Transcriptional Regulation by v-ErbA, the Oncogenic Counterpart of Thyroid Hormone Receptor (TR)

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Ως εμεγαλυνθεις τα εργα Σου Κυριε! Παντα εν σοφια εποιησας

Ψαλμ 104 (103) :24

O Lord, how magnified are thy works! In wisdom have thou made them all

Psalm 104 (103):24

Contens

Abbreviations

Aa	amino acid
AEV	avian erythroblastosis virus
AEL	avian erythroleukemia
AF-2	activation function-2
AIB1	amplified in breast cancer 1
AP-1	activating protein-1
APL	acute promyelocytic leukemia
CA II	carbonic anhydrase II
CBP	CREB binding protein
CEF	chicken embryonic fibroblasts
CREB	cyclic AMP element binding protein
GRIP-1	glucocorticoid recptor interacting protein-1
CRSP	cofactor required for Sp1
DBD	DNA binding domain
DR	direct repeat
DRIP	VDR interacting proteins
EGFR	epidermal growth factor receptor
EKLF	erythroid krüppel like factor
EMSA	electrophoretic mobility shift assay
Epo	erythropoietin
ER	estrogen receptor
GR	glucocorticoid receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRE	hormone responsive element
HS	DNase I hypersensitive site
HSC	hematopoietic stem cell
LBD	ligand binding domain
LCR	locus control region
MAP kinase	microtubule associated protein
MEL	mouse erythroleukemia cells
MoMLV	molony murine leukemia virus
NAT	negative regulator of activated transcription
NCoR	nuclear receptor corepressor
NF-κB	nuclear factor-kappa B
NR	nuclear receptor
PAGE	polyacrylamide gel electrophoresis
P/CAF	p300/CBP associating factor
P/CIP	p300/CBP interacting protein
PIC	pol II transcription preinitiation complex
PKA	protein kinase A
PLZF	promyelocytic leukemia zinc finger
PML	promyelocytic leukemia
Pol II	RNA polymerase II
PPAR	peroxisome proliferator-activator receptor
PR	progesterone receptor
Pu	purine
RA	retinoic acid
RAC3	receptor-associated coactivator
RAR	retinoic acid receptor
Rpd3	reduced phosphate dependency
RXR	retinoid X receptor
SCF	stem cell factor
Sin3	Swi5p independent
SMCC	SRB/MED-containing cofactor complex
SMRT	silencing mediator for retinoic acid and thyroid receptors
SRC-1	steroid receptor coactivator 1
STAT	signal transducer and activator of transcription
T3	3,3',5-triiodo-$_L$-thyronine
TAF	TBP associated factor
TBP	TATA-box binding protein
td-359	transformation deficient 359

TFIIB	transcription factor for pol II, B
TGF	transforming growth factor
TIF2	transcriptional intermediary factor
TPA	12-O-tetradecanoylphorbol 13-acetate
TR	thyroid receptor
TRAM-1	thyroid hormone receptor activator molecule
TRAP	thyroid receptor associating proteins
TRE	thyroid responsive element
TSA	trichostatin A
TSH	thyroid stimulating hormone
VDR	vitamin D3 receptor
VRE	v-ErbA responsive element

Chapter 1

Introduction

Chapter 1

Introduction

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INTRODUCTION

1. Nuclear hormone receptors in disease

Nuclear hormone receptors (NRs) are transcriptions factors that play a major role in homeostasis, development and differentiation. Not surprisingly, malfunctioning forms of nuclear receptors underlie neoplasias as well as a variety of endocrine diseases. Generalised thyroid hormone resistance syndrome (TRS) and vitamin D-resistant rickets type II are caused by mutated nuclear hormone receptors (Hughes *et al.*, 1988; Nagaya *et al.*, 1992). Several types of breast and prostate cancer are associated with mutated nuclear receptors (Suzuki *et al.*, 1996; Tilley *et al.*, 1995) (reviewed in Tenbaum and Baniahmad, 1997). A glucocorticoid receptor (GR) mutant was detected in patients with Cushing syndrome (reviewed in Malchoff *et al.*, 1993). Malignancies of the hematopoietic tissue, such as human acute promyelocytic leukemias (APL) or avian erythroleukemia (AEL) are also correlated with translocation products or heavily mutated, malfunctioning NRs (Kakizuka *et al.*, 1991) (reviewed in He *et al.*, 1999; Stunnenberg *et al.*, 1999). The majority of the mutant receptors causing these diseases affect the balance between proliferation and differentiation of cells by interfering with the action of the normal receptor counterparts. However, the exact molecular mechanisms are not yet understood. The best studied leukemia is the avian erythroleukemia, which provides an excellent model system to unravel the molecular bases and the etiology of hematopoietic malignancies. A better understanding of the function(s) of v-ErbA, a causative agent of AEL, may lead to important findings that can be applied to other mutated transcription factors responsible for a variety of clinical situations.

2. Hematopoiesis and leukemia

In vertebrates, hematopoiesis or blood formation occurs in distinct phases and anatomic sites during development. The origin of blood is the hematopoietic stem cell (HSC) defined as a cell competent to sustain long term hematopoiesis in a recipient individual (Figure 1). In mammals and avians the HSC is located in the blood islands

of the yolk sac (reviewed in Orkin, 1996; Shivdasani and Orkin, 1996). Hematopoiesis occurs in two major steps: the primitive or embryonic and the definitive. Primitive hematopoiesis is a transient wave of cells emerging from mesoderm of the yolk sac. Definitive hematopoiesis is initiated in the aorta-gonad-mesonephros (AGM) region and the fetal liver, and after birth, it occurs in the bone marrow where stem cells are produced (Medvinsky and Dzierzak, 1996; de Bruijn *et al.*, 2000). The cells arising from HSC, called pluripotent stem cells, are critical for the production of all hematopoietic cells in vertebrates. Their unique feature is their ability to self-renew i.e. they have the capacity to proliferate for many generations without entering a differentiation pathway. More importantly, the pluripotent stem cells are able to keep a delicate balance between proliferation and differentiation; they maintain their population as well as generate appropriate numbers of more mature cells that will differentiate into various lineages to generate erythrocytes, myeloid cells or lymphocytes. The arising so-called multipotent cells retain the ability to proliferate, but their differentiation spectra become gradually restricted, leading to the development of committed progenitors. Committed progenitors have only limited self-renewal capacity and ultimately differentiate into mature cells of the various hematopoietic lineages (reviewed in Beug *et al.*, 1996; Keller, 1992; Orkin, 1996) (Figure 1).

Leukemia is manifested by disturbance of the delicate balance between proliferation and differentiation. In leukemia, differentiation of stem cells is limited and cells proliferate aberrantly. Interestingly, several studies have shown that this misbalance can occur in all cell stages i.e. in pluripotent, multipotent and committed hematopoietic progenitors. All these cells, and in particular the committed progenitors, differentiate during their proliferation. Conceivably, it is thought that leukemia-associated mutated proteins function in such a way that they promote the ability of the progenitors to divide without further progress along the differentiation pathway (reviewed in Beug *et al.*, 1996).

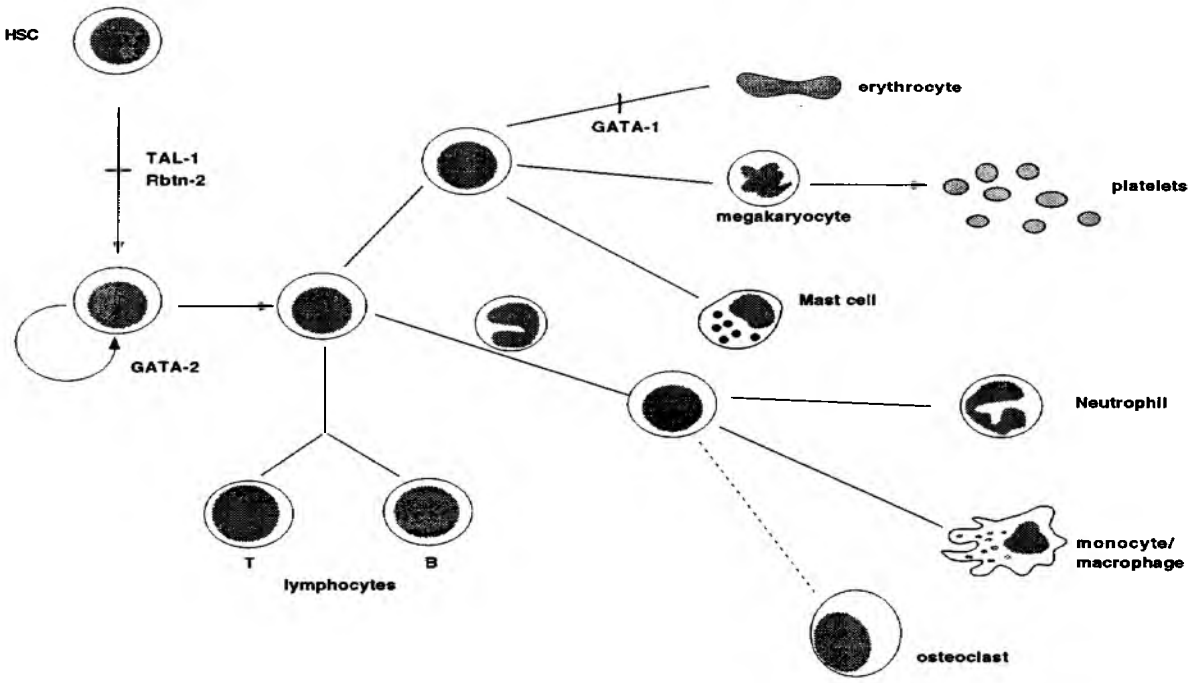


Figure 1 Schema depicting the stages where selected transcription factors exhibit essential functions in erythroid differentiation of the hematopoietic stem cell (HSC). Positioning is based on the earliest block in differentiation resulting from absence of the designated factor. Only factors with a demonstrated role in hematopoietic development (as suggested by interference with cell-specific differentiation in knockout mice) are included. Adapted from (Shivdasani and Orkin, 1996).

3. Factors involved in hematopoiesis

3.1. Extracellular signals

The fate of a given cell depends on the signals that it receives as well as on its unique response to those signals. A number of polypeptide growth and differentiation factors transfer the extracellular signal into the cell and ultimately regulate the activity of several transcription factors. Factors specific for the hematopoietic tissue include erythropoietin (Epo), transforming growth factors (TGFα and TGFβ), stem cell factor (SCF) and interleukines (ILs). These factors transmit their signal via binding to their cognate cellular receptors, which are mainly tyrosine kinases and interleukin receptors.

In the absence of Epo receptor, lineage commitment of erythroid progenitors occurs but terminal differentiation into definitive erythrocytes is not attained because the cells undergo apoptosis (Lin *et al.*, 1996). SCF, the ligand of c-Kit, regulates early hematopoiesis and provides short-term self-renewal capacity. SCF in collaboration with TGFα (the ligand for the tyrosine kinase receptor c-ErbB-1) can significantly prolong the self-renewal potential of erythroid progenitors even in the absence of Epo (Hayman *et al.*, 1993). The signal of both tyrosine kinases and interleukin receptors is mediated through multiple pathways that include Ras, MAP kinase and Jak/Stat pathways. Receptor signalling culminates in the modification of transcription factors leading to activation or repression of key target genes (Beug *et al.*, 1996). Consequently, ectopic expression of cytokines or constitutive activation of their receptors disturb the regulation of certain genes and are often implicated in leukemias (Li *et al.*, 1990; reviewed in McCubrey *et al.*, 2000; Touw *et al.*, 2000).

3.2. Transcription factors

Ensuing research on the mechanisms governing differentiation of hematopoiteic cells revealed a number of widely-expressed as well as cell-specific transcription factors. In the following section I will mainly focus on transcription factors that play a critical role in the differentiation of the HSC into erythrocytes (Figure 1).

Tal-1/SCL, a basic helix-loop-helix (bHLH) transcription factor, has been identified in translocation products in human acute T-cell leukemias. Tal-1/SCL is detected among early hematopoietic progenitors. Tal-1/SCL expression in *Xenopus* embryos precedes that of any other known hematopoietic-specific gene in the earliest sites of blood formation. It has also been suggested that Tal-1/SCL plays a role in late erythroid differentiation (reviewed in Shivdasani and Orkin, 1996). Like Tal-1/SCL, the Lim-domain nuclear protein rbtn2/Ttg2/LMO2 is critical for the early stages of differentiation of the hematopoietic tissue. The rbtn2/Ttg2/LMO2 protein is misregulated in human T-cell acute leukemia. The rbtn2/Ttg2/LMO2 and Tal-1/SCL knockout phenotypes are similar and mice die from anemia due to lack of erythrocytes (Warren *et al.*, 1994). The zinc-finger protein GATA-1 is a central regulator of erythroid-specific gene expression and differentiation, and binds to a GATA DNA

sequence. Within hematopoietic cells, expression of GATA-1 occurs in the erythroid, megakaryocyte, eosinophil, mast cell lineages and multipotent progenitors (Evans and Felsenfeld, 1989; Martin *et al.*, 1990; Romeo *et al.*, 1990). Erythroid precursors lacking GATA-1 show a maturation arrest at the proerythroblast stage suggesting a major role for GATA-1 in survival and terminal erythroid differentiation of these cells (Pevny *et al.*, 1995; Weiss *et al.*, 1994). GATA-1 negative embryonic stem (ES) cells fail to contribute to mature red blood cells, but are fully able to develop into other hematopoietic lineages or other tissues (Pevny *et al.*, 1991). Moreover, differences in the intracellular levels of GATA-1 have been shown to be critical for lineage selection and commitment within the hematopoietic cells (reviewed in Shivdasani and Orkin, 1996). GATA-2, another member of the GATA-family is also critical in the development of hematopoietic cells. It is detected in erythroid and other hematopoietic progenitors but its expression decreases as GATA-1 expression increases with erythroid differentiation (Yamamoto *et al.*, 1990). In chicken erythroid precursors, forced expression of GATA-2 but not GATA-1 or GATA-3 (another member of the GATA-family), promotes proliferation and blocks differentiation (Briegel *et al.*, 1993). An erythroid specific CACCC-binding protein designated erythroid Kruppel-like factor (EKLF) is expressed at all stages of erythropoiesis and is critical for the transcription of erythroid-expressed genes and particularly globins (Miller and Bieker, 1993; Nuez *et al.*, 1995; Perkins *et al.*, 1995). The transcription factor PU.1 has been suggested to play a critical role in lineage commitment (reviewed in Shivdasani and Orkin, 1996). The c-myb gene is highly expressed in progenitor cells of myeloid, erythroid and lymphoid lineages and is downregulated during maturation /differentiation of these cells. The AML-1 gene is frequently deregulated in leukemias. Both c-myb- and AML-1-lacking mice display normal primitive hematopoiesis, whereas definitive hematopoiesis is severely impaired (reviewed in Blobel, 2000). Thus, several transcription mechanisms with competing or synergistic activities operate to ultimately define lineage selection and hematopoietic development.

3.3. Translocation products and mutated nuclear receptors in leukemias

Of particular interest for this thesis are mutated nuclear receptors and chromosomal translocation products. Fusions of a wide variety of genes with markedly distinct functions are now known to be involved in leukemogenesis. For example, the fusion products of Bcr-Abl, Dek-Can, AML-1-ETO or MLL transclocated to a number of chromosomes are signal transducers, nucleoporins, transcription factors or putative chromatin regulators (Fornerod *et al.*, 1995; Kabarowski *et al.*, 1994; Miyoshi *et al.*, 1993; Ridge and Wiedemann, 1994). Among these translocation products, the retinoic acid receptor (RARα) coupled to other transcription factors yielding PML-RARα or PLZF-RARα block differentiation of hematopoietic progenitors. PML-RARα and PLZF-RARα are involved in nearly 80% of the human acute promyelocytic leukemias (APLs) (Alcalay *et al.*, 1991; Borrow *et al.*, 1990; Chen *et al.*, 1993b; de The *et al.*, 1990a; Grignani *et al.*, 1993; Kakizuka *et al.*, 1991; Rousselot *et al.*, 1994). Nuclear receptors carrying point mutations or deletions are also involved in malignant transformation of hematopoietic cells. Murine bone marrow cells expressing a dominant negative RARα lacking its ligand-dependent activation function-2 (AF-2) (see paragraph 4.2.2), are blocked at the stage of lymphohematopoietic progenitors (Tsai *et al.*, 1994; Tsai and Collins, 1993). v-ErbA, a viral variant of thyroid hormone receptor TRα produced by the Avian Erythroblastosis Virus (AEV), is a paradigm of a mutated nuclear receptor implicated in avian erythroleukemia (Graf and Beug, 1983).

The studies described in this thesis are focused on the role of v-ErbA in transcriptional regulation. In order to get more insight into the function of v-ErbA it is a prerequisite to understand in detail the principles that govern the physiological functions of TRα, the cellular counterpart of v-ErbA, and nuclear receptors in general.

4. Nuclear hormone receptors (NRs)

In 1913 a scientific report appeared claiming that 'certain lipids' are essential for growth (McCollum and Davis, 1913; Osborne and Mendel, 1913). Since then, a large number of small lipophilic molecules, referred to as hormones, have been

discovered to play an important role in cell differentiation, homeostasis, development and organ physiology. These hormones, including steroids, retinoids, thyroid hormones and vitamin D3, were shown to translocate to the nucleus to regulate transcription. A milestone in hormone research was the discovery that these lipophilic molecules bind to their cognate nuclear hormone receptors. Consequently, the receptors undergo a conformational change and bind with high affinity to specific sites in chromatin to regulate transcription (reviewed in Mangelsdorf *et al.*, 1995).

4.1. Classification of nuclear hormone receptors

The cloning of the glucocorticoid receptor (GR) and the estrogen receptor (ER), and the realisation that they were highly related to the protein product of the v-ErbA oncogene suggested that these proteins belong to the same family of transcription factors dubbed nuclear receptors (NR) (Evans, 1988; Green *et al.*, 1986; Hollenberg *et al.*, 1985; Miesfeld *et al.*, 1984; Weinberger *et al.*, 1985). At present, this family has grown to greater than 200 members. For the purpose of this thesis I will follow the classification of NRs based on the dimerisation and the DNA binding properties as proposed by Stunnenberg (reviewed in Stunnenberg, 1993). Accordingly, NRs are classified in three different subfamilies (Figure 2A): Class I includes the classical steroid receptors such as GR, ER, progesterone receptor (PR), mineralocorticoid receptor (MR) and androgen receptor (AR). Class I NRs form ligand induced homodimers and bind to hexameric DNA motifs, called half-sites, arranged as palindromic repeats (Figure 2A, B). Class II NRs, including TR, RAR, vitamin D3 receptor (VDR), ecdysone receptor (EcR) and peroxisome proliferator-activator receptor (PPAR) heterodimerise with retinoid X receptor (RXR), which is itself a member of class II. Unlike class I NRs, class II receptors predominantly recognise DNA half-site motifs arranged as direct repeats (Figure 2A, B). Class III comprises, amongst many others, ROR, chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and Rev-Erb that bind to direct repeats as homodimers or as monomers to extended DNA half-sites. The Class III NRs are also arbitrarily referred to as orphan receptors because either they are not likely to have ligand or their ligand

has not been identified yet (Figure 2) (reviewed in Stunnenberg, 1993; Mangelsdorf *et al.*, 1995).

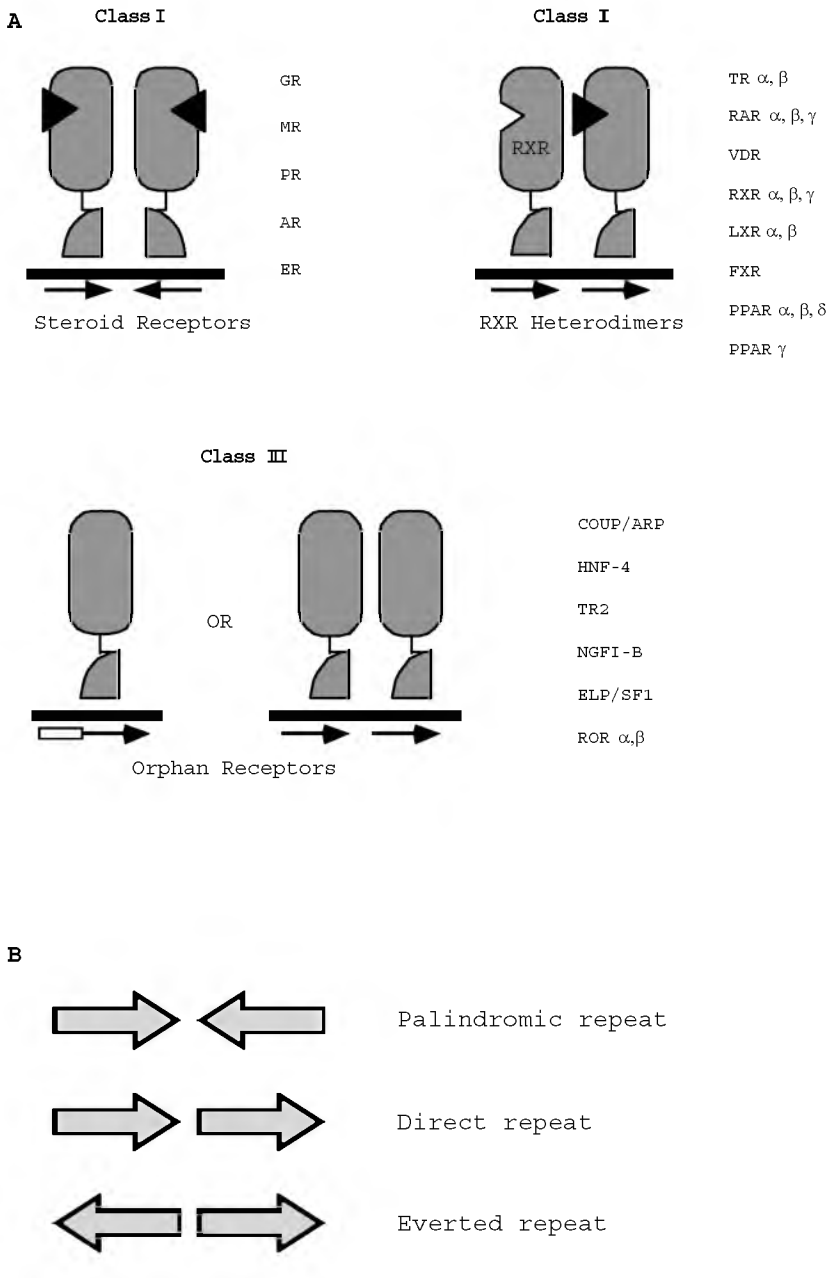


Figure 2 (**A**) Classification of the nuclear hormone receptors (NRs). Depending on their dimerisation and DNA binding properties NRs can be grouped into three classes: the steroid hormone receptors (I), the RXR heterodimers (II) and the orphan receptors (III). Representative receptors of each group are listed. (**B**) Schematic representation of DNA binding sites for NRs with half-sites orientation.

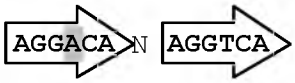
Binding site	Type	Receptor
	DR 3 osteopontin	RXR-VD R
	DR 4 MoMLV	RXR-T R
	DR 1 AcylCoA	RXR-PPAR
	DR 1 ApoAI	RXR-RXR RAR-RXR
	DR 1 CRABP I	RAR-RX R
	DR 2 CRABP I	RXR-RA R
	DR 5 RAR β2	RXR-RA R

Table 1 Class II nuclear receptors binding sites. Indicated are the promoters in which the binding sites are located and the receptor hetero-/homodimers that have been reported to bind and *trans*-activate through the respective binding sites. The binding sites are derived from the following promoters: the rat Cellular Retinoic Acid Binding Protein I (CRABP I) (Durand *et al.*, 1992), Acyl CoA oxidase (Tugwood *et al.*, 1992), Osteopontin (Noda *et al.*, 1990), Moloney Murine Leukemia Virus LTR (MoMLV) (Sap *et al.*, 1990), RAR-β2 (de The *et al.*, 1990), Apo AI (Zhang *et al.*, 1992b). Nucleotides deviating from the consensus sequence $AG^{G}/_{T}TCA$ are highlighted.

4.2. General structure of Class II nuclear receptors

NRs, commonly, have a tripartite structure. A highly conserved DNA binding domain (DBD) separates the variable N-terminal region from the conserved C-terminal domain (Figure 3). The features that have provided the criteria to classify members of the NR superfamily are the DBD, which recognises similar DNA sequences and the C-terminal, ligand binding domain (LBD). On the basis of intron-exon boundaries and structure-function similarities, NRs consist of six regions referred

to as A to F (reviewed in Evans, 1988; Green and Chambon, 1988; Mangelsdorf and Evans, 1995). In the following I will describe the structure and function of the class II NRs.

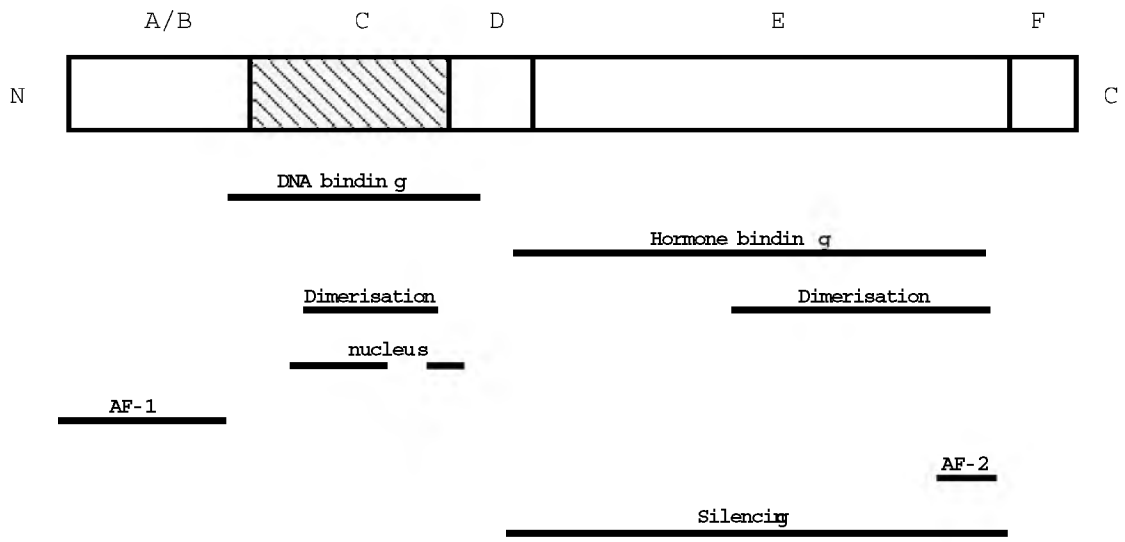


Figure 3 Schematic representation of a typical nuclear hormone receptor with its functional domains. NRs are composed of a highly conserved DNA binding domain (DBD) or C-region, a variable amino-terminus (A/B) region and a conserved carboxy-terminus (D, E and F region). The DBD is responsible for sequence specific binding to DNA responsive elements and harbours dimerisation as well as nuclear localisation functions. The N-terminus harbours *trans*-activation function-1 (AF-1). The C-terminus contains multiple hormone-dependent functions including hormone binding, *trans*-activation function-2 (AF-), silencing function, dimerisation and nuclear localisation properties. The black bars show the localisation of each receptor function.

