western blot analysis revealed the binding of roughly equal amounts of gag-c-ErbA and v-ErbA to the DNA beads using T3-treated and untreated extracts. Furthermore, the NCoR/HDAC3/TBL-1 corepressor proteins co-purified effi-ciently via gag-c-/v-ErbA from extracts prepared from untreated cells (Figure 3B). Using extracts prepared from HD3-V3 cells grown in the presence of T3, binding of NCoR, HDAC3 and TBL-1 to the DNA-affinity resin was significantly diminished. In contrast, the coactivator protein TRAP220, a component of the TRAP–Mediator complex (Yuan *et al.*, 1998) could be recovered. Sur-prisingly, SRC-1, another coactivator reported to interact with liganded TR (Onate *et al.*, 1995), did not bind to ligand-activated gag-c-ErbA although it could readily be detected in HD3-V3 extracts. Similar analyses using HD3 cell extracts (lacking a ligand responsive TR receptor) revealed that v-ErbA was unable to recruit either SRC-1 or TRAP220 (data not shown), in line with the fact that v-ErbA is unable to bind ligand.

To provide proof for the ligand-dependent recruitment of TRAP220, ChIP experiments were performed with chromatin preparations from untreated and T3-treated
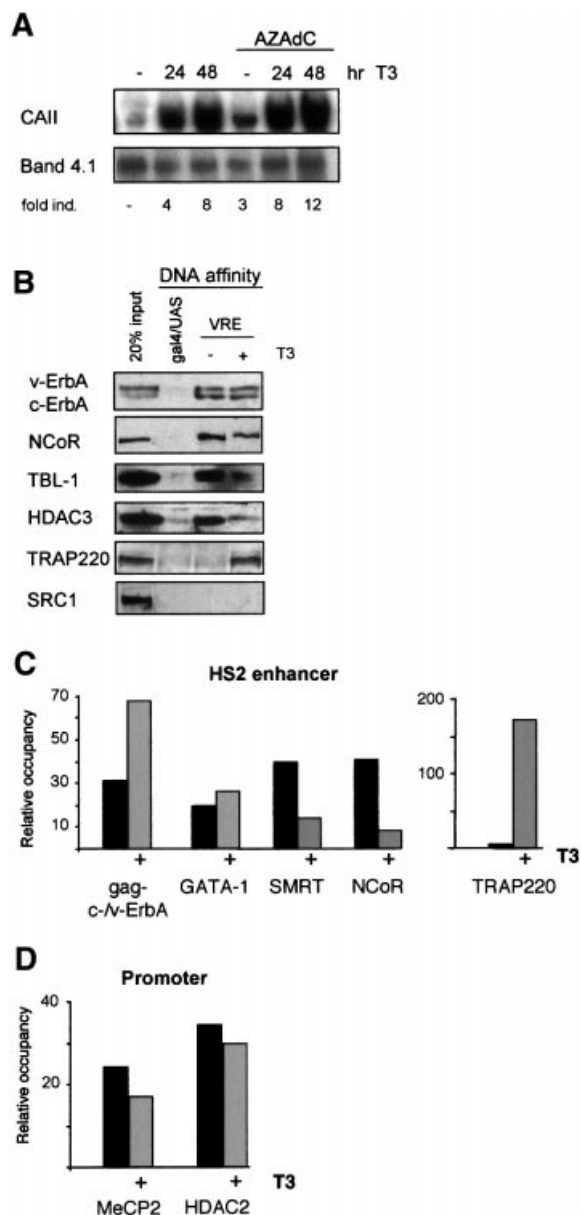

Fig. 3. Thyroid hormone-induced *CAII* activation induces cofactor exchange at the HS2 enhancer region. (**A**) Northern blot showing activation of *CAII* transcription in HD3-V3 cells treated with T3 (300 nM), AZAdC (500 nM) or both. Band 4.1 served as a control. (**B**) DNA-affinity pull-down experiment with oligomerized VRE oligonucleotides in extracts from HD3-V3 cells either untreated or stimulated for 24 h with 300 nM T3. Western blot analysis shows the association/release of indicated corepressor/coactivator subunits with respect to c-/v-ErbA bound to the VRE. (**C**) HS2 enhancer ChIP experiment with soluble chromatin isolated from untreated and T3-treated (24 h, 300 nM) HD3-V3 cells using antibodies directed against the indicated proteins. HS2 PCR-signals were quantified as in Figure 1D. (**D**) as (C), but primer sets that amplify the promoter region were used to quantify the recovered DNA.

HD3-V3 cells. Ligand addition caused a 2-fold increase in the occupancy of the HS2 by gag-c-/v-ErbA (Figure 3C). The relative occupancy of the HS2 by GATA-1 was not affected by ligand treatment of the cells. A 4- to 5-fold decrease in the binding of NCoR and SMRT to the HS2 region was observed after the addition of ligand. Note that v-ErbA, expressed to similar levels as gag-c-ErbA, is unable to respond to ligand and will sustain low level
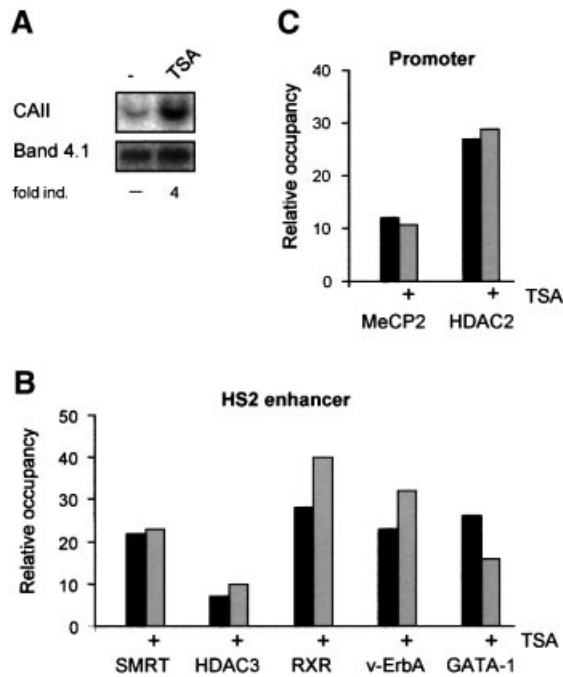
Fig. 4. Corepressor binding after TSA treatment. (**A**) Northern blot showing activation of *CAII* expression by treatment of HD3 cells with TSA (24 h, 100 nM). Band 4.1 served as a loading control. (**B**) ChIP experiments with indicated antibodies, using chromatin isolated from untreated and TSA-treated HD3 cells. The recovered DNA was quantified in real-time PCR as in Figure 1D. (**C**) as (B), but primer sets that amplify the promoter region were used to quantify the recovered DNA.

Fig. 5. The promoter of the repressed *CAII* gene is hyperacetylated. (**A**) ChIP experiment with acetylated H3 and H4 antibodies. The ChIP input signal was set at 100% and all other signals were calculated compared to this signal. (**B**) as (A), but chromatin from untreated and AZAdC-treated HD3 cells was used.

recruitment of NCoR–SMRT complexes even in the presence of T3. Using a TRAP220-specific antibody, a strong increase in TRAP220 occupancy of the HS2 enhancer (up to 150-fold) could be observed in T3-treated compared with untreated cells (Figure 3C). Consistent with the *in vitro* data we could not find evidence for the recruitment of the p160-coactivators SRC-1 or AIB1 to ligand-bound gag-c-ErbA in ChIP experiments (data not shown).

An intriguing question is whether the MeCP2–HDAC2 complex that binds the hypermethylated CpG island remained associated upon ligand addition. ChIP experiments did not reveal significant differences in the association of MeCP2 and HDAC2 with the CpG island before or after ligand administration (Figure 3D). This suggests that the MeCP2–HDAC2 complex at the promoter might continue to repress transcription in the presence of T3, an interpretation that is supported by northern blot analyses; addition of T3 plus AZAdC activated *CAII* transcription in an additive manner (Figure 3A). Thus, T3 and AZAdC activate *CAII* transcription via distinct and independent pathways.