4.2.1. The DNA binding domain (DBD)

The central DNA binding domain (DBD) is the most conserved part between NRs and it targets the receptor to specific DNA sequences known as hormone responsive elements (HREs) (reviewed in Evans, 1988; Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995). The observation that class I NRs bind as homodimers to DNA half-site sequences arranged as palindromes invariably spaced by three nucleotides (Beato, 1989) was initially extrapolated to the class II NRs. However,

subsequent studies revealed that the class II receptors recognise the same hexameric DNA core-motif 5'-$^{A}/_{G}G^{T}/_{G}$TCA-3', referred to as half-site, mostly arranged in a direct repeat configuration (Brent *et al.*, 1989; de The *et al.*, 1990b). Specificity in DNA binding of a class II receptor is achieved by varying the spacing between the half-sites (Table 1): Direct repeats spaced by 1 nucleotide (DR1) constitute binding sites for PPAR, DR3 is a binding site for the VDR and DR4 constitutes a thyroid hormone responsive element (TRE). The binding sites for RAR-RXR are directs repeats spaced by 1, 2 or 5 nucleotides (DR1, DR2 and DR5, respectively). Since DRs are asymmetric elements, heterodimer complexes are asymmetric units and the receptors DBDs are arranged in a head to tail fashion. On DR3, DR4, DR2 and DR5 elements, RXR occupies the 5' half-site and the partner (VDR, TR and RAR respectively) occupies the 3' half-site (Jin and Pike, 1996; Kurokawa *et al.*, 1993; Mader *et al.*, 1993; Naar *et al.*, 1991; Umesono *et al.*, 1991; Vivanco Ruiz *et al.*, 1991) (reviewed in Desvergne, 1994; Mangelsdorf and Evans, 1995). RXR can bind both as homodimer and as heterodimer with RAR on a DR1, but in contrast to its position on a DR2 and DR5, RXR occupies the downstream half-site (Kurokawa *et al.*, 1995) (reviewed in (Leblanc and Stunnenberg, 1995).

Apart from the above-mentioned 'classical' HREs, other arrangements of the hexameric half-sites have been identified to constitute functional HREs for class II NRs, such as palindromic (PAL) or everted repeats-like elements (Figure 2B). It should be emphasised that many natural HREs deviate from the idealised consensus sequences (reviewed in Mangelsdorf and Evans, 1995).

The constraints for binding to a specifically spaced and/or oriented responsive element are dictated by the DBD (Mader *et al.*, 1993; Perlmann *et al.*, 1993). The DBD of NRs contains two highly conserved zinc-fingers with four cysteine residues co-ordinating the binding of one Zn^{2+}-ion (Figure 4A). The first amino-terminal or proximal zinc-finger recognises specific bases of the half-sites. The C-terminal part of the first zinc-finger comprises helix 1 (C1). A part of the helix motif C1, designated P-box, makes direct contacts with the major groove of DNA (Figure 4A, B) (reviewed in Freedman and Luisi, 1993; Nelson *et al.*, 1996; Nelson *et al.*, 1995a).

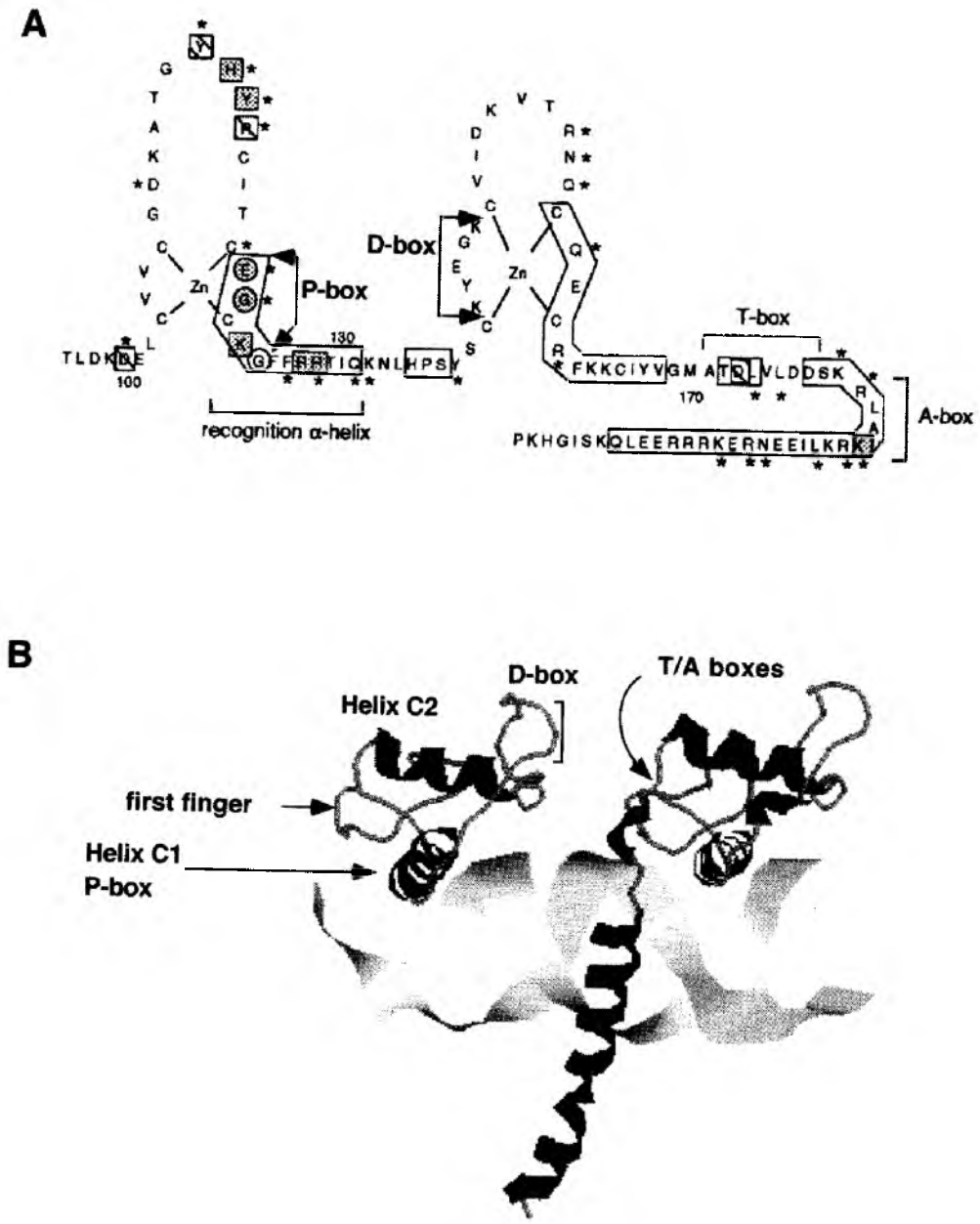


Figure 4 The DNA binding domain of hTRβ. (**A**) Schematic diagram of TRβ DNA binding domain (DBD). α-helices are boxed, the P-box amino acids are circled, amino acids involved in protein-DNA contacts are enclosed in shaded circles or squares, amino acids involved in specific protein interaction with RXR are enclosed in striped squares and amino acids contacting the sugar-phosphate backbone of the DNA are indicated with asterisks (Nelson *et al.*, 1996). (**B**) Cocrystal structure of the DBDs of TRβ and RXR on DNA. Helix C1 is perpendicular to C2 and contacts the major groove of DNA. D-box sequences are involved in dimerisation. TRβ is sitting 5' to RXR (PDB Id: 2NLL; F.Rastinejad, T.Perlmann, R.M.Evans & P.B.Sigler; PDB Deposition: 20-Nov-1996).

The P-box is defined by the five amino acids EGCKG and is conserved in all members of the class II receptors family. A short amino acid sequence located at the N-terminus of the second or distal zinc-finger was originally shown to be involved in protein-protein interactions of the steroid, class I NRs, hence termed dimerisation-box (D-box) (Figure 4A) (Umesono and Evans, 1989). However, for the class II NR heterodimers, the various spacing between the half-sites dictates that different parts of the proteins face each other to form the dimer interface (Perlmann *et al.*, 1993; Rastinejad *et al.*, 2000; Zechel *et al.*, 1994a; Zechel *et al.*, 1994b). The last four amino acids of the distal finger together with the eight downstream residues constitute helix 2 (C2) (Figure 4A). The helices C1 and C2 are oriented nearly perpendicularly and form the core of the DBD. (Figure 4B). The T/A box which is a helix downstream of the C2 contacts the minor groove (Luisi *et al.*, 1991; Rastinejad *et al.*, 1995; Schwabe *et al.*, 1993) (reviewed in Mangelsdorf and Evans, 1995; Rascle *et al.*, 1997). In addition to the DBD, amino acids in the N- and C-termini of the receptors are also required for efficient DNA binding (Chen *et al.*, 1993a; Judelson and Privalsky, 1996; Lee *et al.*, 1993; Rastinejad *et al.*, 1995; Wong and Privalsky, 1995).

4.2.2. The ligand binding domain (LBD)

The C-terminus of NRs, also referred to as E region, is essential and sufficient for hormone binding and hence is called the ligand binding domain (LBD). The LBD is also important for dimerisation, interaction with coactivators and corepressors, and integrates *trans*-activation function (AF-2), *trans*-repression and nuclear translocation properties (Figure 3) (Baniahmad *et al.*, 1992; Barettino *et al.*, 1994; Dang and Lee, 1989; Forman *et al.*, 1989; Glass *et al.*, 1989; Hamy *et al.*, 1992; Marks *et al.*, 1992) (reviewed in Thormeyer and Baniahmad, 1999).

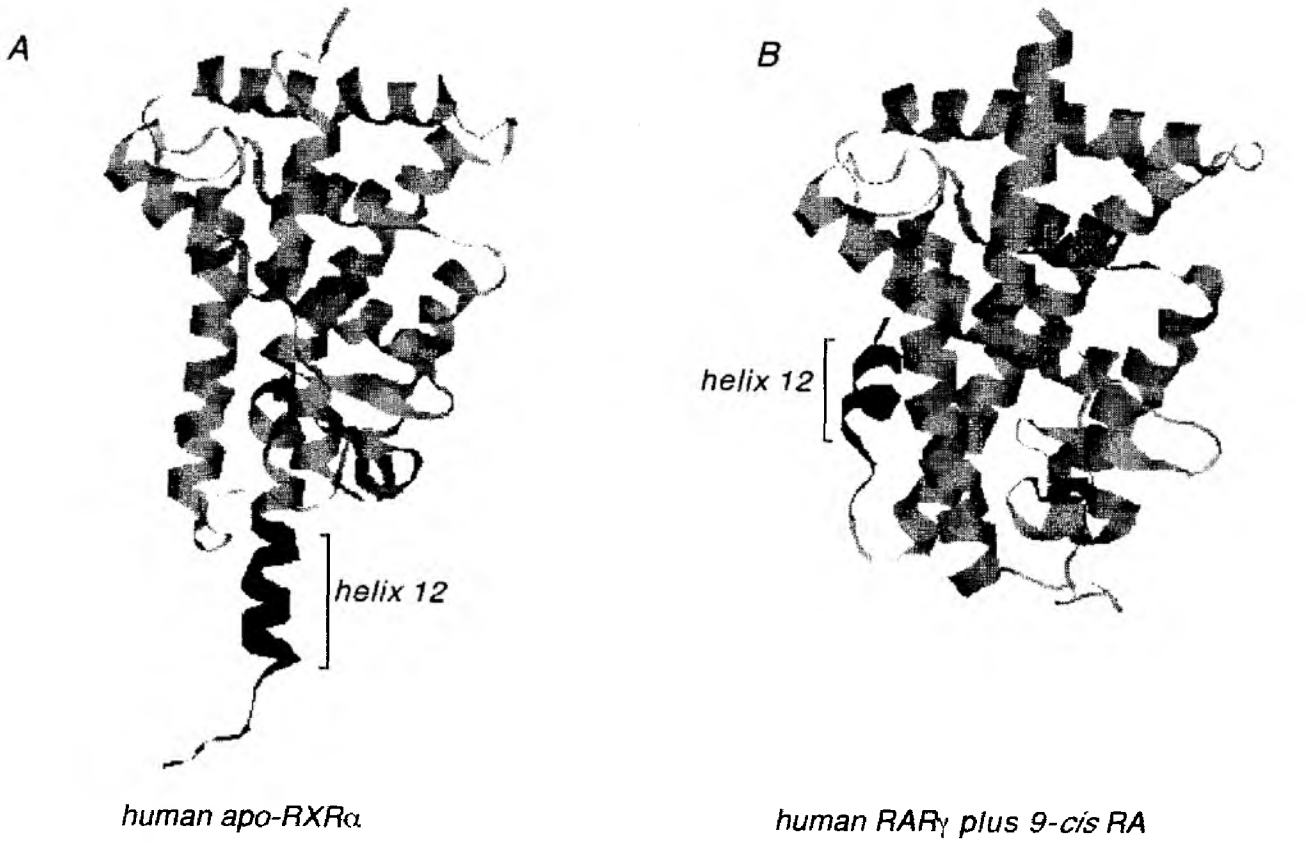


Figure 5 Crystal structure of nuclear receptor ligand binding domains. (**A**) The LBD of the human apo-RXRα (PDB Id: 1LBD; W.Bourguet & D.Moras; PDB Deposition: 22-May-1996). (**B**) Human RARγ liganded with 9-*cis* retinoid acid (PDB Id: 3LBD; B.P.Klaholz, J.-P.Renaud, A.Mitschler & D.Moras; PDB Deposition: 4-Feb-1998). Note the different position of helix 12 in each situation.

Determination of the 3D structure of a number of NRs (RXRα, TRα, RAR, ER, PR and PPARγ) revealed that they share a similar overall fold (Bourguet *et al.*, 1995; Brzozowski *et al.*, 1997; Egea *et al.*, 2000; Nolte *et al.*, 1998; Renaud *et al.*, 1995; Shiau *et al.*, 1998; Tanenbaum *et al.*, 1998; Uppenberg *et al.*, 1998; Wagner *et al.*, 1995; Williams and Sigler, 1998). The structure is termed the antiparallel α-helical sandwich and consists of 11 or 12 successive helices grouped into three layers (Figure 5) (reviewed in Mangelsdorf and Evans, 1995). The central layer comprises helices H4, H5, H8 and H9 which are sandwiched by H6, H7, H10 and H11 on one side and by H1-H3 on the other (numbering of helices according to (Bourguet *et al.*, 1995). The structure generates a pocket in which the ligand is buried and the last helix, comprising

the AF-2 domain, acts as the lid of the pocket. In the unliganded apo-RXR, H12 protrudes outwards from the core of the LBD (Figure 5A) (Bourguet *et al.*, 1995), whereas in the agonist-bound LBD of RAR, TR, PR ER and PPAR H12 is folded back (Figure 5B). Modulation of the architecture of the LBD triggered by binding of the ligand have major implications in activation functions of NRs (Baniahmad *et al.*, 1995; Bocquel *et al.*, 1989; Hollenberg and Evans, 1988; Tasset *et al.*, 1990; reviewed in Evans, 1988; Tenbaum and Baniahmad, 1997).

4.2.2.1. Dimerisation of nuclear receptors

Class I NRs bind to their DNA responsive elements exclusively as homodimers. Although class II NRs are also known to bind as homodimers, they mainly form heterodimers with RXR on their cognate DNA elements (reviewed in Beato *et al.*, 1995; Mangelsdorf and Evans, 1995). Heterodimerisation of TR was first suggested from experiments showing that binding of TR to thyroid responsive elements (TREs) required the presence of a partner found in cell extracts (Forman *et al.*, 1992; Lazar *et al.*, 1991; Yen *et al.*, 1992). Subsequently, RXR was shown to be the partner not only of TR but also of RAR and VDR. Heterodimerisation with RXR not only strongly enhances the DNA binding affinity of RAR, TR and VDR but it also modulates their *trans*-activation function (Bugge *et al.*, 1992; Cheskis and Freedman, 1994; Hallenbeck *et al.*, 1992; Jin and Pike, 1996; Kliewer *et al.*, 1992; Yu *et al.*, 1991; Zhang *et al.*, 1992a). TR binds with highest affinity as heterodimer with RXR to direct repeats, while on everted and palindromic repeats TR preferentially binds as homodimer (Andersson *et al.*, 1992; Harbers *et al.*, 1996; Harbers *et al.*, 1998; Katz and Koenig, 1993; Kurokawa *et al.*, 1993; Wahlstrom *et al.*, 1992).

4.2.3. The N-terminal part of nuclear receptors

The N-terminal or A/B domain is evolutionary the least conserved part, mostly varying in length and sequence composition amongst the receptors. An ill-defined, but strong *trans*-activation function (AF-1) is localised in the N-terminus of most receptors; the AF-1 is hormone-independent (Figure 3). Frequently, AF-1 is cell type- and promoter-specific such as that of RXRγ that was shown to be active in a muscle-specific manner (Bocquel *et al.*, 1989; Dowhan and Muscat, 1996; Hollenberg and

Evans, 1988; Tasset *et al.*, 1990) (reviewed in Stunnenberg, 1993; Mangelsdorf and Evans, 1995; Rascle *et al.*, 1997; Stunnenberg *et al.*, 1999).

4.3. Transcriptional regulation by NRs - Many actors on performance

NRs were thought to be the major players of transcriptional regulation because a number of HREs are found in promoters, enhancers and locus control regions (LCRs), i.e in regulatory regions of several genes. Originally, it was proposed that class II NRs only functioned as activators in the presence of their cognate hormone and that they were neutral in its absence. However, more recent data support a model that unliganded class II NRs repress transcription (reviewed in Mangelsdorf and Evans, 1995; Glass and Rosenfeld, 2000).

The transcriptional activity of NRs has been shown to be dependent on their association with TBP (TATA-box binding protein) and TFIIB (transcription factor IIB) (Blanco *et al.*, 1995; Chen and Privalsky, 1997; Hadzic *et al.*, 1995; Tong *et al.*, 1995). NRs also interact with a number of co-factors that serve as mediators or signal integrators to polymerase II (pol II) basal transcriptional machinery. The co-factors belong to two classes and are associated with NRs in a ligand-dependent manner. The first class comprises the coactivators, which interact mainly with the AF-2 domain of liganded NRs and are shown to enhance the ligand-mediated transcriptional activation (Masuyama *et al.*, 1997) (Nolte *et al.*, 1998) (Westin *et al.*, 1998) (Blanco *et al.*, 1998) (Feng *et al.*, 1998) (Ren *et al.*, 2000) (Rachez *et al.*, 2000). The second class of cofactors comprises the so-called corepressors, which are associated with NRs only in the absence of ligand and they are postulated to enhance the ability of receptors to repress transcription. Thus, class II NRs act as 'on-off' switches of transcription. In the absence of ligand they bind to DNA and repress transcription while upon ligand binding they change conformation and activate transcription (reviewed in Glass and Rosenfeld, 2000).

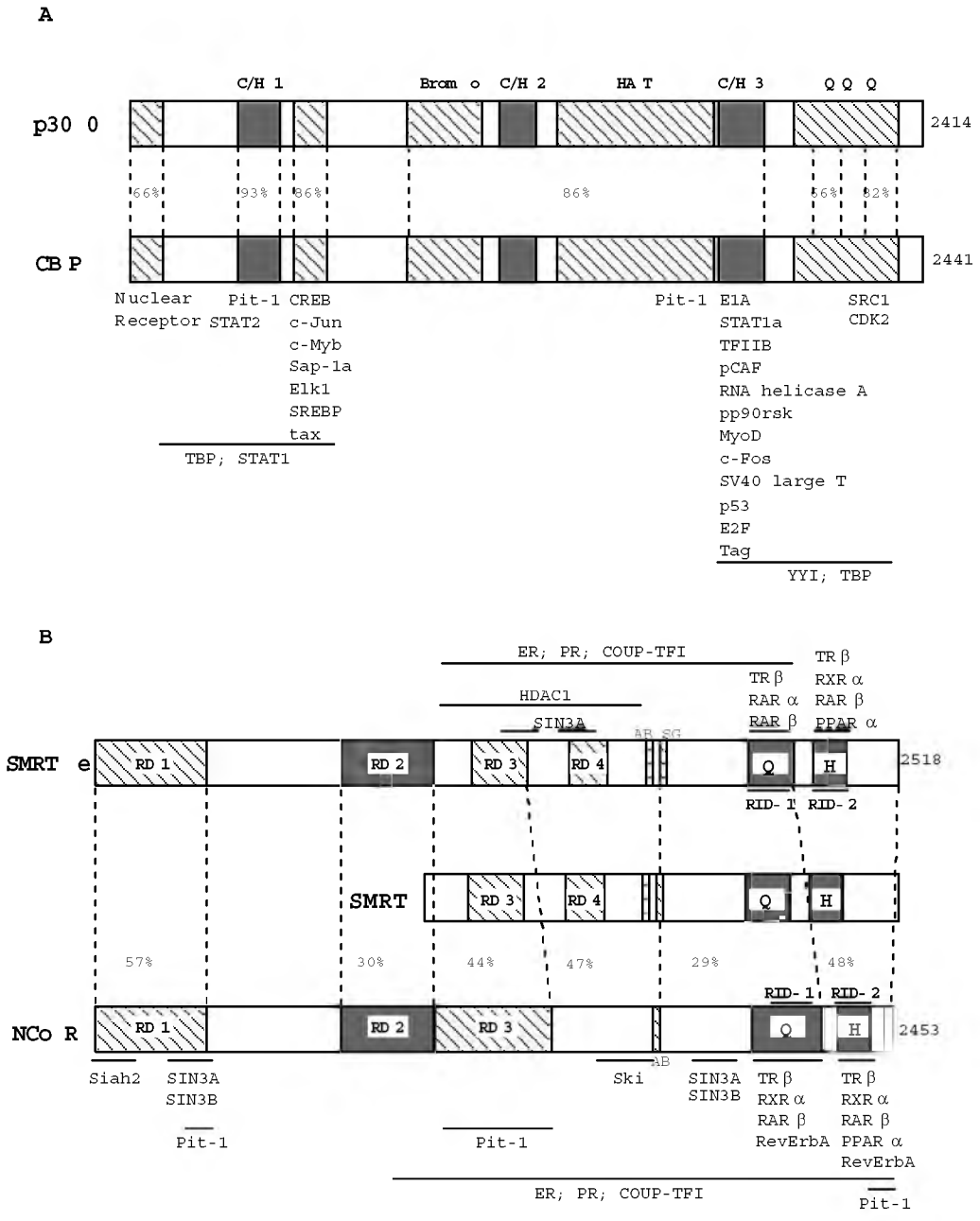


Figure 6 Structural features of nuclear receptor coactivators and corepressors. Conserved motifs or regions important for enzymatic activity are in bold text. Factors known to interact with these proteins are shown in plain text. Homology is represented as identity. (**A**) The structure of p300 versus CBP; AB, acidic basic; HAT, histone acetyltransferase activity; QQQ, glutamine rich; C/H, cysteine rich; Bromo, bromodomain. (**B**) SMRT versus SMRTe (SMRT extended) and NCoR. RD, repression domain; NR, nuclear receptor interacting domain; SG, serine/glycine rich; H, α-helical (adapted from Collingwood *et al.*, 1999).

4.3.1. Coactivators

The first coactivator identified to interact with NRs in a ligand-dependent manner was SRC-1 (Cavailles *et al.*, 1994; DiRenzo *et al.*, 1997; Halachmi *et al.*, 1994; Onate *et al.*, 1995). Subsequent studies led to the identification of three genes that encode SRC-1-related factors, TIF2/GRIP-2/NCoA-1 and p/CIP/AIB-1/ACTR/RAC/TRAM-1 (Anzick *et al.*, 1997; Chen *et al.*, 1997; Hong *et al.*, 1997; Kamei *et al.*, 1996; Li *et al.*, 1997a; Takeshita *et al.*, 1997; Torchia *et al.*, 1997). The SRC family of coactivators contain a highly conserved N-terminal basic helix-loop-helix (bHLH) domain that mediates protein-protein interactions. The C-termini of SRC-1 and ACTR possess histone acetyltransferase (HAT) activity (Spencer *et al.*, 1997). Ligand-mediated transactivation of RAR, PPAR, TR and VDR has been proposed to be critically dependent on their interaction with SRC-1 (Kurokawa *et al.*, 1998; Llopis *et al.*, 2000; reviewed in Glass and Rosenfeld, 2000).

p300/CBP (CREB-binding protein) belong to the NR coactivators with HAT activity (Korzus *et al.*, 1998). p300/CBP directly bind to the LBD of NRs in the presence of ligand and serve essential coactivator roles not only for NRs but also for many other classes of sequence specific transcription factors (Figure 6A) (Kamei *et al.*, 1996; Torchia *et al.*, 1997; reviewed in Glass and Rosenfeld, 2000). Substrates for acetylation by p300/CBP are not only free histones or histones assembled into nucleosomal complexes but also non-histone proteins such as p53, GATA-1 and EKLF (Boyes *et al.*, 1998; Ogryzko *et al.*, 1996; Zhang and Bieker, 1998). Hyperacetylation of histones facilitates transcription factor access and hence boosts transcription (reviewed in Collingwood *et al.*, 1999).

Another coactivator displaying HAT activity is p/CAF (p300/CBP-associating factor) (Yang *et al.*, 1996). It has been shown that p/CAF directly associates with RXR-RAR heterodimer and potentiates ligand-dependent transcriptional activation (Blanco *et al.*, 1998). p/CAF is an essential component of the multisubunit coactivator complex, referred to as PCAF in mammals (Ogryzko *et al.*, 1998).

A complex of at least nine proteins has been found to be associated with liganded TR termed TRAP (TR-associating proteins) (Fondell *et al.*, 1996b). A very similar complex of vitamin D3 receptor (VDR)-interacting proteins (DRIP) has also been purified (Rachez *et al.*, 1998). Surprisingly, several constituents of the

TRAP/DRIP and the highly related ARC (activator recruited cofactor) complex are very similar to protein components of the recently identified CRSP, NAT and SMCC mediator complexes (Ito *et al.*, 1999; reviewed in Hampsey and Reinberg, 1999; Malik and Roeder, 2000). These complexes, which were isolated independently, are reportedly required for *in vitro* transcriptional activation from chromatin templates by a number of other transcription factors including SREBP, NF-κB and VP16 (Gu *et al.*, 1999; Ito *et al.*, 1999; Naar *et al.*, 1999; Ryu *et al.*, 1999; Sun *et al.*, 1998). Purified DRIP enhances ligand-dependent transcriptional activation of VDR in a chromatinised template (Rachez *et al.*, 1999). Purified TRAP enhances *in vitro* transcriptional activation of TR in a chromatin-free system while the effects on chromatinised templates are not yet reported (Fondell *et al.*, 1999). Interestingly, TRAP/DRIP/ARC complex do not contain intrinsic HAT activity (Rachez *et al.*, 1999; Yuan *et al.*, 1998), which raises the question whether the various NR coactivators function at different stages of the gene-activation process.

4.3.2. Corepressors

v-ErbA was the first NR shown to repress transcription (Damm *et al.*, 1989), and subsequently repressive function was confirmed for most unliganded class II receptors. A search for proteins interacting with unliganded receptors led to the identification of the corepressor molecules NCoR (NR corepressor) (Horlein *et al.*, 1995; Kurokawa *et al.*, 1995) and the highly related SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) (Chen and Evans, 1995) or TRAC-2 (TR associated cofactor) (Sande and Privalsky, 1996). While NCoR is a 270 kDa protein (Horlein *et al.*, 1995) SMRT was initially reported to be a 170 kDa protein with high homology to the C-terminus of the NCoR sequence (Figure 6B) (Chen and Evans, 1995). Recently, SMRT variants of 270 kDa originating from alternative splicing have been cloned (SMRTe) with high overall homology to NCoR (Ordentlich *et al.*, 1999; Park *et al.*, 1999). Both NCoR and SMRT show a conserved bipartite structure: their N-termini contain at least three autonomous repressor domains (Chen and Evans, 1995; Horlein *et al.*, 1995). Their C-termini contain two distinct receptor interaction domains (RID) (Li *et al.*, 1997b; Seol *et al.*, 1996; Zamir *et al.*, 1996) (Figure 6B). Corepressors are shown to potentiate the repressive function of receptors and to posses

intrinsic function to repress transcription that is transferable to heterologous DNA-binding domains. It should be emphasised that corepressors interact only with unliganded NRs and they dissociate upon ligand binding (reviewed in Hu and Lazar, 2000).

Besides binding to NRs, NCoR also interacts with the general transcription factor TFIIB and the TBP associated factors $TAF_{II}32$ and $TAF_{II}70$. Association of NCoR with TFIIB has been postulated to result in repression of transcription by preventing the productive interaction between TFIIB and $TAF_{II}32$ (Muscat *et al.*, 1998). NCoR/SMRT also interact with and serve as corepressors for a number of transcription factors unrelated to NRs such as MyoD, a myogenic factor, Mad, a helix-loop-helix (HLH) repressor molecule and CBF-1/RBP-JK, a mammalian homologue of the drosophila protein *suppressor of hairless*. Thus, NCoR/SMRT may play a more general role in gene regulation than only facilitating the repressive function of NRs (Bailey *et al.*, 1999; Heinzel *et al.*, 1997; Kao *et al.*, 1998; reviewed in Glass and Rosenfeld, 2000).

Further insight into the mechanisms of repression by NRs was obtained by the discovery that SIN3A and SIN3B, the mammalian homologues of yeast Sin3, interact with NCoR and SMRT (Alland *et al.*, 1997; Ayer *et al.*, 1995; Heinzel *et al.*, 1997; Nagy *et al.*, 1997; Schreiber-Agus *et al.*, 1995). SIN3A/3B proteins are components of corepressor complexes that contain histone deacetylases (RPD3 in yeast and HDAC1/HDAC2 in higher eukaryotes) as well as a number of other proteins (Alland *et al.*, 1997; Hassig *et al.*, 1997; Heinzel *et al.*, 1997; Kadosh and Struhl, 1997; Laherty *et al.*, 1998; Laherty *et al.*, 1997; Nagy *et al.*, 1997; Taunton *et al.*, 1996; Vidal *et al.*, 1991; Zhang *et al.*, 1997b). Deacetylation of histones renders chromatin structure in a repressive state, thereby limiting transcription factor accessibility. Recruitment of HDACs by NRs via corepressors appear to play a critical role in NR repressive functions (reviewed in Collingwood *et al.*, 1999). More recently, distinct silencing domains of NCoR have been demonstrated to directly interact with HDAC3 or members of the novel class II family of HDACs (HDAC4, HDAC5, HDAC7 and HDAC8) implying a redundant or combinatorial deacetylase-dependent repression (Grozinger *et al.*, 1999; Hu *et al.*, 2000; Huang *et al.*, 2000; Kao *et al.*, 2000; Wen *et al.*, 2000). TBL (transducin beta-like protein 1) has very recently been shown to be a

component of a SMRT/HDAC3 complex, and its expression potentiated repression by TRβ. Mutations in TBL or TRβ have been found in patients suffering from deafness, thus linking defective repressive function of TRβ to deafness (Guenther *et al.*, 2000).

Two other nuclear receptor corepressors are Alien and Sun-CoR. Alien is a 41 kDa protein and although not related to NCoR/SMRT it might utilise a repression mechanism similar to that of NCoR/SMRT (Dressel *et al.*, 1999). Sun-CoR is 16 kDa in size and might be specific for RevErb. Notably, Sun-CoR interacts with NCoR/SMRT (Zamir *et al.*, 1997; Zamir *et al.*, 1996).

4.3.3. Determinants of coactivator and corepressor binding

In the last few years it has become clear that ligand binding is the driving force for changes in conformation of class II NRs and subsequently for cofactor association (reviewed in Glass and Rosenfeld, 2000). Crystal structure studies showed that upon ligand binding a conformational change of the AF-2 domain results in the formation of a surface suitable for interaction with coactivators. A short α-helical motif LXXLL present in many coactivators docks to a hydrophobic cleft on the surface of the LBD that is bounded on one side by the AF-2 in helix H12 and on the other by the end of helix H3 (Brzozowski *et al.*, 1997; Darimont *et al.*, 1998; Nolte *et al.*, 1998; Renaud *et al.*, 1995; Shiau *et al.*, 1998; Wagner *et al.*, 1995).

The interaction interface of NRs for corepressors NCoR/SMRT has been controversial. Originally, helix H1 of the LBD was thought to provide the interface for interaction with corepressors (Figure 8). Mutations in a small region within H1, termed the CoR-box, affected the interaction with NCoR/SMRT (Chen and Evans, 1995; Horlein *et al.*, 1995; reviewed in Hu and Lazar, 2000). However, CoR-box mutations also prevent interactions with RXR suggesting that the CoR-box might not directly interact with corepressors but rather serve a more global structural role (Collingwood *et al.*, 1997; Zhang *et al.*, 1997a; reviewed in Hu and Lazar, 2000). There is evidence that H12, plays a major role in corepressor recruitment; yet, ongoing studies also implicate helices H3, H4 and H5 in the corepressor interaction interface (Figure 8) (Baniahmad *et al.*, 1995; Hu and Lazar, 1999; Li *et al.*, 1997b; Lin *et al.*, 1997; Tagami *et al.*, 1998; Zhang *et al.*, 1999; Zhang *et al.*, 1997a).

Recently, a conserved $LXX^{I}/_{H}IXXX^{I}/_{L}$ sequence has been discovered within the receptor interaction domain (RIDs) of the corepressors (Hu and Lazar, 1999; Nagy *et al.*, 1999; Perissi *et al.*, 1999). This $LXX^{I}/_{H}IXXX^{I}/_{L}$ motif forms an extended helix and is referred to as the CoRNR box. The CoRNR box has been reported to bind to a hydrophobic pocket formed by the LBD of NRs. In the liganded state H12 changes position and the same hydrophobic pocket, is now occupied by the coactivator helical motif LXXLL (Hu and Lazar, 1999; Nagy *et al.*, 1999; Perissi *et al.*, 1999). Since the ligand-activated pocket has exactly the length of the coactivator helix, closure upon ligand binding would inhibit the binding of the extended corepressor helix. It is suggested that the opening/closure of H12 might represent the molecular mechanism for ligand-dependent displacement of the corepressor complex (reviewed in Glass and Rosenfeld, 2000).

4.3.4. Transcription and chromatin structure

The repeating unit of eukaryotic chromosomes is the nucleosome consisting of DNA wrapped around an octamer of histone proteins. Nucleosomes were initially thought to be merely compact and featureless inducers of DNA bending. Recent observations suggest that nucleosomal structure is dynamically/enzymatically regulated, thus playing an important role in transcriptional activation or repression. Indeed, eukaryotic transcription is an interlaced network of transcription factors, chromatin and chromatin-modifying complexes (Figure 7) (reviewed in Felsenfeld, 1992; Kadonaga, 1998; Kingston *et al.*, 1996).

The nucleosomal structure is important for the transcriptional regulation by NRs because a number of NR cofactors exhibit chromatin-modifying activity. The current view is that class II NRs regulate transcription by exerting either repressive or activation function to the polymerase II basal transcriptional machinery. Repression by NRs is attained in two ways. First, corepressors bound to NRs actively inhibit the formation and activity of the Pol II transcription preinitiation complex (PIC). This notion is consistent with findings showing that NCoR inhibits the productive interaction between general transcription factors and TFIIB (Fondell *et al.*, 1996a; Fondell *et al.*, 1993; Tong *et al.*, 1995). Second, via association with HDACs,

corepressors can render chromatin structure in a closed, repressive state and hence (positive) transcription factors accessibility is diminished.

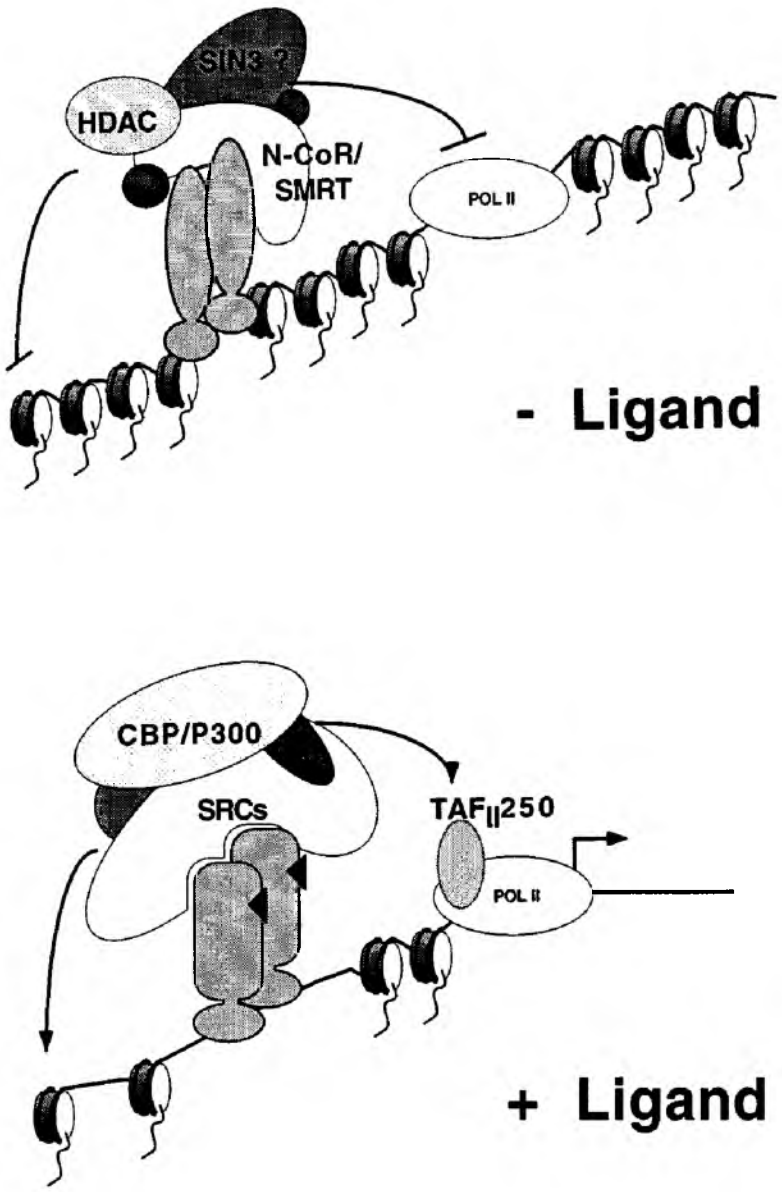


Figure 7 Normal gene regulation controlled by class II nuclear receptors involves corepressors, coactivators, histone deacetylases and chromatin assembly. The receptors can recognise and bind specific DNA elements (HREs) in their unliganded form. Association of corepressors (NCoR/SMRT) inhibit transcription presumably via a non-productive interaction with the constituents (TFIIB) of the preinitiation complex (PIC), while histone deacetylases locally trigger a 'closed' chromatin state. Specifically binding of liganded RXR-TR or RXR-RAR to HREs will recruit coactivator complexes containing HAT activity (CBP, SRC-1) to promote formation of PIC and transcription.

Ligand binding induces a conformation change in the receptors that permit dissociation of the corepressors. It is postulated that for activation of transcription by NRs *in vivo,* chromatin remodelling complexes are recruited to the promoter initially. The yeast SWI/SNF complex or its mammalian homologue BRG-1 posses an ATP-dependent ability to cause local changes in chromatin structure, thus, facilitating the binding of sequence specific transcription factors to nucleosomal DNA (Owen-Hughes *et al.*, 1996). Interactions of receptors on chromatinised templates with BRG-1, SWI/SNF or ISWI (imitation SWI) are implicated in the transcriptional activation function of GR, ER and RAR (Chiba *et al.*, 1994; Di Croce *et al.*, 1999; Ichinose *et al.*, 1997; Muchardt and Yaniv, 1993; Ostlund Farrants *et al.*, 1997; Yoshinaga *et al.*, 1992). SWI/SNF-related complexes may relieve the repressive actions of chromatin and set the stage for a second chromatin-dependent step of gene activation requiring factors with HAT activity. It is postulated that recruitment of the PCAF complex might stimulate transcription by remodelling nucleosomal structure. Finally, the action of additional complexes such as TRAP/DRIP might be required to activate transcription (reviewed in Glass and Rosenfeld, 2000). The fact that TRAP/DRIP do not posses intrinsic HAT activity might reflect that they act late at the actual activation step. Such a sequential model is supported by the observation that thyroid hormone (T3)-mediated transcription was stimulated in the presence of p300 at a step subsequent to chromatin disruption (Li *et al.*, 1999) and that the coactivators CBP and p160s are released late in RAR-dependent promoter activation (Chen *et al.*, 1999).

Considering the above, it is remarkable that the transition of a NR between repressed and activated states is controlled by biochemical and enzymatic symmetry exerted by HDACs or HATs (reviewed in Glass and Rosenfeld, 2000). Should this symmetry exist for all the stages during repression, as it has been proposed for activation, more corepressor complexes with various enzymatic activities are still to be discovered.

5. Why v-ErbA ?

The goal of the studies in this thesis was to investigate the role of nuclear receptors in transcriptional repression. We have chosen v-ErbA as a model system because its repressive activity has significant physiological implications and has been directly correlated with oncogenicity. Avian erythroblastosis virus (AEV), which encodes apart from v-erbA, a second oncogene, v-erbB, is a paradigm of many retroviruses that induce malignancies by the co-operation of two oncogenes. Other examples include the E26 virus encoding v-myb and v-ets (Graf *et al.*, 1992) and the avian Mill-Hill-2 myelocytomatosis (MH-2) virus encoding v-mil and v-myc (Bechade *et al.*, 1985; Jansen *et al.*, 1983). The v-ErbA oncoprotein has a number of structural and functional similarities to the RAR translocation products PML-RARα and PLZF-RARα such as DNA binding, repression of gene transcription and ligand unresponsiveness. Since PML-RARα and PLZF-RARα fusion proteins cause human acute promyelocytic leukemias (APL), interrelated studies will be significant for understanding the molecular bases of gene regulation in malignancies (reviewed in Stunnenberg *et al.*, 1999). At the outset of this work substantial progress had already been made in understanding the transformation function of v-erbA, as compared to other oncogenenic systems. Neveretheless, knowledge on molecular mechanisms responsible for transcriptional repression and consequently oncogenicity was rudimentary.

In terms of availability of materials, the chicken system has a number of conveniences. Primary erythroid progenitor cells suitable for the intended study (chapter 2) were at that time only available from chicken. Mouse primary cells could not be driven into differentiation in a synchronised manner and human primary cells could not be obtained in large amounts. Finally, v-ErbA-expressing erythroid progenitors are amenable to manipulations required to establish novel derivative cell lines.

In the following sections the current knowledge about v-ErbA is presented. Structural similarities and differences between v-ErbA and its cellular homologue, TRα, with implications in v-ErbA function will be discussed.

6. Avian Erythroblastosis Virus (AEV)

AEV is an acutely leukemogenic retrovirus that induces fatal erythroleukemia in young chicks. The target cell for AEV transformation is a committed erythroid progenitor (Samarut and Gazzolo, 1982). The *in vitro* transformed erythroblast-like cells are rapidly proliferating and are tightly arrested in an immature state of differentiation (Graf and Beug, 1983). The AEV virus encodes two oncogenes: v-ErbB, a mutated *trans*-membrane receptor for epidermal growth factor (EGFR) and v-ErbA, a mutated nuclear thyroid hormone receptor (TRα) (Downward *et al.*, 1984; Sap *et al.*, 1986).

v-erbB alone is sufficient for transformation of fibroblasts and erythroblasts *in vivo* and *in vitro*. Both v-ErbB and overexpressed c-ErbB/EGFR, activated by TGFα, induce abnormal self renewal of erythroid progenitors (Beug *et al.*, 1985; Lax *et al.*, 1988; Pain *et al.*, 1991). In contrast to c-ErbB, v-ErbB renders erythroid progenitors independent of the erythroid growth factor Epo (Beug *et al.*, 1982). However, a significant proportion of these v-ErbB-expressing cells escapes the differentiation arrest to spontaneously mature into erythrocytes (Beug *et al.*, 1992). Cells expressing only v-ErbB could neither extensively self-renew nor could they be tightly arrested in differentiation unless certain batches of serums were used (Beug *et al.*, 1995). Subsequently, it was found that these serum batches contained factors such as estrogens and glucocorticoids. Finally, v-ErbB appeared to co-operate with ligand activated receptors (GR and ER) to transform cells (Bauer *et al.*, 1997; Hayman *et al.*, 1993; Schroeder *et al.*, 1993; Wessely *et al.*, 1997).

v-erbA affects the differentiation phenotype of erythroid cells in a fashion quite different from that of v-erbB. *In vitro*, v-ErbA expression drastically broadens the narrow limits of pH and ionic concentrations required for v-erbB-transformed erythroblasts to survive in culture. v-ErbA has no distinct transforming capacity of its own *in vivo* (Frykberg *et al.*, 1983; Gandrillon *et al.*, 1989; Sealy *et al.*, 1983) unless its expression is forced to extremely high levels (Casini and Graf, 1995). However, v-ErbA blocks the spontaneous differentiation occurring in a subpopulation of v-erbB-transformed erythroblasts and increases the growth rate of these cells. Thus, v-erbA leads to a much more severe leukemic phenotype as compared to a v-erbB only-induced leukemia (Kahn *et al.*, 1986; Forrest *et al.*, 1990a; Graf *et al.*, 1977; Hihara *et*

al., 1983; Kahn *et al.*, 1984; Miles and Robinson, 1985). Evidence for the co-operation between v-ErbA and v-ErbB came from experiments performed with cells expressing only v-ErbA. *In vitro* transformation of these v-ErbA cells was dependent on the presence of additional factors or components present in chicken serum. One of these serum components is most likely an avian ligand for c-ErbB. This conclusion was based on the prime observation that certain normal chicken sera stimulate tyrosine autophosphorylation of chicken c-ErbB/EGFR (Pain *et al.*, 1991; Schroeder *et al.*, 1992) (reviewed in Beug *et al.*, 1996). The current view is that v-ErbA co-operates with ligand-activated tyrosine kinases such as TGFα-loaded c-ErbB or stem cell factor (SCF)-activated c-kit to arrest differentiation. Constitutively active, transforming oncoproteins such as v-ErbB, v-sea, v-src, v-H-ras and v-ets were also shown to co-operate with v-ErbA to lead to a severe leukemic phenotype (Kahn *et al.*, 1984; Kahn *et al.*, 1986; Metz and Graf, 1992; Pain *et al.*, 1991; Schroeder *et al.*, 1990).

The precise mechanism by which v-erbA contributes to erythroblast transformation has been elusive for quite long. However, repression of the transcription of certain genes is thought to contribute to its oncogenicity. Three target genes have been suggested to be directly repressed by v-ErbA: the Carbonic Anhydrase II gene (CA II), the erythrocyte anion transport protein gene (Band 3) and the δ-aminolevulinate synthase gene (ALA-S) (Pain *et al.*, 1990; Zenke *et al.*, 1988). Re-expression of CA II partially abrogates the v-ErbA-induced arrest of differentiation (Fuerstenberg *et al.*, 1992), suggesting that repression of CA II is an essential step in erythroleukemia.

CA II catalyses the reversible hydration of CO_2 into bicarbonate anion: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. The high cytosolic concentration of HCO_3^- drives its transport out of the erythrocyte in exchange of an entering Cl^- anion by the membrane protein band 3. Many of the protons (H^+) generated are taken up by deoxyhemoglobin. One proton binds to hemoglobin for two molecules of O_2 that are released. This uptake of protons helps to buffer the pH in metabolically active tissues (Stryer, 1995). Ion concentrations of blood plasma usually fluctuate, for example in an exercising skeletal muscle lactic acid contributes to the H^+ pool. Thus, the exchange of HCO_3^- (equal to $OH^- + CO_2$) for Cl^- dictates the direction of the CA II catalyzed reaction and finally regulates the cytosolic pH to remain near neutral (reviewed in Geers and Gros, 2000; Lindskog,

1997; Tashian, 1989). In conclusion, CA II is important for the major task of the erythrocytes i.e. to provide O_2 to the periphery.

7. The v-ErbA protein

7.1. Structure of the protein

As stated previously, the v-erbA oncogene is a viral variant of the c-erbAα proto-oncogene, chicken TRα. It originates from the fusion of the c-erbA coding region with the viral gag gene during the genesis of AEV. The v-ErbA protein is a 75-kDa-fusion product of gag and c-ErbA/TRα sequences. In the v-ErbA protein the first 12 amino acids of TRα have been replaced by 255 residues of gag (Figure 8, Figure 9) (Debuire *et al.*, 1984; Henry *et al.*, 1985; Sap *et al.*, 1986; Vennstrom *et al.*, 1980; Weinberger *et al.*, 1986) (reviewed in Beug *et al.*, 1994). Nine amino acids corresponding to the AF-2 region of TRα are also deleted. In addition, there are 13 point mutations distributed along the receptor moiety. Two of these mutations are localised in the DNA binding domain; mutation at position 61 changes the class II EGCKG P-box into EGCKS, affecting the specificity and affinity for DNA binding (Bonde *et al.*, 1991; Nelson *et al.*, 1994; Nelson *et al.*, 1995a; Nelson *et al.*, 1995b; Smit-McBride and Privalsky, 1994). The other mutation at position 78 is located within the D-box. Two mutations are localised in the residual N-terminal domain and nine mutations are in the E domain. The mutations in the E domain together with the deletion of the AF-2 domain are responsible for the inability of the oncoprotein to bind hormone and impair its dimerisation properties (Barettino *et al.*, 1993; Munoz *et al.*, 1988; Zenke *et al.*, 1990).

In contrast to TRα, which is exclusively nuclear, v-ErbA is localised both in the nucleus and the cytoplasm (Bigler and Eisenman, 1988; Boucher *et al.*, 1988; Boucher and Privalsky, 1990). The 12 N-terminal amino acids of TRα that are absent in v-ErbA have been demonstrated to be crucial for nuclear localisation (Andersson and Vennstrom, 1997). Mutations in the DBD and N-terminal part of LBD domains have

also been implicated in the subcellular distribution of v-ErbA (Boucher and Privalsky, 1990).

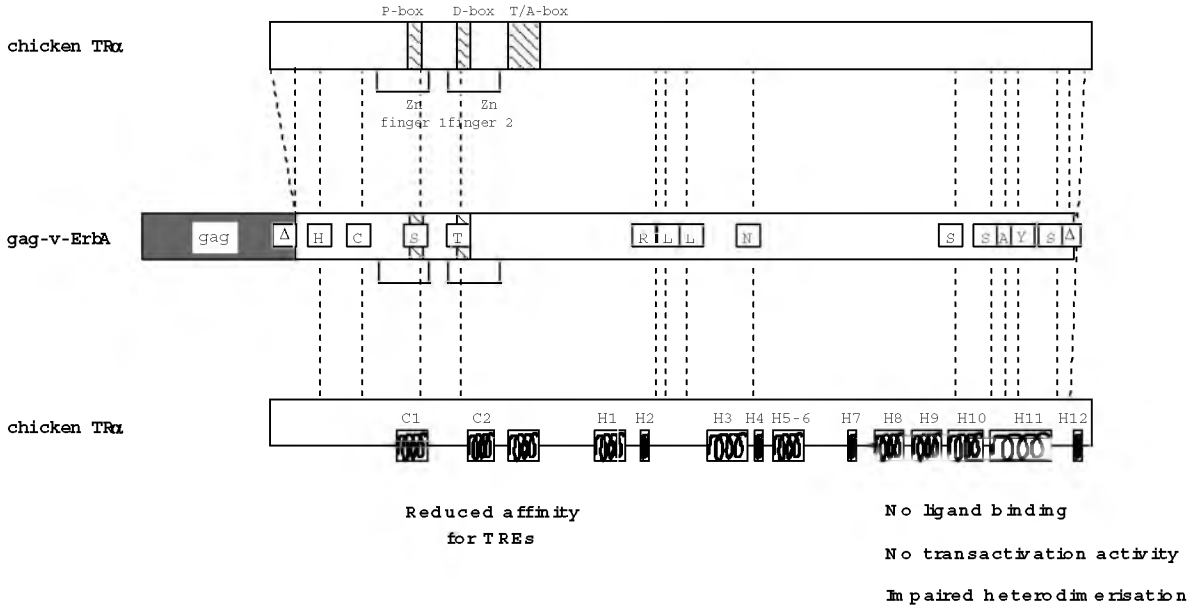
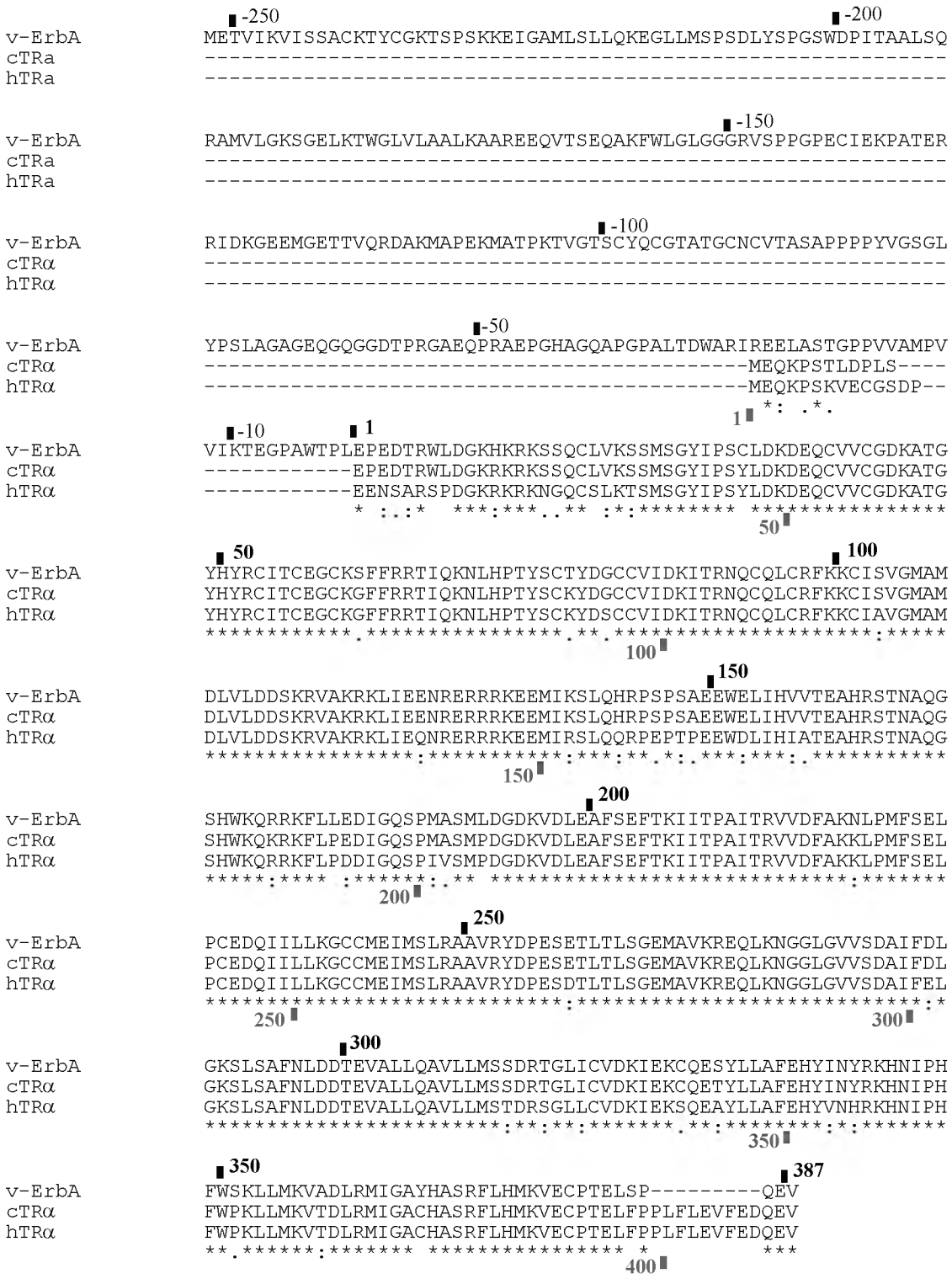


Figure 8 Schematic representation of cTRα and v-ErbA. In TRα are shown regions that are either structurally or functionally defined. For clarity the c-ErbA molecule has been depicted twice. The upper part displays the zinc-finger regions and the P, D and T/A boxes. The lower part displays the regions that form an α-helical structure (Rastinejad *et al.*, 1995). Between the two c-ErbA representations are shown (boxed) the v-ErbA amino acids that are different from cTRα. A boxed Δ represents deletion.