Taken together, the data show that T3 addition triggered the 'on–off' switch, i.e. dislodging the NCoR–SMRT-containing corepressor complex and recruiting a TRAP220-containing coactivator complex to the enhancer.

The repressed CAII promoter is hyperacetylated despite corepressor binding

The HDAC inhibitor TSA efficiently activates transcription from the *CAII* gene in HD3 cells (Figure 4A; Ciana
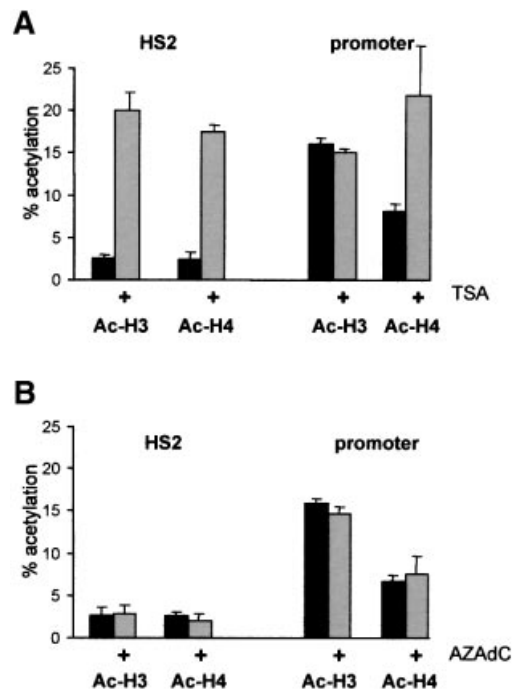
et al., 1998). To investigate whether *CAII* activation by TSA affects binding of corepressor complexes at the enhancer and promoter, ChIP assays were performed using chromatin isolated from untreated and TSA-treated HD3 cells. Figure 4B shows that TSA did not significantly affect binding of GATA-1, v-ErbA, RXR or the NCoR–SMRT complex to the HS2 enhancer region. Furthermore, TSA did not affect promoter methylation and binding of MeCP2–HDAC2 to the promoter region (Figure 4C; data not shown).

To assess the pattern of histone acetylation–deacetylation in the *CAII* locus, ChIP assays were performed with antibodies directed against the acetylated tails of histones H3 and H4. Nucleosomes at the HS2 enhancer were hypoacetylated in proliferating HD3 cells and TSA treatment caused a 7- to 8-fold increase in H3- and H4-acetylation consistent with the inactivation of the HDAC activity associated with the NCoR–SMRT complex (Figure 5A). Note that the percentage of histone acetylation is plotted in Figure 5A. The relative concentration of *CAII* promoter fragments present in CsCl-purified chromatin before immunoprecipitation was arbitrarily set to 100%. Surprisingly, histone H3 and H4 associated with the start site region appear to be hyperacetylated in proliferating HD3 cells where the *CAII* promoter is repressed. Acetylation of histones in the promoter region is generally accepted as a hallmark of an active promoter. TSA treatment did not increase further the acetylated state of histone H3 associated with the start site whereas acetylation of histone H4 was elevated 2-fold (Figure 5A). Thus, the *CAII* promoter appears to be hyperacetylated despite the presence of a HDAC-containing MeCP2 complex in close proximity to the start site of transcription.

To investigate whether dissociation of the MeCP2–HDAC2 complex from the promoter affected the acetylation state of nucleosomes at the promoter and at the enhancer, HD3 cells were treated with the methylation inhibitor AZAdC. The MeCP2–HDAC2 complex dissociated from the promoter following AZAdC treatment (Figure 2C) but this did not significantly alter the level of acetylation of histone H3 and H4, neither at the promoter nor at the enhancer (Figure 5B). These results suggest that increasing histone acetylation at the enhancer is not a prerequisite for derepression of the *CAII* gene.