Figure 9 Multiple sequence alignment between the gag-v-ErbA protein, the chicken TRα and the human TRα protein using the programme CLUSTAL X (1.62b). Here we have numbered the v-ErbA protein by a negative numbering of the 252 amino acids belonging to the gag domain and then by a positive numbering the 387 ErbA specific amino acids (top numeration). The human c-ErbA/TRα protein is numbered below its sequence. In order to know the position of an amino acid in the chicken c-ErbA/TRα sequence one has to either add 12 from the v-ErbA numbering or to subtract 2 from the human c-ErbA numbering. * indicates identical amino acids. gag-v-ErbA protein is a composite sequence made from v-ErbA (Genbank accession M32090, Damm *et al.*, 1987) and p19 protein, accession S35743 (NCBI accession 420624; direct submission) sequences, chicken TRα, Swiss-Prot accession P04625; (Sap *et al.*, 1986) and human TRα protein, Swiss-Prot accession P21205; (Pfahl and Benbrook, 1987).


-250
-200
v-ErbA METVIKVISSACKTYCGKTSPSKKEIGAMLSLLQKEGLLMSPSDLYSPGSWDPITAALSQ
cTRa ----------
hTRa ----------
-150
v-ErbA RAMVLGKSGELKTWGLVLAALKAAREEQVTSEQAKFWLGLGGGRVSPPGPECIEKPATER
cTRa ----------
hTRa ----------
-100
v-ErbA RIDKGEEMGETTVQRDAKMAPEKMATPKTVGTSCYQCGTATGCNCVTASAPPPPYVGSGL
cTRα ----------
hTRα ----------
-50
v-ErbA YPSLAGAGEQGQGGDTPRGAEQPRAEPGHAGQAPGPALTDWARIREELASTGPPVVAMPV
cTRα ----------MEQKPSTLDPLS----
hTRα ----------MEQKPSKVECGSDP--
1
-10
1
v-ErbA VIKTEGPAWTPLEPEDTRWLDGKHKRKSSQCLVKSSMSGYIPSCLDKDEQCVVCGDKATG
cTRα ----------EPEDTRWLDGKRKRKSSQCLVKSSMSGYIPSYLDKDEQCVVCGDKATG
hTRα ----------EENSARSPDGKRKRKNGQCSLKTSMSGYIPSYLDKDEQCVVCGDKATG
50
50
100
v-ErbA YHYRCITCEGCKSFFRRTIQKNLHPTYSCTYDGCCVIDKITRNQCQLCRFKKCISVGMAM
cTRα YHYRCITCEGCKGFFRRTIQKNLHPTYSCKYDGCCVIDKITRNQCQLCRFKKCISVGMAM
hTRα YHYRCITCEGCKGFFRRTIQKNLHPTYSCKYDSCCVIDKITRNQCQLCRFKKCIAVGMAM
100
150
v-ErbA DLVLDDSKRVAKRKLIEENRERRRKEEMIKSLQHRPSPSAEEWELIHVVTEAHRSTNAQG
cTRα DLVLDDSKRVAKRKLIEENRERRRKEEMIKSLQHRPSPSAEEWELIHVVTEAHRSTNAQG
hTRα DLVLDDSKRVAKRKLIEQNRERRRKEEMIRSLQQRPEPTPEEWDLIHIATEAHRSTNAQG
150
200
v-ErbA SHWKQRRKFLLEDIGQSPMASMLDGDKVDLEAFSEFTKIITPAITRVVDFAKNLPMFSEL
cTRα SHWKQKRKFLPEDIGQSPMASMPDGDKVDLEAFSEFTKIITPAITRVVDFAKKLPMFSEL
hTRα SHWKQRRKFLPDDIGQSPIVSMPDGDKVDLEAFSEFTKIITPAITRVVDFAKKLPMFSEL
200
250
v-ErbA PCEDQIILLKGCCMEIMSLRAAVRYDPESETLTLSGEMAVKREQLKNGGLGVVSDAIFDL
cTRα PCEDQIILLKGCCMEIMSLRAAVRYDPESETLTLSGEMAVKREQLKNGGLGVVSDAIFDL
hTRα PCEDQIILLKGCCMEIMSLRAAVRYDPESDTLTLSGEMAVKREQLKNGGLGVVSDAIFEL
250
300
300
v-ErbA GKSLSAFNLDDTEVALLQAVLLMSSDRTGLICVDKIEKCQESYLLAFEHYINYRKHNIPH
cTRα GKSLSAFNLDDTEVALLQAVLLMSSDRTGLICVDKIEKCQETYLLAFEHYINYRKHNIPH
hTRα GKSLSAFNLDDTEVALLQAVLLMSTDRSGLLCVDKIEKSQEAYLLAFEHYVNHRKHNIPH
350
350
387
v-ErbA FWSKLLMKVADLRMIGAYHASRFLHMKVECPTELSP---------QEV
cTRα FWPKLLMKVTDLRMIGACHASRFLHMKVECPTELFPPLFLEVFEDQEV
hTRα FWPKLLMKVTDLRMIGACHASRFLHMKVECPTELFPPLFLEVFEDQEV
400

← **Figure 9**

7.2. Dimerisation of v-ErbA and DNA binding properties

Whether v-ErbA binds to HREs as a homodimer or as a heterodimer with RXR is still a controversial and debated issue. The apparent discrepancies may be due to the use of different experimental approaches (*in vitro* binding versus transfected cells) and different origins of the proteins (*in vitro* translated, baculovirus expressed, vaccinia virus expressed products or nuclear extracts from transfected cells). Due to a point mutation in the LBD at position 351, which changes the proline 363 in cTRα into a serine in v-ErbA, the v-ErbA oncoprotein has decreased affinity for RXR (Barettino *et al.*, 1993; Chen and Privalsky, 1993). Reversion of this serine into a proline in a selected mutant of AEV, AEV-r12, restored the ability to heterodimerise with RXR and strongly enhanced the inhibitory effect of the v-ErbA mutant protein on RA- and T3-induced gene expression in erythroblasts (Barettino *et al.*, 1993; Bauer *et al.*, 1997). Furthermore, the v-ErbA-r12 mutant functioned as a superactive oncogenic protein in erythroblasts (Damm *et al.*, 1987).

Given that v-ErbA and TR have both similarities and differences in their DBDs, it could not be taken for granted that they share the same DNA responsive elements. In addition, the mutation at position 32 in v-ErbA is located in the upstream of the DBD zinc-fingers region, which comprises a very potent determinant of the DNA sequence specificity of class II NRs (Chen *et al.*, 1993a; Judelson and Privalsky, 1996; Zechel *et al.*, 1994a). There is evidence that sequences that mediate activation by T3 are not necessarily v-ErbA responsive elements (Subauste and Koenig, 1995). v-ErbA can bind as heterodimer with RXR on most natural TREs but with lower affinity than RXR-TR heterodimers (Barettino *et al.*, 1993; Bonde and Privalsky, 1990; Damm *et al.*, 1989; Hermann *et al.*, 1993; Judelson and Privalsky, 1996; Sap *et al.*, 1989; Subauste and Koenig, 1998; Wahlstrom *et al.*, 1996; Yen *et al.*, 1994). The optimal element for RXR-v-ErbA heterodimer is reportedly a direct repeat (DR4), while for v-ErbA homodimers it is an everted repeat (Subauste and Koenig, 1998). v-ErbA homodimers can also bind to a DR4 but with lower affinity than v-ErbA heterodimers. Similarly, the RXR-v-ErbA heterodimer can bind to an everted repeat element but with lower affinity than v-ErbA homodimers (Judelson and Privalsky, 1996; Subauste and Koenig, 1998). An element in a silencer region of chicken lysozyme gene has been shown to convey strong repression upon v-ErbA binding. Mutational analysis

showed that this element, designated F2, was an everted repeat-type element with two half-sites spaced by 6 nucleotides (TGACCCcagctgAGGTCA) (Baniahmad *et al.*, 1990). Transcriptional repression by v-ErbA can be conveyed via both DR4 and everted repeat elements, yet, RXR potentiates repression only via the DR4 (Subauste and Koenig, 1998).

RXR-v-ErbA can also bind to DR3 and DR5 but not to DR1 and DR2 (Chen and Privalsky, 1993; Hermann *et al.*, 1993). The binding to DR5 elements depends highly on the fine structure of the element. DR5 from the mouse RARβ gene composed of PuGTTCA motifs, binds v-ErbA weakly either as a monomer or as a heterodimer with RXR, whereas strong binding is achieved on a AGGTCA-type DR5 motif (Chen and Privalsky, 1993).

There are also studies showing that v-ErbA, like TR, can bind to DNA as a monomer to the decameric DNA sequence 5'-T(A/G)AGGTCACG-3' (Subauste and Koenig, 1995). In addition, the oncoprotein has been shown to bind on some TREs as heterodimer with TRα (Yen *et al.*, 1994).

In summary, the DNA binding specificity of v-ErbA is determined by a number of parameters such as protein-protein interactions and the relative position of the molecules on the responsive element (Subauste and Koenig, 1998). Consequently, the architecture of the v-ErbA responsive element (VRE) affects the DNA binding selectivity and affinity and appears to be crucial for the v-ErbA repressive function on transcription (Judelson and Privalsky, 1996; Wahlstrom *et al.*, 1996).

7.3. Phosphorylation

The v-ErbA oncoprotein has two serine residues at positions 16/17 (equivalent to serines 28/29 in cTRα), which are substrate sites for phosphorylation by PKA (Goldberg *et al.*, 1988). These phosphorylation sites do not exist in any mammalian thyroid hormone receptor (Lazar *et al.*, 1988; Weinberger *et al.*, 1986) nor in the avian c-ErbAβ/cTRβ receptor (Forrest *et al.*, 1990b; Showers *et al.*, 1991; reviewed in Rascle *et al.*, 1997). In v-ErbA, phosphorylation of Ser 16/17 is a prerequisite for its function as an oncogene and for repression of transcription of erythroid genes such as CA II and band 3 (Glineur *et al.*, 1990). It has been postulated that v-ErbB triggers the

phosphorylation of v-ErbA which is important for the v-ErbB/v-ErbA-induced block of differentiation. However, an Ala 16/17 mutant of v-ErbA can efficiently repress reporter gene transcription from a prototypic thyroid responsive element in transient transfection experiments (Glineur *et al.*, 1990). It is notable that Ser 16/17 are located in a region that has been implicated in receptor DNA sequence recognition (Chen *et al.*, 1993a; Wong and Privalsky, 1995). Recently, it has been reported that phosphorylation of the serines 16/17 inhibits the binding to DNA of the v-ErbA monomer but not of the homodimer or the heterodimer with RXR, suggesting differential protein/DNA contacts between receptor monomers and dimers (Tzagarakis-Foster and Privalsky, 1998).

7.4. Binding of v-ErbA to cofactors

Due to mutations that v-ErbA contains in the LBD and the deletion of the AF-2 (activation function-2), it cannot bind ligand and hence v-ErbA cannot exert any activation function. Thus, it appears unlikely that v-ErbA can interact with coactivators. In fact, a direct interaction of v-ErbA with p300 or any other coactivator protein has not been reported.

In contrast, a number of studies have shown interaction of v-ErbA with the corepressors NCoR/SMRT *in vitro* (Busch *et al.*, 2000; Hong *et al.*, 1998; Lin *et al.*, 1997). v-ErbA was the first nuclear receptor suggested to convey repression of transcription. A transformation deficient variant of v-ErbA, termed td359-v-ErbA, which carries a mutation in helix H1 of the LBD (Pro 144→Arg), failed to repress transcription in transient transfection assays (Damm *et al.*, 1987; Damm and Evans, 1993). Subsequent experiments showed that H1 was important for interaction with corepressors (Chen and Evans, 1995; Horlein *et al.*, 1995; Li *et al.*, 1997b). Recent findings have demonstrated that v-ErbA exhibits a novel tripartite structural requirement, i.e. helices 1, 5/6 and 8 of the LBD are simultaneously required for corepressor association and repressive function (Busch *et al.*, 2000; Busch *et al.*, 1997). Very recently, a corepressor complex from *Xenopus* oocytes extracts containing NCoR/HDAC3 but not SIN3 has been shown to associate with v-ErbA (Urnov *et al.*, 2000). Although there are no structural data yet available for the interaction between

v-ErbA and corepressors, the fact that the v-ErbA-LBD bears mutations, as compared to TR, points to distinct structural requirements for v-ErbA and TRα with possible implications for their transcriptional repression properties.

7.5. Models for v-ErbA-mediated transcriptional regulation

v-ErbA was initially demonstrated to constitutively inhibit the transcriptional activation of genes mediated by T3 (Baniahmad *et al.*, 1992; Damm *et al.*, 1989; Pain *et al.*, 1990; Sap *et al.*, 1989; Zenke *et al.*, 1990). Subsequent studies showed that v-ErbA also represses genes activated by liganded RAR (Chen and Privalsky, 1993; Sande *et al.*, 1993; Sharif and Privalsky, 1991). The mechanisms underlying the antagonistic function of v-ErbA on TR/RAR remain largely unclear due to a lot of conflicting data. A number of models have been proposed, yet none of them alone can explain all the findings in the literature. In the following I present an overview of models that have been proposed during the last two decades (Figure 10).

7.5.1. Binding site occlusion model

According to the binding site occlusion model, v-ErbA exerts its repressive and oncogenic function by occluding liganded TR from binding to its cognate sites (Figure 10A) (Damm *et al.*, 1989; Pain *et al.*, 1990; Sap *et al.*, 1989; Sharif and Privalsky, 1991). Since v-ErbA cannot bind ligand, occlusion of TR results in inhibition of T3-dependent transcriptional activation. This model emerged from studies swapping DBDs between v-ErbA and TRα, which showed that v-ErbA is unable to bind ligand and to exert any activation function (Barettino *et al.*, 1994; Munoz *et al.*, 1988). Moreover, substitution of the LBD of v-ErbA with that of c-ErbA resulted in proteins able to drive full differentiation program of transformed erythroblasts after administration of T3 (Disela *et al.*, 1991; Schroeder *et al.*, 1992; Zenke *et al.*, 1990). This notion was further supported by the observation that overexpression of TR and addition of ligand could overcome the block of differentiation of erythroid progenitors caused by v-ErbA (Disela *et al.*, 1991). However, the finding that v-ErbA binds to conventional TREs with lower affinity than TR, due to mutations in the P- and D-boxes of the DBD, puts the occlusion model under dispute (Barettino *et al.*, 1993;

Nagl *et al.*, 1995; Nelson *et al.*, 1995b). Restoring the P-box mutation in v-ErbA restored wild type TRα DNA binding affinity but, unexpectedly, abolished the ability of v-ErbA to inhibit RAR-dependent transcriptional activation as well as to transform erythroid progenitors (Bauer *et al.*, 1997; Sharif and Privalsky, 1991). Therefore, it seemed likely that *in vivo* v-ErbA might function through distinct responsive elements that are not necessarily the known TREs and that other mechanistic models had to be postulated.

7.5.2. Sequestration of auxiliary factors required for TR/RAR activation function

It has been reported that v-ErbA binds to most TREs as a heterodimer with RXR (Judelson and Privalsky, 1996; Subauste and Koenig, 1998; Wahlstrom *et al.*, 1996). Given that heterodimerisation of TR or RAR with RXR is required for their efficient DNA binding and function (Bugge *et al.*, 1992; Hallenbeck *et al.*, 1992; Jin and Pike, 1996; Kliewer *et al.*, 1992; Yu *et al.*, 1991; Zhang *et al.*, 1992a), it is conceivable that sequestration of RXR by v-ErbA could impair the function of TR and RAR (Figure 10B). However, a number of observations strongly oppose a sequestration model. Firstly, v-ErbA contains a mutation (Pro 351 to Ser) that impairs its ability to heterodimerise with RXR (Barettino *et al.*, 1993; Chen and Privalsky, 1993). Secondly, *in vitro* v-ErbA binds as heterodimer with RXR to most TREs but with lower affinity than RXR-TR (Hermann *et al.*, 1993; Subauste and Koenig, 1998; Wahlstrom *et al.*, 1996). Finally, restoring the heterodimerisation ability of v-ErbA (r12-v-ErbA) yields a superactive oncogene (Barettino *et al.*, 1994; Damm *et al.*, 1987), suggesting that wild type v-ErbA is not able to efficiently sequester RXR.

7.5.3. Squelching of co-factors

Squelching of class II NR coactivators appears unlikely to contribute to repressive/oncogenic function of v-ErbA, because interactions between activator and receptor require the receptor AF-2 domain (Figure 10C) (reviewed in Glass and Rosenfeld, 2000). The nine amino acids deletion comprising the AF-2 together with point mutations of the LBD render v-ErbA unable to interact with coactivators (Barettino *et al.*, 1994; Munoz *et al.*, 1988). Swapping experiments of v-ErbA LBD

with that of TRα yielded proteins able to drive full differentiation of transformed erythroblasts after T3 administration, linking the repression function of v-ErbA with its inability to be ligand-activated (Barettino *et al.*, 1994; Disela *et al.*, 1991; Forrest *et al.*, 1990a; Lin *et al.*, 1997; Munoz *et al.*, 1988; Sap *et al.*, 1989; Zenke *et al.*, 1990).

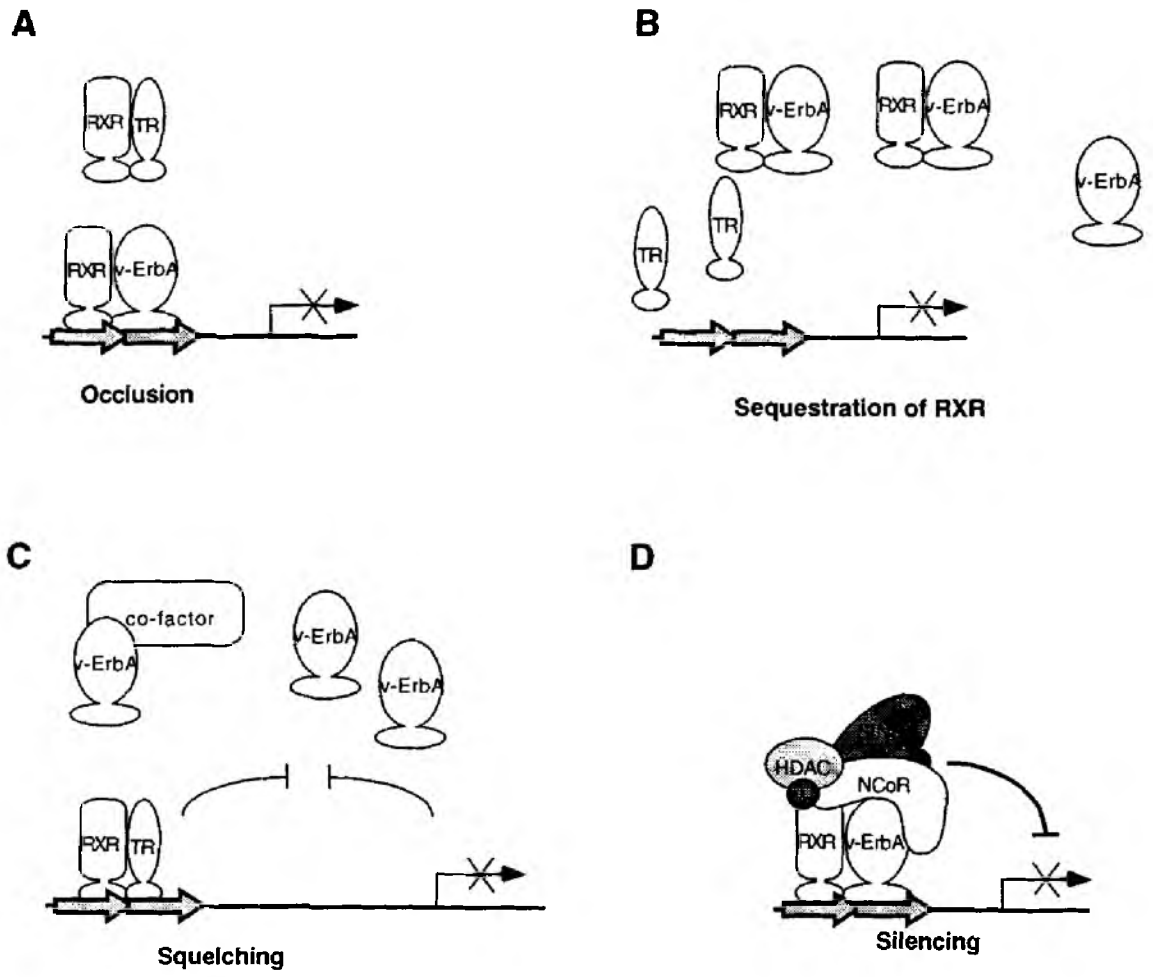


Fig 10 At least four models exist to explain the ability of v-ErbA to repress TR mediated activation via a DNA responsive element. (**A**) Occlusion: v-ErbA, as a dimer, occludes liganded TR from its cognate binding sites. (**B**) Sequestration of RXR: Since v-ErbA is overexpressed, it binds RXR in solution, thus, forbidding TR to heterodimerise with RXR. Subsequently, no functional RXR-TR heterodimers are formed that could bind to TREs to activate transcription. (**C**) Squelching: v-ErbA squelches positive co-factors required for the enhanced transcriptional activation by liganded RXR-TR. (**D**) Silencing: v-ErbA directly and constitutively interacts with corepressors resulting in silencing of transcription.

7.5.4. HRE-independent model: The AP-1 case

So far I have described models interpreting the repression function of v-ErbA mediated in *cis* i.e. via binding of v-ErbA to a DNA responsive element. Nevertheless, there are also data showing that DNA binding-independent interactions between NRs and other proteins can also account for their repressive function. For example, interference of NRs with the AP-1 transcriptional pathways has been initially reported for the glucocorticoid receptor and subsequently verified for TR and RAR (reviewed in Pfahl, 1993). Liganded TR inhibits AP-1 activation function presumably by preventing its binding to the TPA responsive elements. In the absence of hormone, TR cannot interfere with the AP-1 activation pathway (Desbois *et al.*, 1991; Nicholson *et al.*, 1990; Saatcioglu *et al.*, 1997; Salbert *et al.*, 1993; Schmidt *et al.*, 1993; Schule *et al.*, 1991; Zhang *et al.*, 1991). Since AP-1 responds to mitogenic signals responsible for sustaining cell proliferation, and given that hormones are differentiation signals, it is conceivable that a cross-talk between the two pathways at AP-1 sites functions as a sensitive balance between proliferation and differentiation. v-ErbA is not able to interfere with the AP-1 pathway, most probably because of deletion of the AF-2 domain responsible for ligand and AP-1 interaction (Schmidt *et al.*, 1993). It is likely that v-ErbA supports the proliferation program driven by AP-1, reinforcing the notion that arrest of differentiation is regulated by various pathways (Desbois *et al.*, 1991).

7.5.5. Constitutive corepressors binding

The discovery of the corepressors NCoR/SMRT shed new light on the mechanism by which NRs repress transcription, and these findings were extrapolated to the action of v-ErbA in erythroleukemia. The current model is that v-ErbA, when stably bound to DNA, is constitutively associated with NCoR/SMRT (reviewed in Stunnenberg *et al.*, 1999). NCoR/SMRT are thought to facilitate the repressive activity of v-ErbA in two ways. First, NCoR/SMRT prevent the formation and activity of the preinitiation complex (PIC) and second, via recruitment of HDACs, they facilitate a condensed, deacetylated and hence repressive chromatin structure in the neighbourhood of the v-ErbA responsive element (Stunnenberg *et al.*, 1999). Recently, a NCoR/HDAC3 containing complex from *Xenopus* oocytes extracts has been shown to enhance transcriptional repression by v-ErbA only on chromatinised templates (Urnov *et al.*, 2000). The constitutive corepressors binding model is further supported

by findings showing that overexpression of TR and ligand administration overcomes the v-ErbA induced block of differentiation (Disela *et al.*, 1991). Nevertheless, 'proof-of-principle' that NCoR/SMRT associate with v-ErbA *in vivo* and instigate repressive activity from a chromatin embedded v-ErbA responsive element (VRE), remains to be demonstrated.

In conclusion, it appears that v-ErbA inhibits transcription through a number of mechanisms that are dependent on the nature of the element, cell type-specific factors and possibly other parameters. More studies are required to unequivocally establish the connection between v-ErbA repression function and oncogenicity.

8. Outline of this thesis

At the outset of this work several studies had demonstrated the role of v-ErbA in the capacity of AEV to transform cells. Several features and mutations of v-ErbA had been characterised with respect to its oncogenicity, which were correlated with the ability of v-ErbA to repress transcription. However, little was known about the molecular mechanism of transcriptional silencing by v-ErbA. From transfection assays, using artificial v-ErbA responsive elements or thyroid responsive elements it was inferred that v-ErbA represses transcription *in cis in vivo*. Hence, there was a clear need for a model target gene with a chromatin embedded v-ErbA responsive element that would enable molecular studies on the mechanisms and factors involved in silencing by v-ErbA.

In chapter 2, the identification of two prime candidate regions (HS1 and HS2) for transcriptional regulation in the locus of carbonic anhydrase II (CA II) gene is described. The discovery of a v-ErbA responsive element (VRE) located within HS2 established that CA II is a true and direct target gene. We showed that v-ErbA binds to this VRE both *in vitro* and *in vivo* and that it is able to repress transcription of the CA II gene. In differentiating cells, v-ErbA binding was alleviated *in vivo* concomitant with a relief of repression of CA II transcription.

To further investigate the function of v-ErbA and the possibility for an interplay between v-ErbA and other factors bound to HS2, we next performed a detailed functional characterisation of HS2 (chapter 3). HS2 was demonstrated to behave as a potent enhancer in the absence of v-ErbA, and GATA-factor binding sites were shown to govern the erythroid-specific enhancer activity of HS2. v-ErbA appeared to neutralise the positive action of factors bound to GATA-sites, a function shared by unliganded TRα. Because v-ErbA is postulated to be the paradigm of an unliganded receptor that represses transcription, we compared its repressive function via the HS2-VRE with that of TRα and found that they are not equivalent in this aspect of gene regulation.

In chapter 4 we set out to investigate whether and which corepressors are involved in repression by v-ErbA *in vivo*. A dominant negative corepressor-approach was taken using a truncated NCoR protein (C-NCoR) that contains the receptor interaction domains but lacks transcriptional silencing function. AEV transformed cells stably expressing C-NCoR were established. Although a physical interaction between v-ErbA and NCoR and/or C-NCoR was demonstrated using immunoprecipitation assays, relief of transcriptional repression of the endogenous CA II gene in the presence of C-NCoR was not observed *in vivo*. The data presented question the role of NCoR in the repressive function of v-ErbA.

In chapter 5, the main conclusions derived from these studies as well as perspectives for future research are discussed.

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Chapter 2

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

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Abstract

v-ErbA, a mutated thyroid hormone receptor alpha (TR), 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 (CA II) 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 CA II transcription. Co-expression of a gag-TRα fusion protein in AEV-transformed cells and addition of ligand derepresses CA II transcription. Treatment of transformed cells with the histone deacetylase inhibitor, trichostatin A, derepresses the endogenous, chromatin-embedded CA II gene, while a transfected HS2-enhancer construct remains repressed. Taken together, our data suggest that v-ErbA prevents CA II activation by 'neutralizing' *in cis* the activity of erythroid transcription factors.

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 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 bon marrow cells expressing a dominant negative RAR 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 (Munoz *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.*, 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 (Onate *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 (CA II) (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 CA II 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 TRβ A gene minilocus which reconstitutes chromatin, permitted analysis of the TR 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 CA II 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, CA II-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 CA II gene, whereas a transfected, repressed HS2-enhancer construct remains unaffected by this treatment.

Results

DNase I hypersensitivity site induction in the CA II locus during erythroid differentiation

To identify regulatory regions in the CA II 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 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, CA II mRNA could not be detected on Northern blots (Figure 1C). Increased DNase I sensitivity was observed in the promoter region of CA II, designated prHS, only in differentiating, primary cells that actively transcribe the gene but not in non-CA II transcribing, proliferating erythroid progenitors (Figure 1B and C). We conclude that in proliferating, primary erythroid progenitors, the CA II locus is primed for expression.

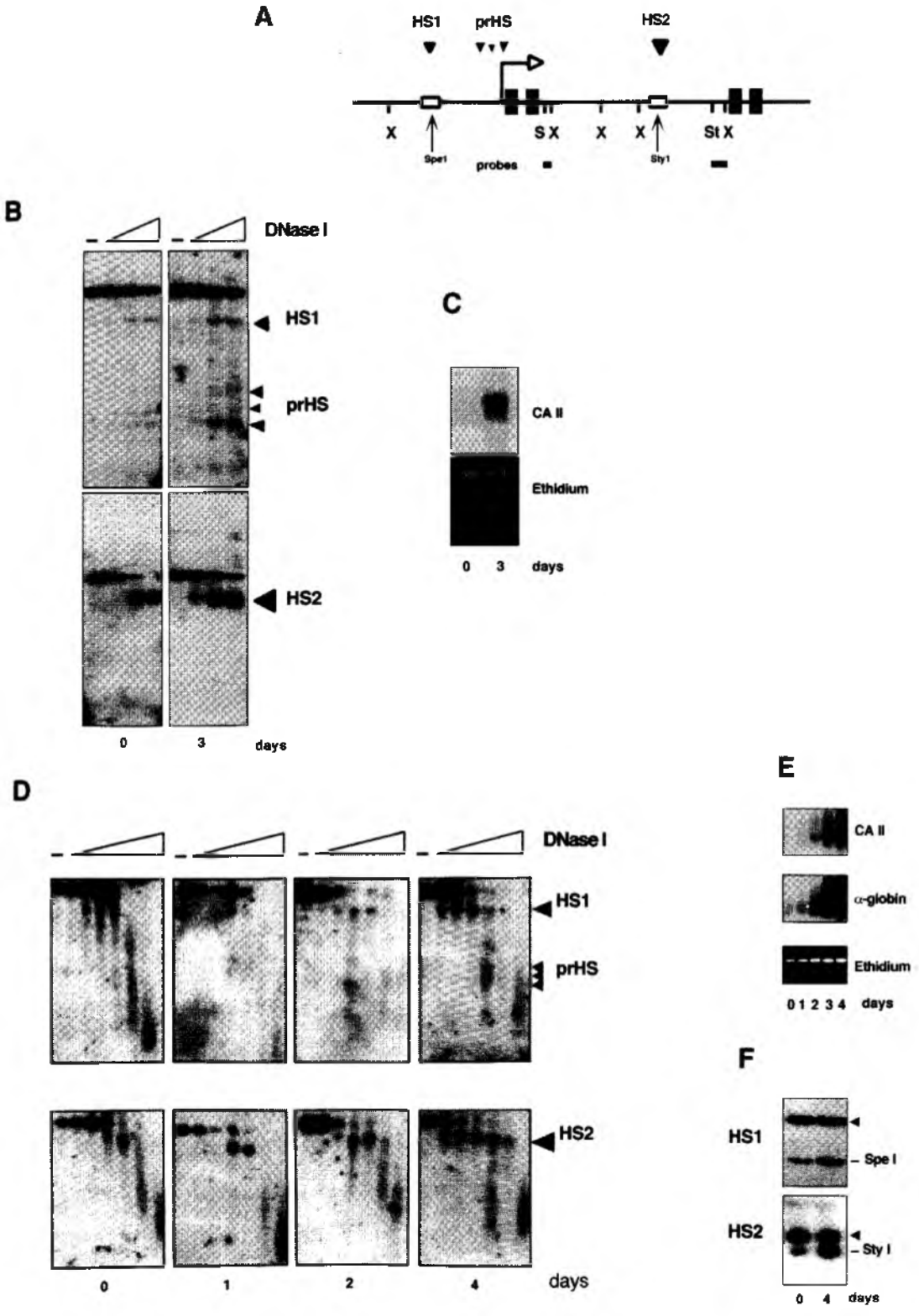
A
HS1
prHS
HS2
X
Spe1
S X
X
X
Sty1
St X
probes
B
DNase I
HS1
prHS
HS2
0
3
days
C
CA II
Ethidium
0 3 days
D
DNase I
HS1
prHS
HS2
0
1
2
4
days
E
CA II
α-globin
Ethidium
0 1 2 3 4 days
F
HS1
Spe I
HS2
Sty I
0 4 days

Figure 1 ➔

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 CA II mRNA and of -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 CA II transcription and in conveying regulation by v-ErbA.

Figure 1 Analysis of the chicken CA II locus during differentiation of primary erythroblasts and v-ErbA transformed HD3 cells. (**A**) Schematic diagram of the CA II genomic locus. Exons 1-4 (black boxes) and the relative positions of the XbaI (X), SacI (S) and StuI (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 SpeI and StyI, respectively, marking the cleaved fragments.

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 CA II 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 complementary 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 CA II gene and within the DNase I hypersensitive region HS2.

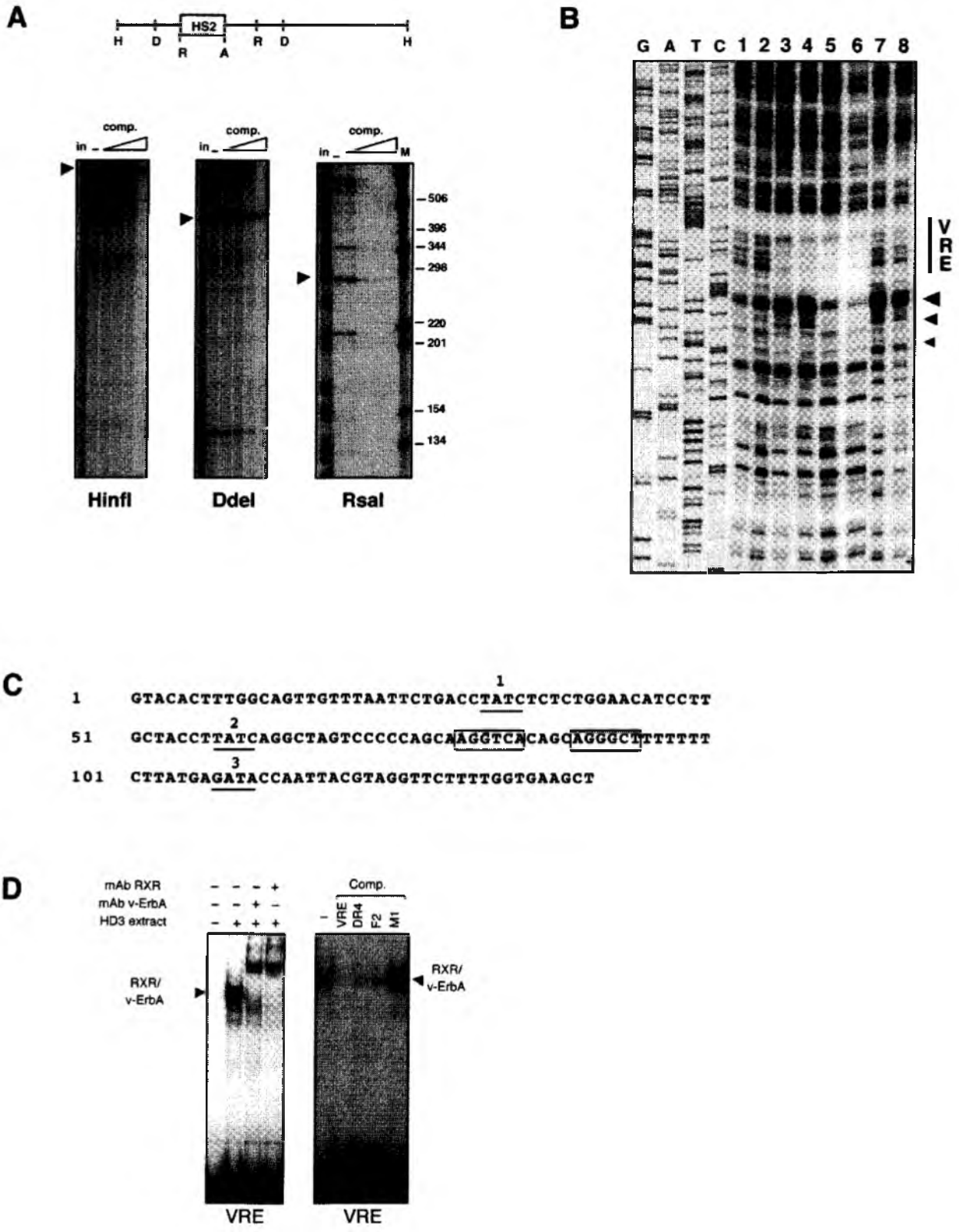
A
HS2
H D R A R D H
comp.
in
M
506
396
344
298
220
201
154
134
HinfI
DdeI
RsaI
B
G A T C 1 2 3 4 5 6 7 8
VRE
C
1 GTACACTTTGGCAGTTGTTTAATTCTGACCTATCTCTCTGGAACATCCTT
51 GCTACCTTATCAGGCTAGTCCCCCAGCAAGGTCACAGCAGGGCTTTTTTT
101 CTTATGAGATACCAATTACGTAGGTTCTTTTGGTGAAGCT
D
mAb RXR
mAb v-ErbA
HD3 extract
Comp.
VRE
DR4
F2
M1
RXR/
v-ErbA
VRE
VRE

Figure 2 ➔

v-ErbA binds to the CA II-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 CA II 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.

Figure 2 Identification and characterization of a v-ErbA binding site in CA II. (**A**) Immunoprecipitation of v-ErbA-DNA complexes. ^{32}P-labeled DNA fragments generated by HinfI (H), DdeI (D) and RsaI (R) digestion of clone pCA IIX/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 RsaI 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 RsaI-AluI 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.

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 CA II 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 CA II promoter and lends support to the notion that v-ErbA represses CA II transcription through the putative HS2 enhancer.

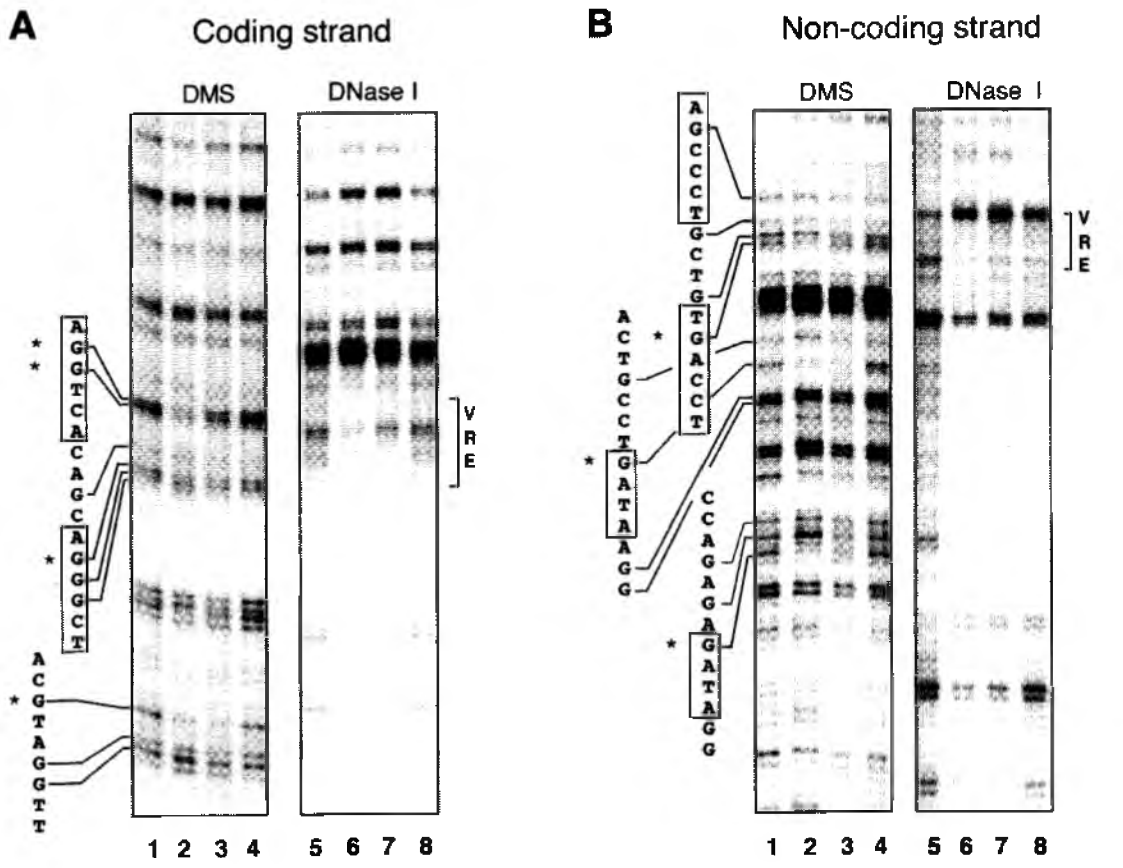

Figure 3 DMS and DNase I *in vivo* footprinting. DMS (lanes 1-4) and DNase I (lanes 5-8) *in vivo* footprinting of (**A**) the coding strand, and (**B**), the non-coding strand, in HD3 cells before (lanes 2 and 6) and after (lanes 3 and 7) induction of differentiation, in chicken embryo fibroblasts (lanes 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 (*).

HS2 is an enhancer

To test whether the HS2 indeed functions as an enhancer, the 137 bp RsaI-AluI 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 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.

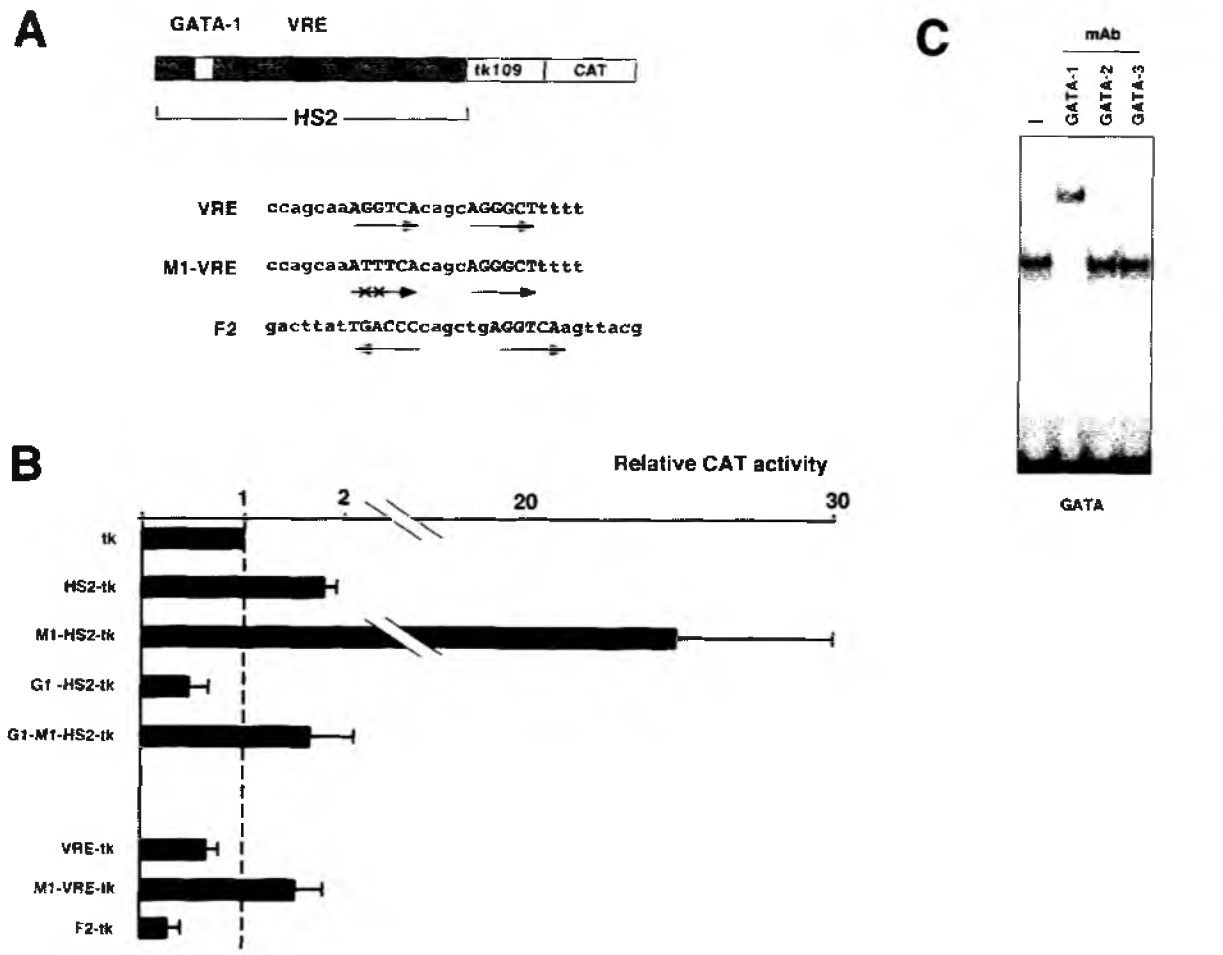

Figure 4 Transcriptional repression in v-ErbA expressing HD3 cells is mediated by the VRE. (**A**) Schematic diagram of the tk reporter constructs containing either fragments of HSV or synthetic oligonucleotides. The nucleotide sequence of the VRE, M1-VRE and F2 oligonucleotides are shown, with arrows indicating the half-sites and the crosses indicating the mutated bases. In addition to the RsaI-AluI 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 32P-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.

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

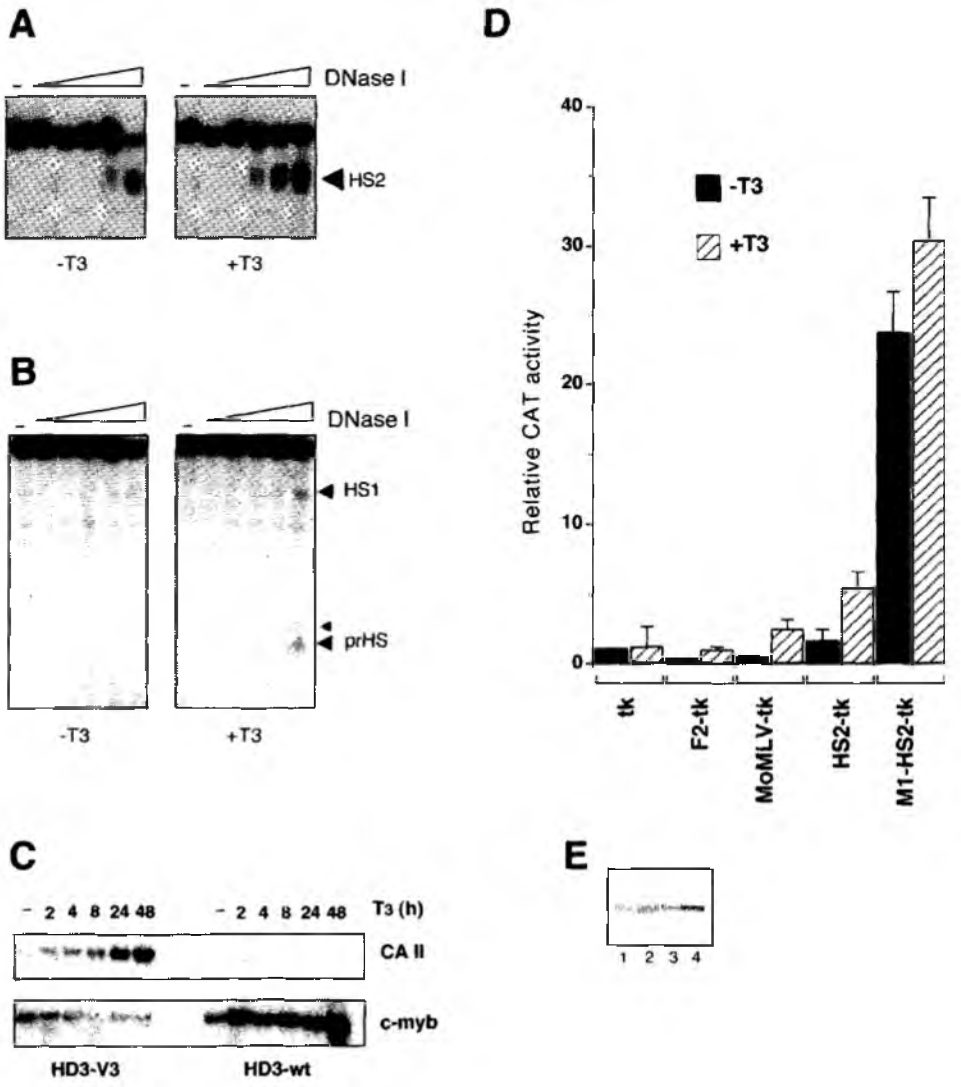

Figure 5 Transcriptional activation and chromatin remodeling instigated by ligand-activated gag-cTR. (**A** and **B**) DNase I hypersensitive site mapping in HD3 cells expressing gag-cTRα (named HD3-V3) before or after 24 h of T3 treatment. (**C**) Northern blot analysis of CA II and c-myb in the course 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.

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 CA II 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 CA II 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 CA II gene and that v-ErbA binds to the VRE *in vitro*, we tentatively conclude that v-ErbA acts to repress the CA II 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 CA II 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 3x(RARE2)-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 CA II gene was activated upon these treatments (Figure 6B). Surprisingly, the TSA treatment alone resulted in significant activation of transcription from the endogenous CA II 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 CA II gene was sensitive to TSA as well as to T3 treatment whereas the transfected HS2 enhancer only responded to ligand, but not to TSA treatment. Similar results were obtained with the HD3 cells (data not shown).

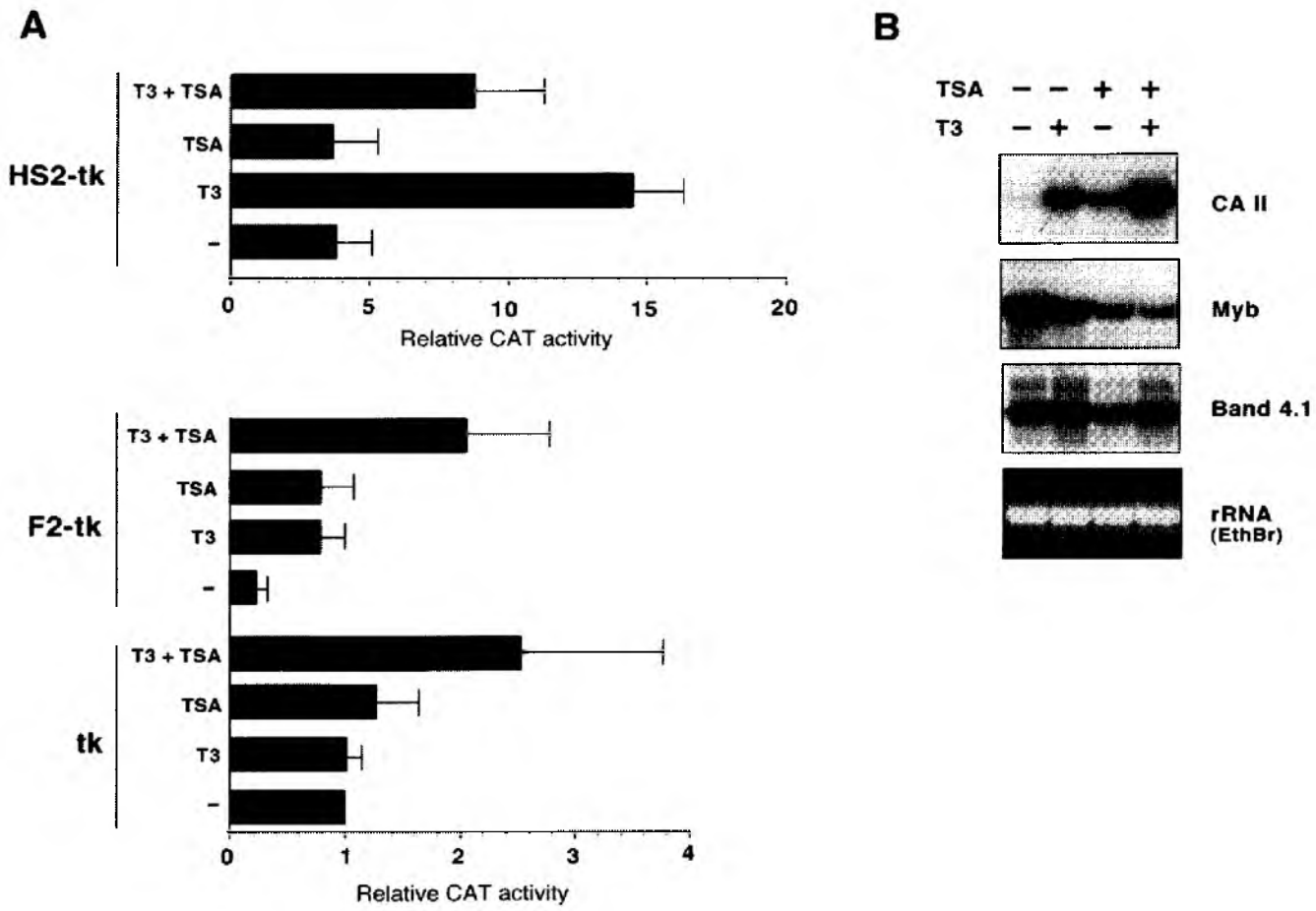

Figure 6 Effect of TSA and T3 treatment of HD3-V3 cells. (**A**) Transient transfection assays with the indicated constructs, with CAT activities expressed relative to the tk reporter alone. (**B**) Northern blot analysis of endogenous genes in the treated cells. Cells were treated with the indicated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

Discussion

v-ErbA acts *in cis* to repress CA II 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 CA II (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 CA II (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 CA II 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 CA II 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 CA II 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 CA II 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 CA II 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 CA II 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 CA II 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.