Taken together, the repressed *CAII* promoter is hyperacetylated despite the association of HDAC2 protein at the CpG island. Furthermore, dissociation of the MeCP2 complex did not alter the extent of acetylation at the promoter. In contrast, TSA caused extensive histone acetylation at the HS2 enhancer in accordance with the inactivation of HDAC3 present at this transcription control region.

Discussion

The *CAII* locus has been extensively studied in our lab and this resulted in the identification and characterization of the DNase hypersensitive region HS2 in the *CAII* locus (Ciana *et al*., 1998). Here we show that v-ErbA represses transcription by recruitment of a specific corepressor complex to the HS2 enhancer *in vivo* and *in vitro*. Furthermore, the promoter binds a methylation-dependent MeCP2–corepressor complex. Recruitment of multiple specific HDAC-containing complexes efficiently silences transcription of *CAII*. Thus, this study provides *in vivo* evidence for a role in corepressor complexes in the regulation of a natural chromosomal target gene.

Repression instigated at the HS2 enhancer

To unravel the molecular mechanisms involved in repression by v-ErbA, we have used *in vitro* as well as *in vivo* approaches to identify the (co)factors involved in *CAII* repression. Using immunoprecipitation and DNA affinity resins, we show that v-ErbA recruits a corepressor complex containing NCoR–SMRT, HDAC3 and TBL-1. TBL-1 is related to the Tup1 and Groucho corepressors with respect to its WD40 domain that has the potential to interact with histone H3 *in vitro*. TBL-1 might therefore serve to bring HDAC3 to chromatin thereby facilitating histone deacetylation (Guenther *et al*., 2000). Several NCoR-containing corepressor complexes have been identified which possess HDAC activity but display distinct protein subunit compositions (Guenther *et al*., 2000; Li *et al*., 2000; Underhill *et al*., 2000; Urnov *et al*., 2000; Wen *et al*., 2000; Jones *et al*., 2001). A number of studies implicated the Sin3–HDAC complex in repression by NCoR–SMRT (Heinzel *et al*., 1997; Lee *et al*., 2000). However, this and other studies (Urnov *et al*., 2000) did not provide evidence for the recruitment of a Sin3–HDAC complex by nuclear hormone receptors either *in vitro* or *in vivo*. Nonetheless, Sin3–HDAC complexes are present in HD3 erythroblasts and are recruited by MeCP2. Furthermore we have shown that neither the class II HDAC4 (Huang *et al*., 2000) nor subunits of the Mi2–NURD complex (Wade *et al*., 1999; Zhang *et al*., 1999) are recruited by v-ErbA.

ChIP experiments established that GATA-1 and v-ErbA–RXR are recruited to the HS2 enhancer *in vivo* in proliferating erythroblasts that express *CAII* at very low levels. Furthermore, a NCoR–HDAC3-containing corepressor complex is in turn recruited by v-ErbA to the HS2 enhancer corroborating and extending the notion that the enhancer activity of the HS2 governed by two adjacent GATA-1 sites is nullified by v-ErbA.

MeCP2- instigated repression at the CAII promoter region

Experiments involving AZAdC revealed that DNA methylation also contributes to repression of *CAII* transcription. AZAdC activated *CAII* expression and caused (partial) demethylation of the hypermethylated promoter CpG island. The methylation pattern within the *CAII* CpG island is disperse, which appears to be a feature common to repressed genes in a number of different cell lines (De Smet *et al*., 1999; Müller *et al*., 2000; Danam *et al*., 2001). Interestingly, preliminary studies indicate that the *CAII* promoter is hypomethylated in primary erythroid cells suggesting that methylation may be due to transformation by AEV or extensive culturing of the cells. Methylation of genes appears to be confined to tissue-specific genes but not housekeeping genes in various cultured cell lines (Antequera *et al*., 1990). The *CAII* gene is an erythroid-specific gene that may not be required in cell culture and hence may have become methylated during extensive culturing of the cells.