CA II-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 CA II 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 CA II, 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 CA II-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 GlySer 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 CA II *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 TRβ A 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 RAR2 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 RAR2 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 RAR 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 XbaI and hybridized with the ^{32}P-labeled riboprobes, SacI-XbaI and StuI-XbaI, 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 SpeI or StyI 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; CA II, 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 clone pCA IIX/N containing a 17 kb fragment of the CA II gene (cloning will be described elsewhere) was digested with HinfI, RsaI and DdeI, respectively, and labeled. DNA (5 pmol) was incubated with HD3 extracts in 20 mM HEPES pH 7.9, 100 mM NaCl, 5 mM MgCl2, 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 0.5xTBE, 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 EF1-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 RsaI-AluI fragment of 137 bp spanning HS2 was inserted into the HindII-BamHI 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 SalI and XhoI termini into the XhoI 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 RsaI 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'-CCAGTCTGTGCCAAGTAGTTC-3', 5'-GCTGAGTTGAAATCACTG-3'; non-coding strand: 5'-TGACAAGCAGGAGAGTAA-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.

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Chapter 3

The v-ErbA oncoprotein quenches the activity of an erythroid-specific enhancer

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Abstract

v-ErbA is a mutated variant of thyroid hormone receptor (TRα/NR1A1) borne by the Avian Erythroblastosis virus causing erythroleukemia. TRα is known to actively repress specific genes in the absence of its cognate ligand, T3 hormone. v-ErbA is unable to bind ligand, and hence cannot act as a positive transcription factor. v-ErbA is thought to contribute to leukemogenesis by actively repressing erythroid-specific genes such as the carbonic anhydrase II gene (CA II). In the prevailing model, v-ErbA occludes liganded TR from binding to its cognate elements and constitutively interacts with the corepressors NCoR/SMRT. We previously identified a v-ErbA responsive element (VRE) within a DNase I hypersensitive region (HS2) located in the second intron of the CA II gene. We now show that HS2 fulfils all the requirements for a genuine erythroid-specific enhancer that functions independent of its orientation and position. We find that the HS2 enhancer activity is governed by two adjacent GATA-factor binding sites. v-ErbA prevents HS2 activity by quenching the positive function of factors bound to GATA-sites. However, v-ErbA does not convey active repression to silence the transcriptional activity intrinsic to the heterologous tk promoter. In contrast, unliganded TR can efficiently quench HS2 enhancer activity as well as actively repress a downstream promoter. We propose that depending on the sequence and context of the binding site, v-ErbA contributes to leukomogenesis by occluding liganded TR as well as unliganded TR thereby preventing activation or repression, respectively.

Introduction

Malignant transformation of hematopoietic cells is caused by the disturbance of a delicate balance between proliferation and differentiation. The correct functioning of members of the nuclear hormone receptor superfamily is required to maintain homeostasis, and to promote differentiation of hematopoietic cells. Not surprisingly, therefore, mutated, aberrant nuclear receptors are found in many types of cancer and

diseases (Goldhirsch and Gelber, 1996). Paradigms are fusion proteins involving retinoic acid receptor (RARα), PML-RARα, PLZF-RARα and NPM-RARα that cause human acute promyelocytic leukemia (APL) (Warrell *et al.*, 1993) and v-ErbA, an oncogenic version of chicken thyroid hormone receptor (TRα), involved in avian erythroleukemia (AEL) (Beug *et al.*, 1996). Fatal avian erythroleukemia is caused by infection with the Avian erythroblastosis virus (AEV) which expresses two co-operating oncogenes (v-ErbB and v-ErbA). v-ErbB is a mutated epidermal growth factor receptor (EGFR) with constitutive tyrosine kinase activity whereas v-ErbA does not bind ligand and hence is proposed to display a constitutive silencing activity. While v-ErbB alone causes a partial differentiation arrest and a delayed, weak erythroleukemia, v-ErbA itself induces disease only when vastly overexpressed (Beug *et al.*, 1996). The transformation promoting activity of v-ErbA lies in its ability to arrest differentiation of v-ErbB expressing erythroblasts by effectively silencing stage-specific erythroid genes such as the carbonic anhydrase II gene (CA II) (Disela *et al.*, 1991).

Due to mutations in the presumed ligand binding domain, v-ErbA can neither bind T3 nor activate transcription. Consequently, v-ErbA is thought to act as a dominant negative oncogenic receptor variant that antagonises T3-mediated transcriptional activation (Sap *et al.*, 1989). TRα (NR1A1) (Nomenclature committee, 1999), acts as a ligand-operated molecular switch; firstly, in the absence of ligand, TR is proposed to repress transcription via association with corepressors such as NCoR/SMRT (Horlein *et al.*, 1995). The corepressors, in turn, recruit histone deacetylase (HDAC)-containing complex(es) that stabilise(s) repressive, 'closed' chromatin structure to assure efficient silencing of downstream genes (Bauer *et al.*, 1998; Heinzel *et al.*, 1997). Second, upon ligand binding, the receptor undergoes a conformational change that causes dissociation of the corepressor complex(es) and permits subsequent association of coactivator complexes such as p300/CBP, SRC and TRAP/SMCC to activate transcription (reviewed in Glass and Rosenfeld, 2000). Hence, the ability of v-ErbA to transform erythroid progenitors can be attributed to its repressive unliganded 'mode' (Horlein *et al.*, 1995). The fact that many of the mutations in v-ErbA abolish activation functions present in genuine TRα (NR1A1), (Barettino *et al.*, 1994) is consistent with the occlusion/corepressor model. Although

attractive, this model awaits *in vivo* verification; thus far, supporting data have been obtained almost exclusively from transient transfection assays, two-hybrid assays and *in vitro* GST-pull down experiments. One observation in particular is difficult to reconcile with this model. v-ErbA, as compared to TR, shows lower affinity and altered DNA specificity for classical thyroid responsive elements (TREs) mainly due to a mutation in the DNA-recognition helix (P-box) (Judelson and Privalsky, 1996). Reversion of this mutation restores wild type (TR) DNA binding affinity. However, rather than acting as a superoncogene (due to increased DNA binding properties), the P-box-reverted v-ErbA variant has lost the ability to transform erythroid cells (Nelson *et al.*, 1994). These observations imply that v-ErbA does not merely function as a constitutive unliganded TR; moreover, the set for v-ErbA binding sites appears to be distinct from or only partially overlapping with that of TR.

In a previous study, we identified a DNase I hypersensitive site (HS2) in the second intron of the chicken carbonic anhydrase II (CA II) gene (Ciana *et al.*, 1998). CA II is a direct target gene of v-ErbA and deregulation of CA II transcription partially accounts for the v-ErbA induced transformation (Fuerstenberg *et al.*, 1992). Our data suggested that HS2 might act as an enhancer. We showed that the v-ErbA oncoprotein bound to a v-ErbA responsive element (VRE), located within the HS2, prevented the activity of the enhancer. Here, we report on a detailed analysis of the CA II-HS2 enhancer that provides insight into the mechanism by which v-ErbA affects transcription of erythroid-specific genes, therefore blocking differentiation during lineage commitment. Using transient transfection assays in AEV-transformed erythroid progenitor (HD3) cells we have assessed *cis*-acting sequences governing the CA II-HS2 function and have defined HS2 as a true tissue-specific enhancer that acts independent of its position and orientation with respect to the promoter. Furthermore, the activation of the HS2 enhancer is dependent on factors bound to GATA-protein binding sites and v-ErbA acts by quenching their activity. Our study reinforces that v-ErbA acts as a dominant negative TR by occluding the VRE and ablating the T3-mediated activation of CA II. However, when the HS2-VRE is linked to the tk promoter, v-ErbA, but not unliganded TR, is inefficient in repressing the transcriptional activity intrinsic to the tk promoter. These data suggest that v-ErbA exerts only part of the full complement of negative, repressive activity intrinsic to TR.

Results

Carbonic anhydrase II HS2 region acts as a true enhancer

Using DNase I hypersensitivity mapping, we have previously identified a novel v-ErbA responsive element (VRE), comprised of two half-sites in a direct repeat configuration spaced by 4 nucleotides (DR4), located in a hypersite in the second intron of carbonic anhydrase II (CA II) gene, termed HS2 (Ciana *et al.*, 1998). Transient transfection assays in AEV-transformed HD3 cells using a HS2-containing reporter (HS2-tk) showed that HS2 does not confer any transcriptional activity to the promoter. However, mutation of the first half-site of the VRE (M1-HS2-tk), that abolished v-ErbA binding resulted in a 30-fold enhancement of transcription (Ciana *et al.*, 1998). These and other observations suggested that the HS2 region likely functions as an enhancer which is repressed by v-ErbA.

Operationally, enhancers are defined as *cis*-acting sequences that activate transcription of promoters in a position- and orientation-independent manner. To test orientation (in)dependency we first placed the M1-HS2 element in both orientations (+ and -) upstream of its cognate CA II promoter (–180 to +51) yielding M1-HS2(+)-CAII and M1-HS2(-)-CAII constructs, respectively. Transient transfection assays in HD3 cells showed that M1-HS2 boosted the level of transcription via the CA II promoter to the same extent as observed for the tk promoter reporter (Figure 1B). Moreover, the HS2 enhancer acted in both orientations. As expected, the HS2 fragment with the wild type VRE did not activate transcription from the CA II promoter (data not shown). Similar results were obtained with M1-HS2 as an enhancer in combination with a longer CA II promoter fragment (1.4 kb, position –1362 to +51) (data not shown).

Since the natural position of the HS2 enhancer in CA II gene is in the second intron (Figure 1A), we tested whether HS2 could enhance transcription from a position downstream of the CA II promoter. Transcription of the resulting constructs (CAII-M1-HS2) was boosted 11- to 16-fold (Figure 1C). Analogously, the M1-HS2 fragment placed in either orientation downstream of the tk promoter also enhanced transcription but to a lesser extent (data not shown).

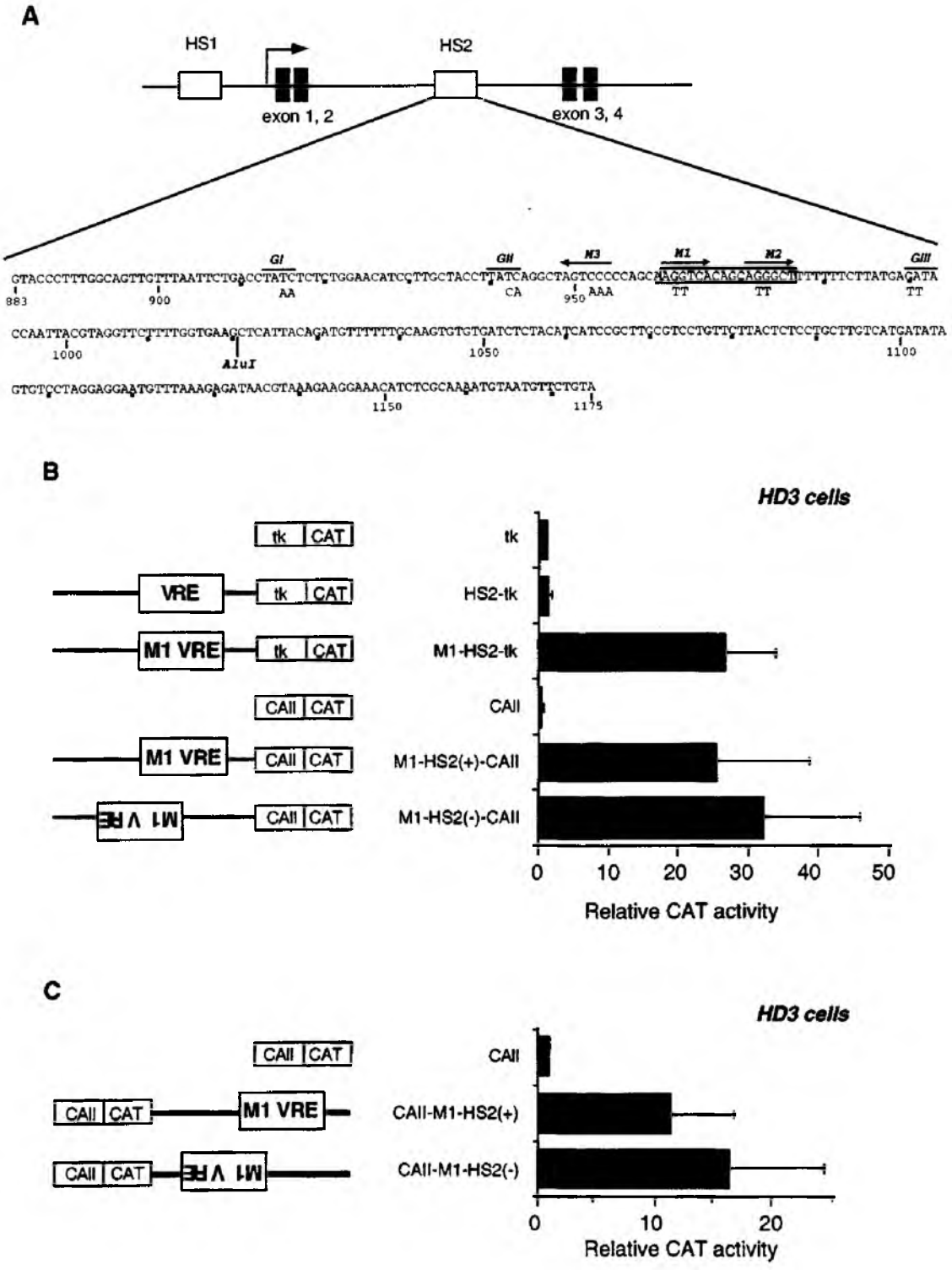
A
HS1
HS2
exon 1, 2
exon 3, 4
GI
GII
N3
M1
M2
GIII
GTACCCTTTGGCAGTTGTTTAATTCTGACCTATCTCTCTGGAACATCCTTGCTACCTTATCAGGCTAGTCCCCCAGCAAGGTCACAGCAGGGCTTTTTTTCTTATGACATA
883
900
AA
CA
950
AAA
TT
TT
TT
CCAATTACGTAGGTTCTTTTGGTGAAGCTCATTACAGATGTTTTTTTGCAAGTGTGTGATCTCTACATCATCCGCTTGCGTCCTGTTCTTACTCTCCTGCTTGTCATGATATA
1000
AluI
1050
1100
GTGTCCTAGGAGGAATGTTTAAAGAGATAACGTAAAGAAGGAAACATCTCGCAAAATGTAATGTTCTGTA
1150
1175
B
HD3 cells
tk CAT
VRE tk CAT
M1 VRE tk CAT
CAII CAT
M1 VRE CAII CAT
M1 VRE CAII CAT
tk
HS2-tk
M1-HS2-tk
CAII
M1-HS2(+)-CAII
M1-HS2(-)-CAII
0
10
20
30
40
50
Relative CAT activity
C
HD3 cells
CAII CAT
CAII CAT M1 VRE
CAII CAT M1 VRE
CAII
CAII-M1-HS2(+)
CAII-M1-HS2(-)
0
10
20
Relative CAT activity

Figure 1 ➔

Taken together, these results unambiguously show that HS2 acts both upstream and downstream of its natural CA II promoter as well as the heterologous tk promoter in an orientation independent fashion and is therefore a *bona fide* enhancer. Furthermore, v-ErbA appears to impair the activity of the potent HS2 enhancer.

HS2 is an erythroid-specific enhancer

To assess whether HS2 acts as a ubiquitous cell-type specific enhancer we performed transfection experiments using the HS2-tk and M1-HS2-tk reporters in a number of different cell lines including mouse erythroleukemia cells (MEL), chicken embryonic fibroblasts (CEF) or mouse fibroblasts (L). The experiments revealed HS2-mediated activation of transcription in MEL cells but not in the non-erythroid CEF or L cells (Figure 2). The HS2 enhancer activity, therefore, appears to be confined to erythroid lineages. In MEL cells, the transcriptional activity obtained with the HS2-tk reporter was 6-fold compared to tk reporter alone. This rather moderate level of enhancement by HS2-tk as compared to M1-HS2-tk is surprising since MEL cells do not express v-ErbA and thus full enhancer activity was anticipated. The lower level of enhancer activity in MEL cells is most probably due to differences inherent to these two erythroid cell lines used (see also discussion).

Figure 1 The HS2 fragment functions as a true enhancer (**A**) Schematic representation of the CA II locus with exons 1-4 (black boxes) and DNase I hypersensitive sites HS1 and HS2 (open boxes). The insert details the 288bp (RsaI-RsaI) fragment with Alu I site defining the 3' boundary of the 135 bp fragment used in most of the following experiments. GI, GII and GIII indicate position of GATA-sites that were mutated and M1, M2 and M3 show the mutated receptor-half-sites. The VRE sequence is boxed. Letters below the sequence indicate the substitution mutations in the various HS2 constructs. (**B**) Transient transfection assays in HD3 cells with CA II promoter reporter constructs carrying the 135 bp M1-HS2 enhancer fragment in both orientations. The values represent the mean value of the relative chloramphenicol acetyltransferase (CAT) activity, as normalised by luciferase values of the internal control, of at least three experiments, with error bars shown. Transcriptional activity of tk-CAT construct is arbitrarily set to 1. (**C**) Transfection assays in HD3 cells with reporters containing the M1-HS2 fragment downstream of the CA II promoter as indicated. Transcriptional activity of CA II reporter is set to 1.

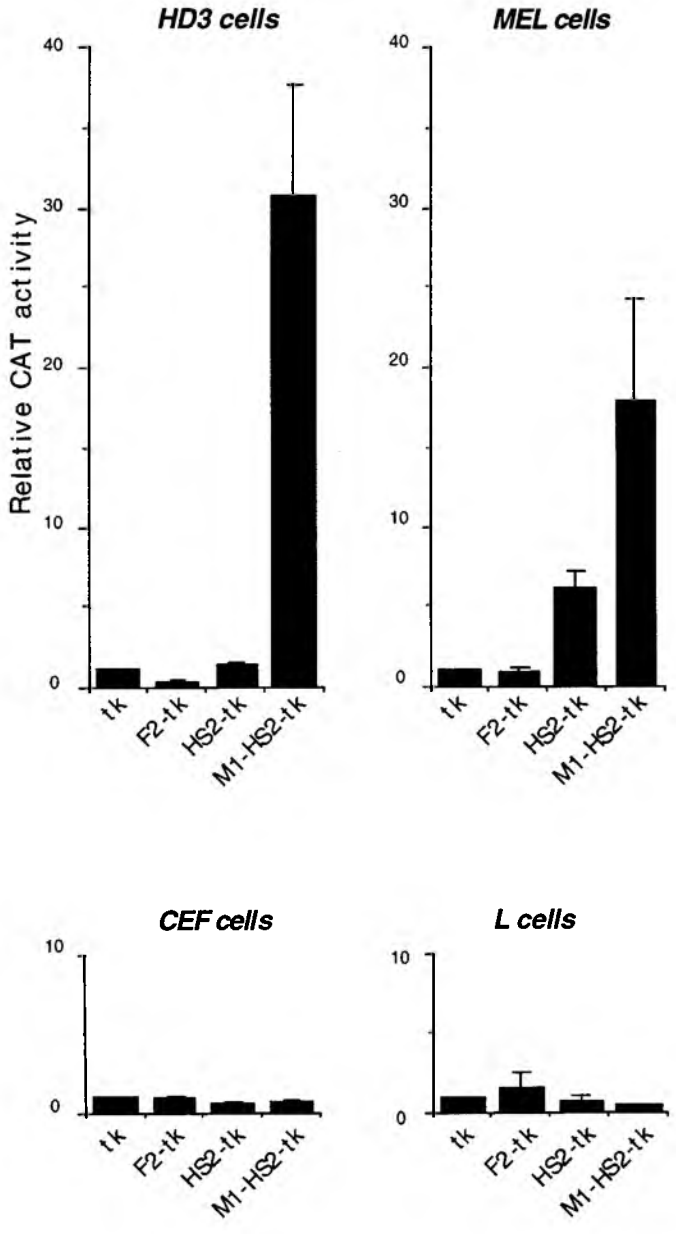

Figure 2 The HS2 enhancer activity is erythroid-specific. Transient transfection assays with the indicated tk reporter constructs in various cell lines as shown. The values represent the mean value of the relative CAT activity of at least three experiments. Transcriptional activity of tk-CAT construct is arbitrarily set to 1.

Delineation of the HS2 enhancer

To dissect the molecular mechanism of repression by the v-ErbA oncoprotein, we set out to identify *cis*-acting sequences that are important for the observed HS2 enhancer activity. Given the presence of high levels of v-ErbA in HD3 cells, fragments spanning the HS2 and carrying the wild type VRE sequence do not display enhancer activity (Ciana *et al.*, 1998). Therefore, we constructed a series of 5' and 3' deletion mutants within the context of M1(883-1175)-tk, a 288bp fragment encompassing the entire HS2, to screen for *cis*-acting sequences (Figure 3). This construct showed enhancer activity comparable to that of the previously analysed, 135 bp containing M1-HS2-tk reporter. Two deletions from the 5' end of the M1(883-1175)-tk fragment significantly reduced the HS2 activity (Figure 3), while further deletion including the VRE (plasmid (979-1175)-tk) resulted in abolishment of the enhancer activity.

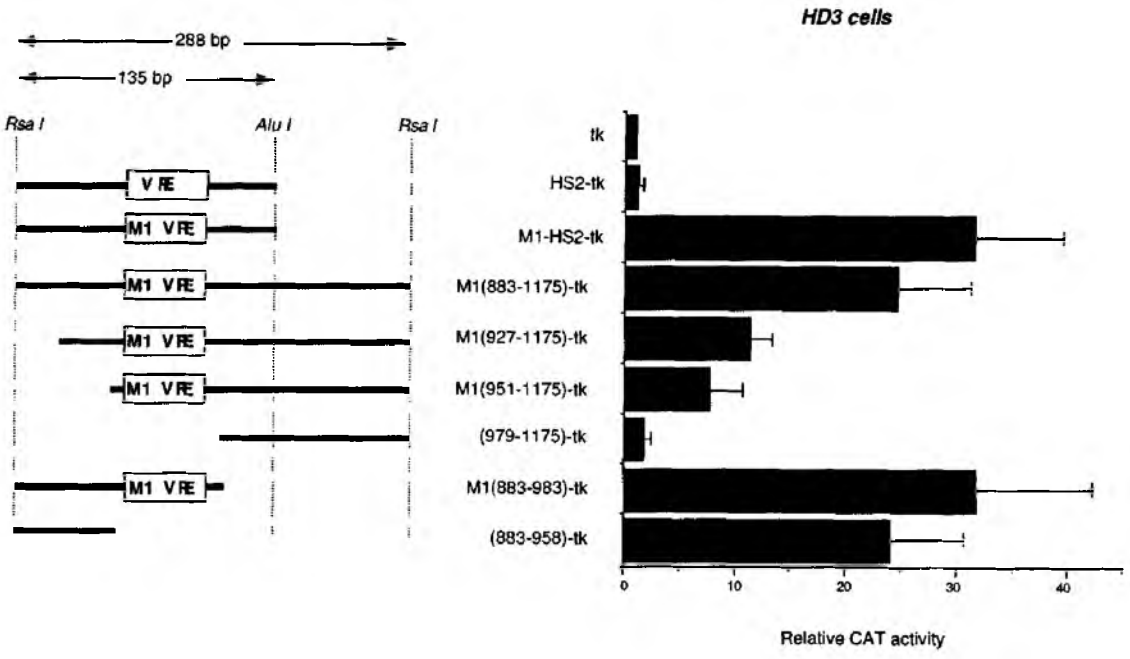

Figure 3 Delineation of the intronic enhancer region HS2 of the CA II gene. Assays of CAT expression of HD3 cells transfected with the indicated fragments of HS2 enhancer cloned in front of tk promoter. The values represent the mean value of the relative CAT activity of at least three experiments. Transcription is expressed relative to tk promoter alone.

Analysis of a 3' deletion mutant of M1-HS2-tk (up to position -983) revealed a transcriptional activity similar to M1-HS2-tk, while further 3' deletion including the VRE (up to position -958) resulted in slightly lower levels of transcription. Taken together, our data show that sequences critical for the enhancer activity are located mostly upstream of the VRE. For reasons of consistency, the 135-bp HS2 region used in our previous study (Ciana *et al.*, 1998), which exhibits the full enhancer activity in HD3 cells, was used in the remainder of the experiments.

GATA-factors binding sites contribute to the HS2 enhancer activity

The obtained overall activity of the HS2 enhancer probably is the net result of repression instigated from the VRE and enhancement from positive (erythroid-specific) factors binding to the enhancer. We have previously shown (Ciana *et al.*, 1998) that two of the three potential GATA-factor binding sites present in the HS2 enhancer fragment (positions 913 and 940 referred to as I and II, respectively) (Figure 1A) were protected in DMS footprint assays *in vivo* and showed DNase I hypersensitive cutting *in vitro*. A third potential GATA-site (III, position 990) was protected only in *in vitro* footprint assays. Using electrophoretic mobility shift assays (EMSA), we also showed that the GATA-1 protein present in HD3 cells binds to GATA-site I. Transient transfection assays confirmed that this GATA-element was required for the HS2 enhancer activity.

To elucidate the function of the two other GATA-sites, we introduced mutations in these sites in the M1-HS2-tk context i.e. in a configuration in which the contribution of the GATA-sites can be monitored. In transient transfection assays in HD3 cells, mutation of either of the GATA-sites I or II (GI-M1-HS2 and GII-M1-HS2, respectively) abolished enhancer activity (Figure 4). In contrast, mutation of the GATA-site III (GIII-M1-HS2) resulted only in a moderate reduction of the enhancer activity. These results are in accordance with the deletion analysis (Figure 1B) showing that DNA sequences downstream of the VRE do not significantly contribute to the overall activity of the enhancer. In conclusion, the HS2 enhancer activity is strongly dependent on the two adjacent GATA-factor binding sites (I and II) located upstream of the VRE. These observations corroborate and extend the notion that v-

ErbA quenches the positive transcriptional activity of factors (possibly GATA-proteins) bound to these GATA-sites.

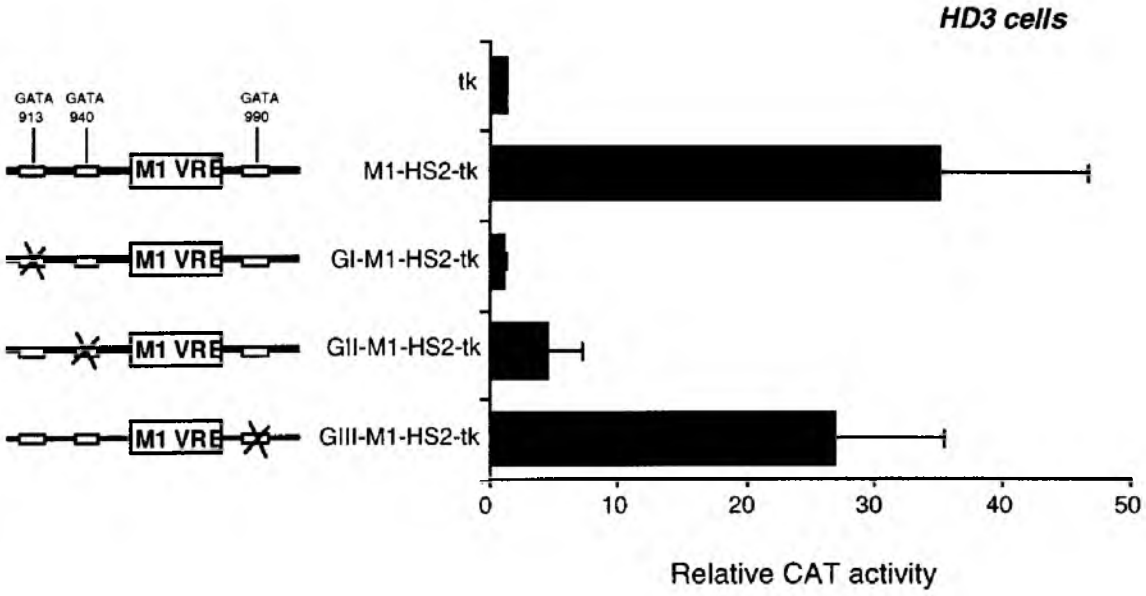

Figure 4 GATA-factor binding sites are required for the HS2 enhancer activity. Transient transfection assays in HD3 cells with tk reporters carrying the M1-HS2 fragment with mutations of the individual GATA-sites. The values represent the mean value of the relative CAT activity of at least three experiments. Transcriptional activity of tk-CAT construct is arbitrarily set to 1.