The mechanism by which methylation represses transcription has long been unclear but recent data suggested the involvement of MBDs that reside in distinct complexes. The MBDs all share a high homology in the methyl-binding domain with the founding member of this family, MeCP2 (reviewed by Bird and Wolffe, 1999; Ballestar and Wolffe, 2001). We have shown that MeCP2 is expressed at relatively high levels in HD3 cells. In our immunoprecipitation experiments, MeCP2 coprecipitated Sin3B and HDAC2 and the involvement of MeCP2–HDAC2 in *CAII* promoter binding *in vivo* was established using ChIP. As a single methylated CpG is able to bind MeCP2 (Nan *et al*., 1993), it is likely that multiple MeCP2 proteins bind to the promoter region and recruit HDAC-containing complexes.

Dynamic regulation at the CAII locus

Our data provide evidence for the involvement of minimally two distinct corepressor complexes in repression of the *CAII* gene. Furthermore we found that dislodging of only one of these complexes suffices to activate *CAII* transcription. In this study, *CAII* gene transcription has been initiated in different ways, either via the recruitment of coactivators (activation) or by release or inactivation of corepressors (derepression). Dissociation of the v-ErbA-bound corepressor complex from the enhancer could be accomplished by treatment of HD3-V3 cells with T3. We provide *in vivo* evidence that T3 activation caused a release of the NCoR–SMRT-containing complex from gag-c-ErbA bound to the HS2 enhancer *in vivo*. T3-activated gag-c-ErbA was shown to recruit the coactivator TRAP220 to the HS2 enhancer. TRAP220 anchors the TRAP complex, which is composed of >25 distinct polypeptides, to liganded TR (Fondell *et al*., 1996;

reviewed in Ito and Roeder, 2001). We did not find evidence for a role of the p160 coactivators SRC-1 and AIB1 in ligand activation instigated by gag-c-ErbA after 24 h of ligand administration, suggesting that p160 and TRAP coactivators do not bind simultaneously. However, the p160 coactivators might bind gag-c-ErbA shortly after hormone treatment as suggested by a model in which coactivators are sequentially recruited (Glass and Rosenfeld, 2000). Ligand administration did not affect association of the MeCP2–corepressor complex with the promoter, indicating that the HS2 enhancer is able to partially overcome repression instigated at the CpG island.

AZAdC treatment caused demethylation and release of the promoter-bound MeCP2–corepressor complex but binding of the enhancer-bound v-ErbA–corepressor complex was not affected. Interestingly, although AZAdC induced *CAII* transcription, acetylation of histone H3 and H4 associated with the promoter was not affected. Thus, dissociation of a closely positioned HDAC-enzymatic activity did not increase H3 and H4 histone acetylation at the *CAII* promoter. It might be that promoter-associated HDAC2 specifically targets histones H2A and H2B without affecting H3 and H4. Another possibility is that specific lysine residues are targeted by HDAC2. In this study antibodies directed against the di-acetylated H3 (K9/K14) and tetra-acetylated H4 tails that do not accurately discriminate between (de)acetylation of specific lysine residues were used. It will be of interest to use antibodies that can detect (de)acetylation of specific lysine residues at the histone tails. Deckert and Struhl (2001) already found that histone acetylation is differentially affected by repressor proteins. Furthermore, experiments in yeast using deletion strains for the HDA1 and RPD3 histone deacetylases show that these deacetylases target specific lysine residues in the H3 and H4 tails (Vogelauer *et al.*, 2000; Wu *et al.*, 2001). In cells from RETT syndrome patients that express a truncated MeCP2 protein in the absence of wild-type MeCP2 protein, an increase in the mono-acetylated histone isoform H4 K16 is observed, indicating that the MeCP2-associated corepressor complex targets specific lysine residues (Wan *et al.*, 2001). Moreover, recent data show that lysine or arginine methylation of histone tails might be important in transcriptional regulation (Nielsen *et al.*, 2001; Noma *et al.*, 2001). The MeCP2- and v-ErbA-targeted corepressor complexes might contain methylase proteins that regulate *CAII* transcription by histone methylation as has been shown for the Rb protein (Nielsen *et al.*, 2001).

How does TSA induce CAII transcription?