HS2 contains multiple half-sites

We have previously shown that v-ErbA binds to the VRE as a heterodimer with RXR (Ciana *et al.*, 1998). The HS2-VRE deviates from a canonical TRE element in the second half-site, an AGGgCt sequence rather than AGGTCA (Figure 1A). Closer inspection of the HS2 sequence revealed additional potential nuclear receptor half-sites. Of particular interest is the non-consensus half-site (position 949-954) located six base-pairs upstream of the VRE, which together with the first half-site of the VRE form an everted repeat (ER) spaced by 6 nucleotides (Figure 1A). Given that ER6-type elements, such as the F2 from the chicken lysozyme promoter, have been shown to be

targets for v-ErbA regulation of transcription (Baniahmad *et al.*, 1990), we investigated the relative contribution of each of these half-sites in the v-ErbA mediated transcriptional repression via HS2 by mutagenesis analysis (Figure 5). Transient transfection assays of the resulting M2-HS2-tk and M3-HS2-tk constructs in HD3 cells revealed enhancement of HS2 transcription suggesting that both the DR4 and the ER6 elements equally contribute to repression of the enhancer activity. Furthermore, we observed that the central consensus half-site alone was sufficient to confer repression (2-fold) (Figure 5), consistent with data from Baniahmad and co-workers (Baniahmad *et al.*, 1990). Thus, all three half-sites contribute to the v-ErbA-mediated repression, but only the central consensus half-site is critical.

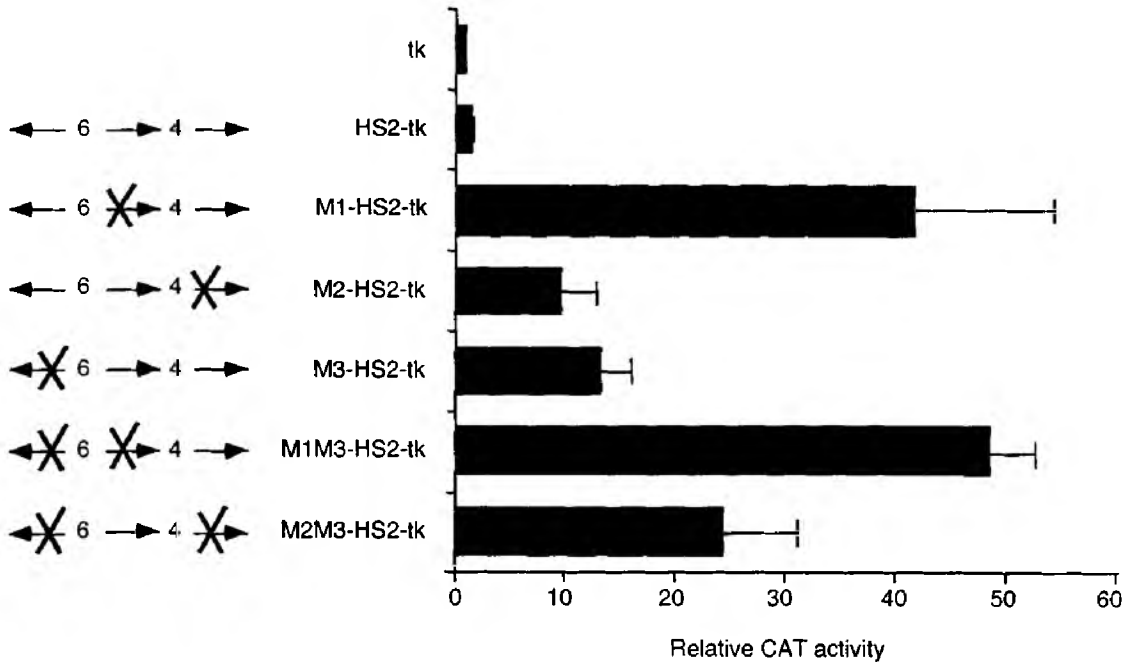

Figure 5 The HS2 enhancer contains multiple half-sites that can mediate repression by v-ErbA. Transient transfection assays in HD3 cells with tk reporters containing mutations of the indicated potential half-sites (see Figure 1A for sequence). The values represent the mean value of the relative CAT activity of at least three experiments. Transcription is expressed relative to tk promoter alone.

VRE can act as a TRE

Given the altered DNA binding specificity of v-ErbA due to the P-box mutation (Judelson and Privalsky, 1996), it was not *a priori* granted that TR could bind to the CA II-VRE and mediate T3-dependent activation. We, therefore, performed EMSA experiments using the VRE oligonucleotide and extracts from HD3 cells or nuclear receptors expressed from vaccinia virus. As shown in Figure 6A, RXR-TR efficiently bound to the VRE (lane 8) suggesting that this element may serve as a TRE. Neither v-ErbA nor RXR alone bound efficiently to the VRE under these conditions (lanes 3-5). Addition of the same amounts of v-ErbA plus RXR (lanes 6 and 7) led to formation of a protein-DNA complex with a mobility similar if not identical to that obtained with HD3 cell extracts (lane 2). The faster migrating complex (lanes 3, 4, 6 and 7) most likely represents a v-ErbA monomer bound to the VRE.

To measure the relative affinities of RXR-v-ErbA and RXR-TR for the VRE, off-rate experiments were performed. As a competitor, unlabelled VRE probe was added in 100- or 1000-fold molar excess to pre-formed complexes and aliquots were loaded on a running gel at the indicated time-points. As shown in Figure 6B (and data not shown), the complex formed by RXR-v-ErbA disappeared immediately upon addition of competitor, whereas under the same conditions the RXR-TR was detectable up to 4 minutes after addition of the competitor. The $t_{1/2}$ for the RXR-v-ErbA complex on the VRE is in the range of seconds while that for RXR-TR is in a range of minutes. These results show that the affinity of RXR-v-ErbA for the HS2-VRE is significantly lower than that of RXR-TR. A gag-TRα receptor fusion (termed V3) expressed in the HD3V3 cells (Disela *et al.*, 1991) appeared to bind to the HS2-VRE in conjunction with endogenous RXR with kinetics similar to that of recombinant RXR-TR (data not shown). These observations suggest that the differential binding behaviour of RXR-v-ErbA and RXR-TR was not due to an intrinsic difference in DNA binding characteristics between recombinant, vaccinia expressed and 'natural' receptors. It should be noted that the RXR-TR complex formed on the MoMLV-TRE (DR4-type) or on a consensus DR4 is more stable than on the VRE (Bugge *et al.*, 1992), emphasising that the VRE is not an optimal binding site for RXR-TR.

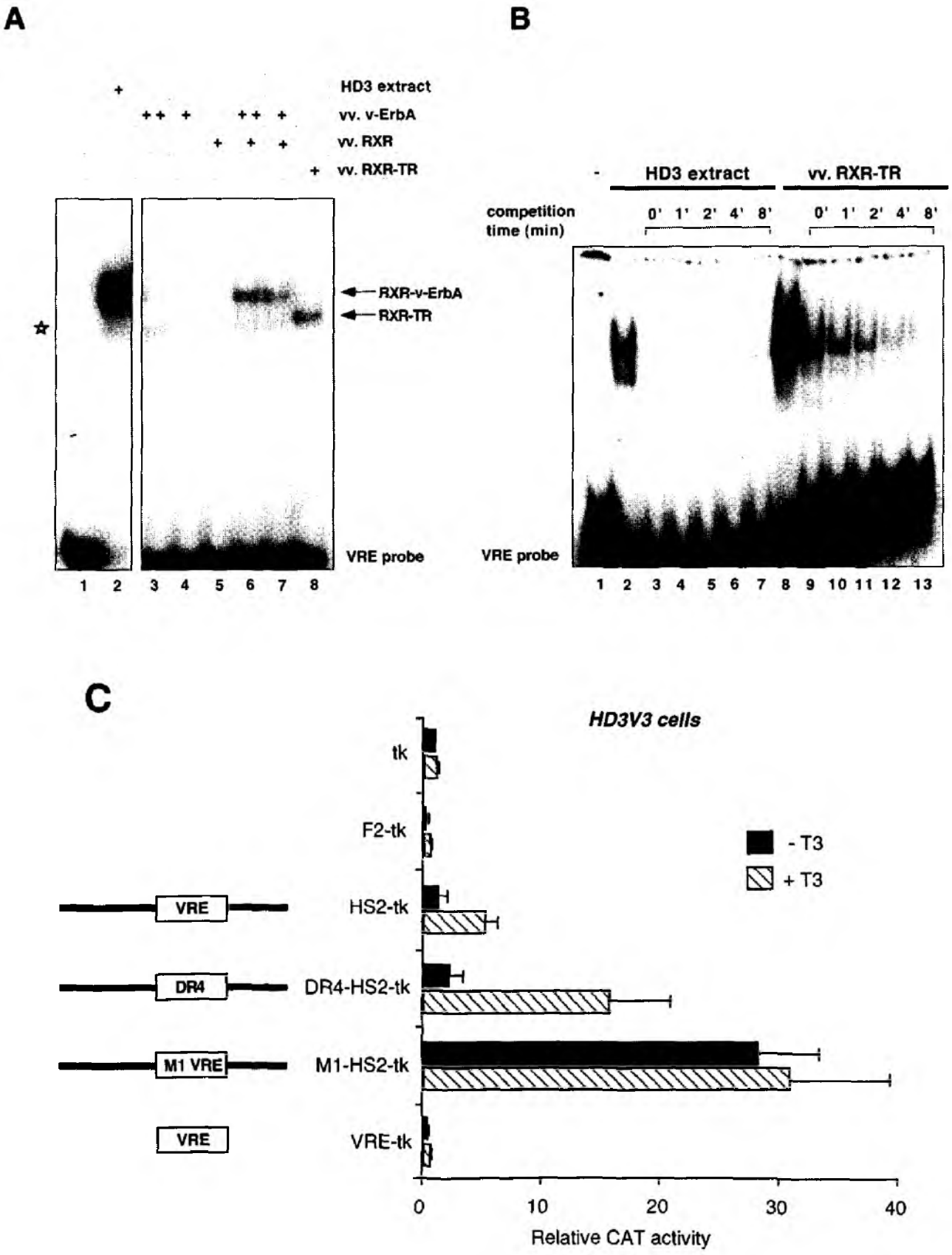
A
HD3 extract
vv. v-ErbA
vv. RXR
vv. RXR-TR
RXR-v-ErbA
RXR-TR
VRE probe
1 2 3 4 5 6 7 8
B
HD3 extract
vv. RXR-TR
competition time (min)
0' 1' 2' 4' 8'
0' 1' 2' 4' 8'
VRE probe
1 2 3 4 5 6 7 8 9 10 11 12 13
C
HD3V3 cells
tk
F2-tk
HS2-tk
DR4-HS2-tk
M1-HS2-tk
VRE-tk
VRE
DR4
M1 VRE
- T3
+ T3
0 10 20 30 40
Relative CAT activity

Figure 6 ➔

To investigate whether the HS2-VRE can mediate T3-induced activation, we performed transient transfection assays in HD3V3 cells in which the level of expression of V3 (gag-TRα) is comparable to that of v-ErbA (Disela *et al.*, 1991). Transfection of the HS2-tk reporter construct in these cells yielded transcription levels comparable to those obtained in HD3 cells, thereby suggesting that HS2 enhancer activity can be quenched by gag-TRα. Addition of T3 boosted the level of transcription 4.5-fold, which is only 25% of the activity obtained with M1-HS2-tk in these cells (Ciana *et al.*, 1998). The impaired ability of the wild type HS2 enhancer to display full activity in response to T3 is most likely due to the high levels of v-ErbA in HD3V3 cells opposing the activation by ligand-bound RXR-V3.

To directly compare the function of the VRE with that of a canonical TRE in the context of HS2 enhancer, we replaced the VRE with a consensus DR4 element by changing the 3' most half-site of the VRE (position 971-976) into AGGTCA yielding DR4-HS2. In HD3V3 cells and in the absence of hormone, transcription of the DR4-HS2-tk construct is repressed to levels similar to that obtained with HS2-tk suggesting that unliganded v-ErbA and/or V3 quenched the enhancer activity via a DR4. Administration of T3 resulted in elevated levels of activation as compared to HS2-tk (7- and 4.5-fold, respectively) (Figure 6C).

Figure 6 Liganded TR binds to and activates transcription from the HS2-VRE. (**A**) A gel mobility shift assay of ^{32}P-labelled synthetic oligonucleotide containing the VRE sequence using HD3 cell-extract or vaccinia-expressed receptor proteins, as indicated. RXR-v-ErbA and RXR-TR dimers are indicated with arrows and v-ErbA monomer with an aterisk. (**B**) Off-rate assay performed with the same probe as in Figure 6A and either HD3 cell-extract or vaccinia-expressed RXR-TR. Lane 1: free probe; lane 2: VRE probe plus HD3 cell-extract; lanes 3-7: as in lane 2 plus 1000-fold of unlabeled VRE probe added as competitor, with aliquots loaded on the gel at the indicated time points; lane 8: VRE probe plus vaccinia-expressed RXR-TR; lane 9-13: as in lane 8 plus unlabeled VRE probe as competitor with aliquots loaded on the gel at the indicated time points. (**C**) Transient transfection assays in HD3V3 cells, expressing gag-TRα and v-ErbA, with the indicated tk reporter constructs. Black bars denote relative CAT activities in the absence of hormone while hatched bars represent CAT values in the presence of T3. Transcription is arbitrarily set to 1 for the to tk promoter alone in the absence of hormone.

To further investigate the ability of the VRE to mediate a T3-response, transfection assays were performed in HD3V3 cells using a reporter plasmid containing the minimal VRE sequence placed in front of the tk promoter (VRE-tk). T3-dependent transcriptional activation of the VRE-tk was merely 1.7-fold, while of the F2 element and of the well-characterised MoMLV-TRE was 6- and 8-fold, respectively (Sap *et al.*, 1989; Ciana *et al.*, 1998; and data not shown).

In summary, these data show that thyroid hormone responsiveness can be mediated by the VRE in the HS2 context. DR4, however, is more potent since it nearly restores enhancer activity as defined by the M1-HS2. Secondly, the VRE by itself possess very little activation potential in cells that co-express v-ErbA and gag-TRα.

v-ErbA and gag-TRα act differently to instigate repression via the HS2 enhancer

Unliganded TR and v-ErbA are thought to repress transcription of T3-regulated genes. Support for this model is provided by experiments using the F2 element from the chicken lysozyme gene promoter; transcription of the F2 element is repressed 6- to 7-fold as compared to tk alone in HD3 cells (Baniahmad *et al.*, 1992). We have so far shown that the wild type HS2 enhancer is 'neutral', i.e. it neither enhances nor represses transcription from the homologous or from a heterologous promoter in HD3 cells. This suggests that positive and negative factors regulating its activity are in balance. To investigate whether v-ErbA could actively repress the transcriptional activity of the heterologous tk promoter we mutated GATA-sites I and II within the wild type HS2 enhancer so that the negative transcriptional activity of v-ErbA would prevail (Figure 7A). Surprisingly, transfection experiments in HD3 cells showed that mutation of these GATA-sites (GI-HS2-tk and GII-HS2-tk reporters) did not reduce transcription significantly below that of the enhancerless tk promoter (less than 2-fold) (Figure 7A). These data suggest that in HD3 cells v-ErbA does not efficiently, if at all, repress the transcriptional activity intrinsic to the heterologous tk promoter.

To test whether the incapacity of v-ErbA to convey transcriptional repression to the linked tk promoter was an intrinsic property of v-ErbA and/or dictated by the VRE element, similar experiments were performed in HD3V3 cells that express equivalent levels of gag-TRα (V3) and v-ErbA. In HD3V3 cells, the levels of transcription of GI-HS2-tk and GII-HS2-tk were strongly repressed as compared to HS2-tk and more

importantly to tk alone (6-fold and 3.5-fold, respectively) (Figure 7A). An even more pronounced effect was obtained when a DR4-HS2 enhancer derivative carrying a mutation in the GATA-site I was tested. The GI-DR4-HS2-tk reporter yielded a 10-fold repression of transcription as compared to tk alone in HD3V3 cells whereas the repression was less than 2-fold in HD3 cells. Finally, in HD3 cells the minimal HS2-VRE element (VRE-tk) yielded 1.3-fold repression of transcription as compared to tk whereas in HD3V3 cells the repression was up to 5-fold. Taken together these results show that v-ErbA in contrast to gag-TRα is unable to convey repression to the tk promoter when bound to the HS2-VRE. This differential activity is dictated by both the HS2 context and the VRE since transcription of the F2-tk is equally repressed in both cell lines (Figure 7A). Taken together, our data indicate that the ability of the v-ErbA oncoprotein to repress is not equivalent to that of unliganded gag-TRα.

Discussion

In this study we have continued to probe the function of the leukemia inducing oncoprotein, v-ErbA, in the control of transcriptional repression. Our detailed analysis of the previously identified regulatory region in the erythroid stage-specific CA II gene (Ciana *et al.*, 1998) now provides a more precise model for the v-ErbA-mediated repression. Firstly, we have shown that the regulatory region of the CA II gene located in the second intron, termed HS2, is a true enhancer that can activate transcription independently of its position and orientation relative to the promoter. The apparent erythroid-specific activity of the enhancer is dictated primarily by two adjacent GATA-factor binding sites. The v-ErbA oncoprotein as well as the unliganded TR can efficiently quench the activity of the HS2 enhancer thereby suggesting that v-ErbA and unliganded TR interfere with the positive function of the erythroid GATA-factors. Secondly, we have shown that the HS2 enhancer can convey T3-mediated activation to a heterologous or CA II promoter (Figure 6C and data not shown), providing additional evidence for the role of T3 in the activation of CA II transcription (Disela *et al.*, 1991). Thirdly, we have provided evidence that v-ErbA bound to VRE, in contrast to unliganded TR, is impaired in repressing the tk promoter.

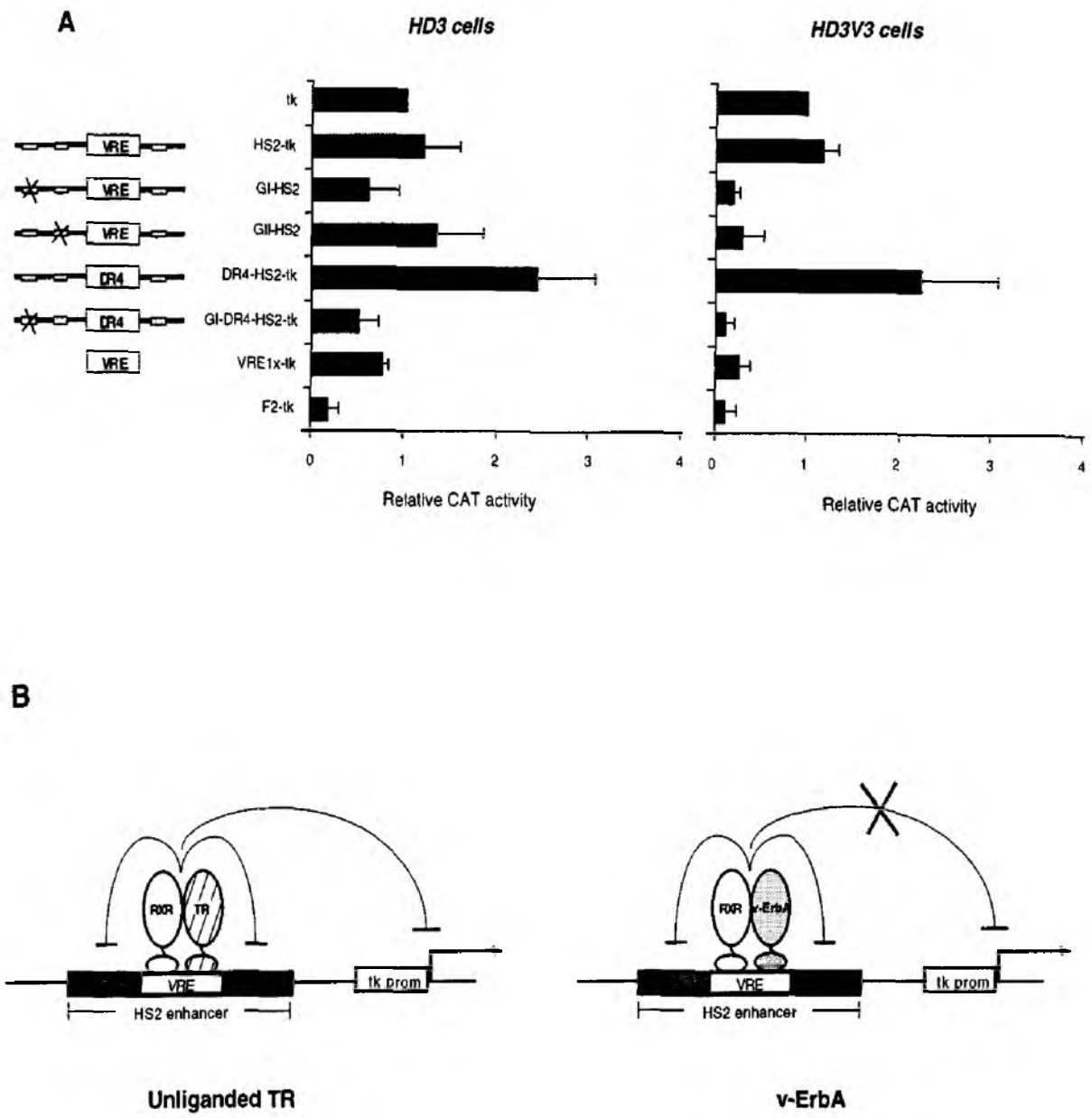

Figure 7 v-ErbA, unlike unliganded gag-TRα, cannot repress the transcriptional activity intrinsic to the tk promoter, when bound to the HS2-VRE. (**A**) Transient transfection assays in HD3 cells (left) and in HD3V3 cells (right) with the indicated tk reporters. The values represent the mean value of the relative CAT activity of at least three experiments. Transcription is expressed relative to tk promoter alone for both cell lines. (**B**) Proposed model whereby unliganded gag-TRα can both quench the HS2 enhancer and the transcription from tk promoter whereas the v-ErbA oncoprotein can only quench the activity of the enhancer.

Mutation and deletion analyses (Figure 3, 4) showed that the HS2 enhancer activity is greatly dependent on two GATA-factor binding sites and that v-ErbA when bound to the VRE can quench their positive transcriptional activity. Interference of nuclear receptor superfamily members with the function of GATA-factors appears to be a recurring theme. For example, the promoter of the slow myosin heavy chain (MyHC) 3 gene contains one GATA-site and one Vitamin D3 responsive element (VDRE). While GATA-factors enhance the transcriptional activity of the MyHC 3 promoter in both ventricular and atrial cardiomyocytes, VDR inhibits transcriptional activity only in ventricular cardiomyocytes (Wang *et al.*, 1998). Moreover, liganded glucocorticoid receptor has been shown to inhibit the function of GATA-1 protein on the promoters of the β-major and β-minor globin genes, which both contain a GATA-site adjacent to a GRE. The functional inhibition of GATA-1 was explained as the result of a direct interaction between GATA-1 and GR (Chang *et al.*, 1993). In pilot immunoprecipitation experiments, we did not observe a direct interaction between GATA-factors and v-ErbA; under the same conditions the interaction between v-ErbA and NCoR could readily be detected (G. B. and H. S. unpublished observations).

The presence of enhancers in introns, regulating transcription of tissue-specific genes, such as the HS2 from the CA II gene is not uncommon. Examples in the hematopoietic system are the 3' enhancer of the chicken adult β-globin and the intronic immunoglobin enhancer (Eμ) (Emerson *et al.*, 1987). The 3' intronic enhancer of the chicken adult β-globin gene binds GATA-factors that could co-operate with GATA-factors bound to a site proximal to the TATA box to instigate transcription of β-globin gene (Emerson *et al.*, 1987). We speculate that a similar mechanism may be in place for the CA II locus since the CA II promoter contains a GATA-motif. Our findings that HS2 can enhance transcription from its own CA II promoter more efficiently compared to the heterologous tk promoter, is in line with this model. Further investigations are required to address the nature of the interference of v-ErbA with GATA-factors activity on the CA II-HS2 enhancer.

In the erythroid MEL cells, the activity of the HS2 enhancer is moderate relative to that obtained in HD3 cells (Figure 2). This apparent discrepancy probably reflects intrinsic differences in the constellation of transcription factors in the cell lines used. In MEL cells, endogenous TR could not be detected and T3-treatment of MEL

cells transfected with HS2-tk did not result in significant activation of transcription (data not shown) implying that the endogenous TR levels in MEL cells are low and not responsible for the diminished HS2 enhancer activity. Moreover, transfection of a F2-tk reporter containing the strong VRE/TRE from the chicken lysozyme gene (Baniahmad *et al.*, 1992) did not yield transcriptional repression in this cell line in the absence of TR co-expression (Figure 2). Given that the HS2 enhancer activity is dependent mainly (if not solely) on erythroid factors bound to GATA-sites (Figure 4), the moderate six-fold enhancer activity of HS2 in MEL cells as compared to HD3 cells may be explained by lower levels of transcriptionally 'competent' GATA-factors. In fact, MEL cells are kept in an undifferentiated state due to overexpression of the transcription factor PU.1, which directly interacts with GATA-1 to inhibit its ability to activate transcription and to drive erythroid cells to terminal differentiation (Rekhtman *et al.*, 1999).

In previous studies we and others showed that TRα is likely to be involved in erythroid differentiation (Disela *et al.*, 1991). We showed that liganded TRα plays a role in CA II activation presumably by increasing the accessibility of the HS2 (Ciana *et al.*, 1998). The notion that v-ErbA occludes liganded TR from its binding site is supported by the fact that substitution of the VRE with a canonical DR4, a better binding site for TR, potentiates the T3 response (Figure 6C). Therefore, the sequence composition of the VRE is critical for the efficiency of the occlusion since v-ErbA occludes liganded gag-TRα more efficiently on the VRE than on the DR4.

As mentioned before, v-ErbA prevents the HS2 enhancer by quenching the GATA-factors activity, a function that is exerted locally within the HS2 region. Gag-TRα can also quench HS2 activity (Figure 7A, compare HS2-tk in HD3 and HD3V3 cells) consistent with the observation that in erythroid cells overexpressing gag-TRα CA II is repressed in the absence of T3 (Disela *et al.*, 1991; Bauer *et al.*, 1998). However v-ErbA, unlike unliganded gag-TRα, was unable to actively repress transcription from the heterologous tk promoter when bound to the HS2-VRE (Figure 7A). Unliganded TRα and TRβ have been reported to interfere with the preinitiation complex (PIC) assembly to actively repress transcription. It has been shown that TR directly interacts with TFIIB (Baniahmad *et al.*, 1993; Fondell *et al.*, 1996; Fondell *et al.*, 1993) while v-ErbA is impaired in such an interaction (Urnov *et al.*, 2000). We

speculate that v-ErbA is unable to interact with TFIIB in the context of the HS2-VRE, and thus cannot interfere with factors governing the basal transcription of the tk promoter. More and direct experiments will be needed to unravel whether and how v-ErbA communicates with TFIIB and are expected to shed more light in understanding the correlation between repression of gene networks by v-ErbA and its oncogenicity.