Whereas activation of *CAII* by AZAdC or thyroid hormone treatment coincides with the release of one corepressor complex from the *CAII* locus, addition of TSA did not alter the binding of these complexes to the locus. TSA treatment induced the anticipated hyperacetylation of histones H3 and H4 at the HS2 enhancer probably due to the inhibition of the activity of the HDAC3-containing corepressor complex. Remarkably, the promoter appears to be hyperacetylated even in its repressed state in untreated proliferating cells, suggesting that the promoter chromatin may be 'open' and primed for transcription. Similar phenomena have been reported for a number of genes in different organisms. For example, in the mouse

yolk sac, the inactive β-globin minor promoter is hyperacetylated (Forsberg *et al.*, 2000) and the promoter of the mouse MMTV-LTR was reported to have a relatively high level of acetylation when the gene is inactive (Sheldon *et al.*, 2001). Hyperacetylated histones are also associated with a selected number of other yeast promoters, suggesting that inactive promoters are not necessarily hypoacetylated (Deckert and Struhl, 2001). If histone acetylation is not the trigger for transcription activation at the repressed *CAII* promoter by TSA, the question arises as to what other mechanisms could be involved. An explanation might be that TSA affects the acetylation state of non-histone components, such as basal transcription factors. Recent data suggest that the activity of the basal transcription factors TFIIE and TFIIF may be regulated by acetylation (Imhof *et al.*, 1997). TSA treatment may also result in acetylation of GATA-1 associated with the HS2 enhancer and activation of this important erythroid-specific transcription factor (Boyes *et al.*, 1998). Interestingly, at the yeast PHO8 promoter, histone acetylation provides a signal for the remodeling of chromatin by SWI–SNF (Reinke *et al.*, 2001). PHO8 promoter acetylation is transient, since acetylation levels at the repressed and activated PHO8 promoter appear to be similar. Thus, histone acetylation might precede activation of a gene, 'priming' it for expression. The acetylated state of the *CAII* promoter might reflect this intermediate state of the promoter, which is still repressed but primed for activation.

Detailed analysis of the mechanisms by which HDAC inhibitors such as TSA activate transcription will be required to provide more insights for effective use of these inhibitors for cancer treatment.

Materials and methods

ChIP and real-time PCR

Chromatin isolation was performed according to Orlando *et al.* (1997). Approximately 3×10^8 HD3 cells were grown in 100 cm^2 dishes, cross-linked by the addition of formaldehyde (1% final concentration), sonicated and chromatin was isolated by CsCl gradients. The equivalent of ~10^7 cells were used for one immunoprecipitation reaction. For chromatin immunoprecipitations, 200 μl chromatin samples were diluted 2-fold in IP buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 150 mM NaCl and protease inhibitors) and precleared with 70 μl of a 50% protein A/G–Sepharose slurry (Santa Cruz) containing 10 μg sonicated salmon sperm DNA and 1 mg/ml bovine serum albumin (BSA) for 1 h with agitation at 4°C. Precleared chromatin was incubated with antibodies overnight at 4°C after which 50 μl of a 50% protein A/G–Sepharose slurry was added and immunocomplexes were recovered. Immunoprecipitates were washed five times subsequently with low salt buffer (as IP buffer), twice with high salt buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 500 mM NaCl and protease inhibitors) and twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted twice from the beads by adding 200 μl 1% SDS in 0.1 M NaHCO$_3$ at room temperature for 15 min. For the reversal of cross-links, 16 μl 5 M NaCl was added and the samples were incubated at 65°C for 4 h, after which the samples were phenol–chloroform extracted and precipitated with 20 μg glycogen as carrier. Samples were dissolved in 50 μl TE.

The precipitated DNA was quantified by real-time PCR with the GeneAmp 5700 Sequence Detection System (PE Biosystems) using the SYBR Green I kit (PE Biosystems). Unless indicated otherwise, a PCR with a background control primer set (NC3/4) was performed which was used as control and was arbitrarily set to 1. All other PCR signals were calculated compared to this. Furthermore, total DNA from the IP input was included in the PCR and all PCR signals were corrected for this. Oligonucleotides used for ChIP analysis: *CAII* HS2: HS7,

5′-TCTGGAACATCCTTGCTA-3′; 3P, 5′-AGCGGATGATGTAGA-GAT-3′; background control: NC3, 5′-GCAGACACTGGCAGGTTTC-3′; NC4, 5′-TATGAGCCTTAGCCTTAG-3′; promoter: 7A, 5′-CGTGC-CCCGCGCACGGAG-3′; 8, 5′-GGCGGGGGGGCAAGAGGCG-3′.

Cell culture

Two derivatives of the AEV-transformed cell line HD3, namely HD3-EpoR and HD3-V3, expressing, respectively, the murine erythropoietin receptor or a gag-chicken TR fusion, were used. Before T3 treatment, HD3-V3 cells were grown for 48 h in medium containing stripped serum; 300 nM T3 was added to the medium where indicated.

Northern blot

Total RNA was extracted using the guanidium–CsCl method; *CAII* and Band 4.1 mRNA levels were detected by northern blot analysis as previously described (Zenke *et al.*, 1990).

Antibodies

The following antibodies were used: 1G10 antibody against v-ErbA, Myc antibody 1-9E10.2 (ascites hybridoma cells ATCC CRL-1729). The polyclonal antibodies NCoR (C-20), HDAC1 (H51), HDAC2 (H54), Sin3A (AK11), Sin3B (AK12), SRC1 (M-341), TRAP220 (M-255) and RXR (ΔN197) were from Santa Cruz. The polyclonal antibodies against ac-H3 (06-599) and ac-H4 (06-598) were obtained from Upstate Biotechnology. Other antibodies used: polyclonal antibody against NCoR (provided by J.Torchia), polyclonal antibodies against MeCP2 (provided by A.Wolffe), monoclonal antibody against GATA-1 (provided by D.Engel), polyclonal antibody against HDAC4 (provided by T.Kouzarides), polyclonal antibody against MTA2 (provided by R.Brouwer), HDAC3 (provided by J.Wong). TBL-1 rabbit polyclonal antibody was raised against amino acids 1–251 of human TBL-1. The CHD4/Mi-2 antibodies are described in Xue *et al.* (1998). SMRT rabbit polyclonal antibody has been described in Li *et al.* (2000).

DNA-affinity binding assay

Double-stranded oligos Gal4: 5′-GATCGGAGGACAGTACTCCG-3′, VRE: 5′-TCGACCCAGCAAGGTCACAGCAGGGCTTTTTTTC-3′. Oligos were multimerized and end-labeled using the large Klenow fragment of *Escherichia coli* DNA polymerase and biotin-11-dCTP (Gibco-BRL), followed by purification on a spin column. Double-stranded oligo (100 μg) was incubated with 1 mg streptavidin–agarose beads (Sigma) in WB1000 (1 M NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol) buffer for 30 min at room temperature. Beads were washed twice with WB1000 and three times with WB150 [150 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 1× protease inhibitor mix, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/μl BSA]. Diluted nuclear extracts (150 mM NaCl) were incubated with 10 μg bio-oligo coupled to streptavidine–agarose beads for 2 h at 4°C on a rotating wheel. Bound proteins were washed five times with buffer WB150, boiled in 1× sample buffer and subjected to immunoblotting.

Immunoprecipitations

Nuclear extracts from HD3 or HD3-V3 cells were prepared according to Dignam *et al.* (1983). Diluted nuclear extracts (200 mM NaCl) were pre-cleared with protein A–agarose beads, the protein extract was then incubated with either anti-v-ErbA, anti-myc or anti-MeCP2 for 4 h at 4°C on a rotating wheel. Bound proteins were washed five times in buffer D (200 mM NaCl, 0.25% NP-40, 10 mM HEPES, 1 mM EDTA, protease inhibitors, 1 mM PMSF, 1 μg/μl BSA), boiled in 1× sample buffer and subjected to immunoblotting.

Acknowledgements

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