Recent data have implicated helix 12 (H12) of the receptors LBD in corepressor binding (Renaud *et al.*, 2000; Zhang *et al.*, 1999). Given that v-ErbA lacks an intact H12, it is possible that distinct binding affinities for corepressors might account at least partially for the differential v-ErbA and TR repression capacity. Our finding that v-ErbA and TR show distinct repression activities on the VRE but not on the F2 element are consistent with observations suggesting repression and corepressor release to be dependent on the architecture of TREs (Olson *et al.*, 1998; Wahlstrom and Vennstrom, 1998).

In conclusion, v-ErbA appears to regulate transcription in at least three ways: firstly, by quenching the activity of GATA-factors bound to the HS2, secondly, by occluding liganded TRα and thirdly by interfering with the repression function of unliganded TRα (Figure 7B). Similar mechanisms might be in place for other erythroid v-ErbA target genes. We propose that v-ErbA, apart from the already known ability to occlude liganded TR, can antagonise the function of unliganded TRα as well when bound to the HS2-VRE, thus preventing the full silencing function of TRα. It is possible that this functional difference in the repressive, unliganded modes of v-ErbA and gag-TRα may contribute to v-ErbA oncogenic activity.

Studies on v-ErbA function are moving our knowledge forward to unravelling the connections between transcriptional repression and oncogenicity. Applying this knowledge will certainly provide novel insights into the molecular mechanisms controlling normal and abnormal erythroid differentiation.

Materials and Methods

Cell culture

The derivatives of the AEV-transformed cell line HD, HD3-EpoR and HD3-V3, expressing the murine erythropoietin receptor or a gag-chicken TRα fusion, respectively, were grown in CFU-E medium (Dolznig *et al.*, 1995). Before T3 treatment, HD3-V3 cells were grown for 48 hours in medium containing stripped serum using the anion-exchange resin AG 1-X8 (BIORAD). Chicken embryo fibroblasts (CEF) were grown in Iscoves' medium (Gibco-BRL) supplemented with 8% fetal calf serum, 2% chicken serum, 100 U/ml penicillin and 100μg/ml streptomycin. MEL cells were a kind gift from Philipsen and were grown in DMEM (Gibco-BRL) supplemented with 10% fetal calf serum, penicillin-streptomycin and non-essential amino-acids (Philipsen *et al.*, 1990). L cells were a kind gift from Baniahmad and were grown in DMEM (Gibco-BRL) supplemented with 10% fetal calf serum and penicillin-streptomycin as described (Baniahmad *et al.*, 1992).

Transient transfection assays

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 internal control, and harvested after 48 hours. In HD3-V3 transfected cells grown in stripped serum-containing culture medium 150 nM T3 was added for the last 24 hours where indicated. CAT and luciferase activities were measured as described previously (Barettino *et al.*, 1993).

MEL cells were transfected by electroporation as described (Philipsen *et al.*, 1990). Briefly $2x10^7$ cells were incubated on ice for 10' with 20 μg of reporter construct together with 5 μg of EF1α-Luc as internal control and 15 μg of empty pSG7 vector to keep the same amount of transfected DNA in all samples. Subsequently, the cells were electroporated in a BIORAD gene pulser at 960 μF and 280 V and after 10' incubation on ice were plated in 10 ml of complete medium. After 48 hours, the cells were harvested for measurement of CAT and lusiferase activities.

CEF cells and L cells were transfected with the calcium phosphate method as described (Barettino *et al.*, 1993). Briefly, $3x10^5$ cells were transfected with 5 μg of reporter construct along with 1 μg of EF1α-Luc as internal control and 5 μg of empty pSG7 vector to keep the same amount of transfected DNA in all samples. After 16 hours the cells were washed with phosphate buffer saline (PBS) and harvested after 24 hours for measurement of CAT and lusiferase activities.

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

Oligonucleotides used for gel retardation

Coding strand:

VRE: 5'-TCGACCCAGCAAGGTCACAGCAGGGCTTTTTTTC-3';

Non coding strand:

VRE: 5'-TCGAGAAAAAAAGCCCTGCTGTGACCTTGCTGGG-3';

Oligonucleotides used for mutagenesis:

pBL5': 5'-TCCCAGTCACGACGTTGTAAA-3' in pBLCAT2;

pBL3': 5'- GTTCGAATTCGCCAATGACAA-3' in pBLCAT2;

mutagenesis-VRE-M1: 5'-CCCTGCTGTGAAATTGCTG-3';

mutagenesis-VRE-M2: 5'-AAAGCAATGCTGTGACCTTG-3'

mutagenesis-VRE-M3: 5'-TTGCTGGTTTACTAGCCTGATAAGG-3';

mutagenesis-GI-GATA: 5'-GTTCCAGAGATTTAGGTCAG-3';

mutagenesis-GII-GATA: 5'-GGACTAGCCTTGTAAGGTA-3';

mutagenesis-GIII -GATA: 5'-CTACGTAATTGGTAAATCATA-3';

mutagenesis-VRE-DR4: 5'-CTCATAAGAAAAAATGACCTGCTGT-3'

Oligonucleotides used for cloning:

Upper strand

M1-927-945: 5'-CCCAAGCTTATCCTTGCTACCTTATCAG-3' (HindIII site underlined)

M1-951-971: 5'-CCCAAGCTTTCCCCCAGCAATTTCACAGCA-3' (HindIII site underlined)

979-998: 5'-GCTCTAGAGCTTTCTTATGAGATACCAA-3' (XbaI site underlined);

1019-1041: 5'-GCTCTAGAGCTCATTACAGATGTTTTTTGC-3' (XbaI site underlined)

CAII upper: 5'-CTTTGATCTGCGCCTCCA-3'

Lower strand

1021-1004: 5'-GCTCTAGAGCTTCACCAAAAGAACCTA-3' (XbaI site underlined)

M1-983-966: 5'-GCTCTAGAGCGAGAAAAAAAGCCCTGCTGT-3 '(XbaI site underlined)

958-938: 5'-GCTCTAGATGGGGGACTAGCCTGATAAG-3' (XbaI site underlined)

CAII lower: 5'-CGGGATCCCGCCAGTGATGGGACCTGGTG-3' (BamHI site underlined)

Gel retardation assay

20 pmoles of labeled VRE -oligonucleotide were incubated with HD3 protein extract (40μg) or vaccinia-virus expressed v-ErbA, TR and RXR proteins for 20' on ice (de Magistris and Stunnenberg, 1988). Binding was performed in 80mM $MgCl_2$, 0.1% Triton-X 100, 0.1mg/ml poly(dI-dC), 1 mg/ml BSA, 0.2 mM EDTA, 34%(v/v) glycerol and 4 mM DTT. Off-rate competition was performed by adding a 100- or 1000-fold molar excess of unlabeled VRE-oligonucleotide and aliquots were applied on pre-cooled and pre-run 0.5xTBE, 5% polyacrylamide gels at the indicated time points.

Plasmids

A RsaI/RsaI fragment of 288 bp spanning the HS2 was inserted into EcoRV site of pBlueScript and subsequently subcloned into SalI/BamHI sites of pBLCAT2 (Luckow and Schutz, 1987), yielded plasmid HS2(883-1175)-tk. M1(883-1175)-tk was generated from HS2(883-1175)-tk by oligonucleotide-directed site-specific mutagenesis (Seraphin and Kandels-Lewis, 1996) using the oligonucleotides pBL5' and pBL3' and the mutagenesis oligonucleotide M1-VRE. The HS2-tk construct was generated from HS2(883-1175)-tk by PCR amplification using primers pBL5'and 1021-1004, digested with SalI/XbaI and inserted into SalI/XbaI sites of pBLCAT2. M1-HS2-tk was generated from HS2-tk by oligonucleotide-directed site-specific mutagenesis using the oligonucleotide M1-VRE.

M1(927-1175)-tk and M1(951-1175)-tk constructs were generated by PCR amplification of M1(883-1175)-tk using primers 927-945 and M1-951-971,

respectively, along with pBL3', digested with HindIII/XbaI and inserted into HindIII/XbaI site of pBLCAT2. (979-1175)-tk construct was generated by PCR amplification of HS2(883-1175)-tk using primers 979-998 and pBL3', digested with XbaI and inserted into XbaI site of pBLCAT2. The proper orientation of the insert was assessed by sequencing analysis. PCR amplification of M1-HS2-tk with primers pBL5' and M1-983-966 or 958-938, digesting with HindIII/XbaI and subsequent cloning into HindIII/XbaI site of pBLCAT2, yielded plasmids M1(883-983)-tk and (883-958)-tk respectively. F2-tk plasmid was a kind gift from Baniahmad (Baniahmad *et al.*, 1992). Annealing of primers VRE-upper and VRE lower and ligation with pBLCAT2 digested with SalI yielded plasmid VRE-tk.

A CA II promoter fragment was PCR amplified using primers CAII-upper and CAII-lower (has a mutation in the ATG of CA II), digested with ApaI, filled in with Klenow and digested with BamHI. The resulting 263 bp promoter fragment, inserted into XbaI, filled in with Klenow and BamHI sites of pBLCAT3, yielded construct CAII. Digesting the CAII with SalI, filling in with Klenow and ligating with an excised insert from M1-HS2 with SalI/XbaI and filled in with Klenow, yielded plasmids M1-HS2-CAII(+) and M1-HS2-CAII(-). For the generation of CAII-M1-HS2(+)/(-) the M1-HS2 insert was excised from M1-HS2-tk construct with SalI/XbaI filled in with Klenow, and ligated with CAII plasmid digested with SmaI.

M2-HS2-tk and M3-HS2-tk were generated from HS2-tk by oligonucleotide-directed site-specific mutagenesis using the mutagenesis oligonucleotides M2-VRE and M3-VRE respectively. M1M3-HS2-tk and M2M3-HS2-tk were generated from M1-HS2-tk and M2-HS2-tk using the M3-VRE oligonucleotide.

M1-HS2 constructs carrying mutations of the GATA-sites were generated from M1-HS2-tk and mutagenesis oligonucleotides GI-GATA, GII-GATA and GIII-GATA. Likewise, the GI- and GII-HS2-tk constructs were created from the HS2-tk. DR4-HS2-tk was constructed with the mutagenesis oligonucleotide VRE-DR4 from HS2-tk construct and from this, using the mutagenesis oligonucleotide GI-GATA, the GI-DR4-HS2-tk plasmid was generated. All constructs were checked using sequencing analysis.

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Chapter 4

The C-terminus of NCoR interacts with v-ErbA in AEV-transformed cells but does not abrogate the repressive activity of v-ErbA

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Abstract

Unliganded type II nuclear hormone receptors such as thyroid hormone and retinoic acid receptors (TR and RAR, respectively) when bound to their cognate *cis*-acting elements are thought to repress transcription via binding to a corepressor complex consisting of NCoR/SMRT, HDACs and other polypeptides. v-ErbA, a mutated thyroid hormone receptor alpha, shows oncogenic activity which correlates with its ability to repress transcription of differentiation-specific genes. In this study, we have assessed the role of NCoR (and SMRT) in silencing transcription by v-ErbA. We show that endogenous NCoR can interact with v-ErbA in immunoprecipitation assays. Using a dominant negative approach, we transiently or stably transfected v-ErbA expressing HD3 cells with the C-terminus of NCoR (C-NCoR) that do not display silencing activity but have retained the ability to interact with nuclear receptors. Data presented here show that expression of C-NCoR did not result in derepression of transcription of the v-ErbA target gene carbonic anhydrase II (CA II) neither did it affect the repression ability of v-ErbA on DR4-containing reporter constructs, notwithstanding that v-ErbA could interact with C-NCoR. Therefore, we conclude that C-NCoR does not functionally act as a dominant negative corepressor *in vivo* under the tested conditions.

Introduction

Nuclear hormone receptors are sequence-specific transcriptional regulators that are involved in the control of cell proliferation, differentiation, physiology as well as in cancer (reviewed in Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995). Their modular structure is well conserved among the members of the family, particularly the DNA binding domain (DBD) and the ligand-binding domain (LBD). Nuclear receptors such as RAR and TR can act both as activators as well as repressors of transcription depending on ligand binding. Extensive studies on the mechanism of transcriptional regulation revealed that the function of nuclear receptors is largely mediated by

coactivators and corepressors that associate with the LBD. Upon ligand binding, the conformation of the receptor becomes permissive to interaction with coactivators such as CBP/p300, PCAF (Blanco *et al.*, 1998) and the p160 family proteins such as SRC-1, GRIP/TIF2, ACTR/RAC3/P/CIP and TRAM1 (reviewed in Horwitz *et al.*, 1996; McKenna *et al.*, 1999; Glass and Rosenfeld, 2000). Recently the coactivator proteins were shown to either posses intrinsic histone acetyltransferase (HAT) activity or to recruit/interact with HATs (reviewed in Glass and Rosenfeld, 2000). Hyperacetylation of histones is correlated with transcriptionally active genes and thus, it is postulated that coactivator recruitment by nuclear receptors contributes to the stabilisation of an 'open', active chromatin structure (reviewed in Pazin and Kadonaga, 1997).

In the absence of ligand, nuclear receptors repress transcription. Originally, it was postulated that the repressive function of receptors was due to their ability to squelch limiting coactivators (Kamei *et al.*, 1996) or auxiliary heterodimerisation partners such as RXR (Barettino *et al.*, 1993; reviewed in Glass and Rosenfeld, 2000). More recently, a search for cofactors involved in the repressive function of nuclear receptors led to the identification of Nuclear receptor Co-Repressor (NCoR) (Horlein *et al.*, 1995; Kurokawa *et al.*, 1995; Zamir *et al.*, 1996), Silencing Mediator for Retinoid and Thyroid hormone receptors (SMRT) (Chen and Evans, 1995), TR-associated cofactor (TRAC2) (Sande and Privalsky, 1996), SunCoR (Zamir *et al.*, 1997) and Alien (Dressel *et al.*, 1999). NCoR is 270 kDa protein and shows a bipartite structure consisting of a repression region at the N-terminus and a receptor interaction region at the C-terminus. The repression region comprises at least three autonomous repression domains that can transfer active repression to a heterologous DNA binding domain (Chen and Evans, 1995; Horlein *et al.*, 1995; Li *et al.*, 1997; Nagy *et al.*, 1997). SMRT was initially reported to be a 170 kDa protein, however, more recently, alternative splice variants encoding a 270 kDa protein have been reported with an analogous bipartite structure like NCoR (Ordentlich *et al.*, 1999; Park *et al.*, 1999). NCoR/SMRT interact with unliganded RAR and TR via two receptor interaction domains (RID) located at the very C-terminus of the corepressors (Horlein *et al.*, 1995; Sande and Privalsky, 1996; Seol *et al.*, 1996; Li *et al.*, 1997; Cohen *et al.*, 1998; Hu and Lazar, 1999; Perissi *et al.*, 1999; Wong and Privalsky, 1998).

NCoR and SMRT are thought to be essential for the transcriptional repression activity of nuclear receptors (Chen and Evans, 1995; Horlein *et al.*, 1995; Kurokawa *et al.*, 1995; Li *et al.*, 1997; Hu and Lazar, 1999). NCoR antibody microinjection into cells blocked active repression mediated by TR (Heinzel *et al.*, 1997). Thyroid hormone resistance syndrome correlates with mutations in the LBD of TRβ that enhance ligand independent interactions of TRβ and NCoR/SMRT (Yoh *et al.*, 1997). The model of corepressors association with class II nuclear receptors to repress transcription has become more complicated since the discovery of mammalian homologues of the yeast Sin3 repressor, which interact with NCoR and SMRT (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997). Purification of the murine SIN3 complex showed interaction of SIN3 with the histone binding proteins RbAp 48/46, the histone deacetylases HDAC1/2 and two small proteins SAP30 and SAP18 (Zhang *et al.*, 1997b; Laherty *et al.*, 1998). The model was proposed that nuclear receptors via their association with corepressors interact with the SIN3 complex, which in turn recruits HDACs. Recruitment of HDACs leads to a more compact, repressive state of chromatin and thus repression by nuclear receptors is attained not only via corepressors but also via 'repressive' chromatin modifications (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997; Wong and Privalsky, 1998; reviewed in Pazin and Kadonaga, 1997; Wolffe, 1997). Recently, novel HDAC factors that belong to the class II family of HDACs as well as HDAC 3, have been shown to directly interact with distinct repression domains of NCoR/SMRT, implying redundant or combinatorial mechanisms for HDAC-dependent repressive activity of nuclear receptors (Grozinger *et al.*, 1999; Huang *et al.*, 2000; Kao *et al.*, 2000; Wen *et al.*, 2000).

The v-ErbA protein is an oncogenic mutated variant of TRα and has been proposed to interact with corepressors (reviewed in Stunnenberg *et al.*, 1999). v-ErbA is encoded by the avian erythroblastosis virus (AEV) which causes sarcoma development and fatal erythroleukemia in chickens. AEV expresses a second oncogene, v-ErbB, which is a mutated epidermal growth-factor receptor (EGFR) with constitutive tyrosine kinase activity. v-ErbB alone causes a delayed, weak form of erythroleukemia. v-ErbA potentiates the AEV leukemic phenotype and its transformation capacity lies in its ability to tightly arrest differentiation of v-ErbB-

expressing erythroblasts by effectively silencing different stage-specific erythroid genes such as the carbonic anhydrase II gene (Zenke *et al.*, 1990; Disela *et al.*, 1991). It is also postulated that v-ErbA competes with TR for binding to thyroid responsive elements (TREs) to represses transcription of T3-regulated genes (Horlein *et al.*, 1995; Sap *et al.*, 1989; reviewed in Stunnenberg *et al.*, 1999; Thormeyer and Baniahmad, 1999). Because of several mutations and a deletion in its C-terminus, v-ErbA cannot bind ligand and has lost known activation functions (Barettino *et al.*, 1994). Ligand unresponsiveness is thought to lead to constitutive interaction of v-ErbA with the NCoR/SMRT corepressor complex.

The interaction interface for NCoR in nuclear receptors was initially reported to be the N-terminal part of the LBD (Helix 1) (Horlein *et al.*, 1995; Chen and Evans, 1995). This was particularly appealing because a transformation deficient molecule of v-ErbA designated td359-v-ErbA (Royer-Pokora *et al.*, 1979), that caries a mutation in the helix 1, is unable to repress transcription of TRE regulated reporters (Damm *et al.*, 1987; Damm and Evans, 1993). Likewise, TRα with engineered mutations at helix 1 fails not only to repress transcription but is unable to interact with SMRT (Chen and Evans, 1995; Horlein *et al.*, 1995). However, more recent data demonstrated that the extreme C-terminus of LBD (helix 12) as well as helices 3, 4 and 5 comprise the critical requirements for the nuclear receptor/corepressor interaction (Baniahmad *et al.*, 1995; Lin *et al.*, 1997; Yoh *et al.*, 1997; Hu and Lazar, 1999; Zhang *et al.*, 1999). The notion that more than one helices of the LBD are involved in corepressor binding is further supported by data demonstrating that three subdomains of v-ErbA on helices 1, 5/6 and 8 co-operatively interact with NCoR and convey the repressive function of v-ErbA (Busch *et al.*, 1997; Busch *et al.*, 2000). However, notwithstanding the flood of reports, 'proof of the principle' for the repressive role of NCoR/SMRT in the repressive function of class II nuclear receptors *in vivo* remains to be shown.

In the present study, we have assessed the involvement of NCoR in v-ErbA-mediated gene repression *in vivo*. We have taken a dominant negative approach by using the C-terminal part of NCoR, which only comprises the receptor interaction domains without any silencing domains. We show that v-ErbA can interact with overexpressed C-NCoR as well as with endogenous NCoR from v-ErbA-expressing erythroid progenitor (HD3) cells. However, transient or stable expression of C-NCoR

did not abrogate the v-ErbA mediated transcriptional repression. Similarly, the mRNA levels of the v-ErbA target gene, carbonic anhydarse II were not derepressed in HD3 cells stably expressing C-NCoR. Our results suggest that NCoR/SMRT are not involved in the v-ErbA-mediated repression under the conditions used and that additional mechanisms apart from transcriptional repression might contribute to the v-ErbA induced oncogenicity.

Results

The N-terminus of the LBD plays a critical role in the v-ErbA repressive function

Using *in vivo* footprinting experiments we have previously shown that v-ErbA binds to a v-ErbA responsive element (VRE) located in an intronic DNase I hypersensitive region of CA II gene, named HS2. Evidence for the function of HS2 as a genuine erythroid-specific enhancer was obtained by mutating the first half-site of the VRE. This mutation unleashed an enhancer activity of 30-fold as a consequence of abolishment of v-ErbA binding (Ciana *et al.*, 1998 and chapter 3). These and other observations led us to conclude that v-ErbA is a strong repressor when bound to the HS2 enhancer thereby neutralising its enhancer activity.

In this study we set out to unravel the molecular mechanism that v-ErbA utilises to repress transcription and whether auxiliary factors such as SMRT or NCoR are required. To consolidate that the observed repression of the HS2 enhancer was instigated by v-ErbA, cotransfection experiments were carried out in mouse erythroleukemia (MEL) cells that do not express v-ErbA. In MEL cells, the level of transcription of HS2-tk reporter was 6-fold higher than that of tk alone, which is consistent with the erythroid specificity of the HS2 enhancer (Figure 1). Transfection of HS2-tk along with a v-ErbA expression vector, resulted in repression of transcription to the level of tk alone, reinforcing the notion that repression of the HS2 enhancer activity was indeed instigated by the v-ErbA protein (Figure 1). A v-ErbA variant carrying a mutation in helix 1 of LBD, designated td359-v-ErbA, was shown to fail to repress transcription from reporters containing artificial TREs (Damm *et al.*,

1987; Damm and Evans, 1993). Insertion of the corresponding mutation in TR abrogated the interaction of TR with NCoR (Chen and Evans, 1995; Horlein *et al.*, 1995). Cotransfection experiments in MEL cells showed that td359-v-ErbA did not convey transcriptional repression on HS2 (Figure 1), while cotransfection of unliganded TR repressed HS2 transcription to the same levels as v-ErbA. These observations are in line with data from others (Damm and Evans, 1993) and show that mutations in helix 1 of LBD of the v-ErbA affect the repressive function. Combined with the recent observation that td359-v-ErbA protein is unable to interact with NCoR/SMRT (E Caldenhoven, unpublished observations), our data suggest that NCoR/SMRT indeed plays a role in the v-ErbA repressive function.

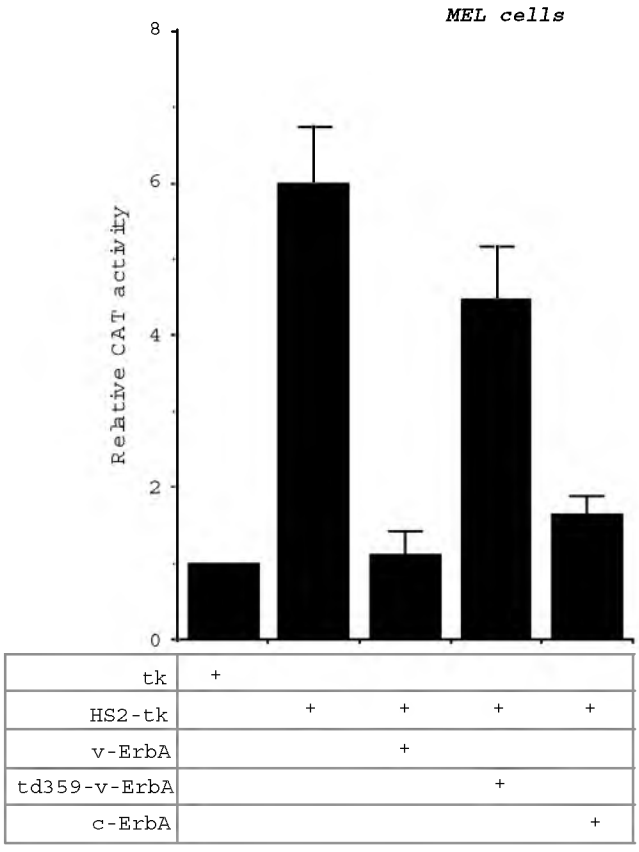


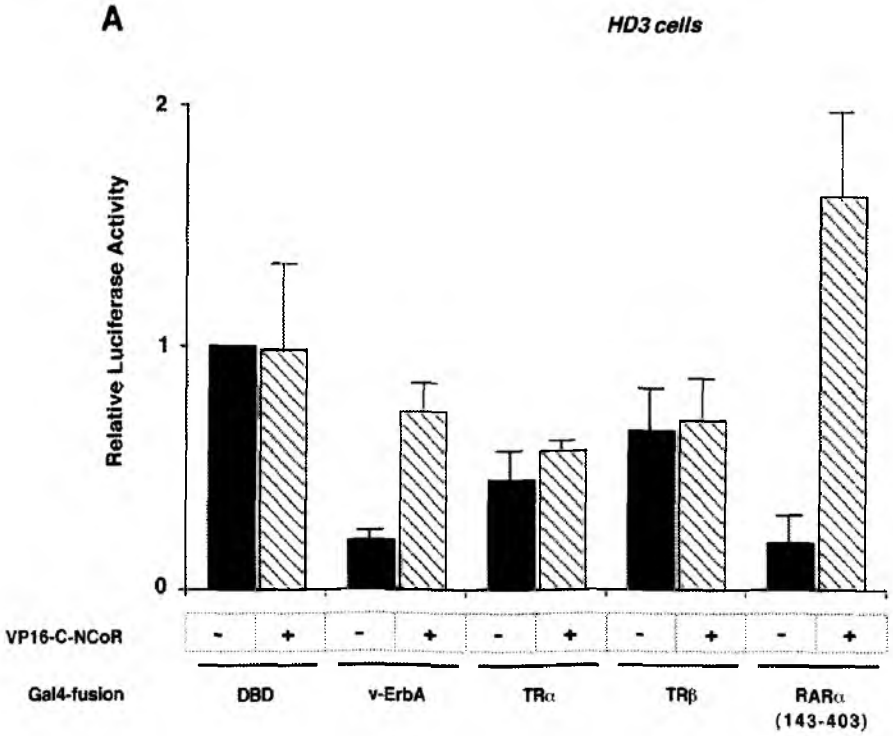
Figure 1 The HS2 enhancer of CA II is repressed by v-ErbA. Transient transfection assays in MEL cells with HS2-tk reporter along with expression vectors for v-ErbA, td359-v-ErbA, c-ErbA (chicken TRα) or empty pSG5 vector. The values represent the mean value of the relative chloramphenicol acetyltransferase (CAT) activity, as normalised by luciferase values of the internal control, of at least three experiments, with error bars shown. Transcriptional activity of tk-CAT construct is arbitrarily set to 1.

v-ErbA interacts with overexpressed exogenous and endogenous NCoR

It has been shown for a number of cell lines that v-ErbA can interact with corepressors in mammalian two hybrid assays (Sande and Privalsky, 1996; Hong *et al.*, 1998; Busch *et al.*, 2000). Therefore we wished to assess whether such a physical interaction between v-ErbA and NCoR can also occur in HD3 cells. We performed two-hybrid assays (Figure 2A and data not shown) using Gal4-DBD fused to receptor LBDs and a VP16 fusion of a C-terminal part of NCoR (aa 1945-2453). In HD3 cells, Gal4-RARα(143-403), a RARα variant that lacks helix H12 (Baniahmad *et al.*, 1992; Damm *et al.*, 1993) and Gal4-v-ErbA caused 5-fold repression of transcription compared to Gal4-DBD alone whereas Gal4-TRα and Gal4-TRβ, caused a 2- to 3-fold repression (Figure 2A black bars). Cotransfection of VP16-C-NCoR resulted in a moderate but significant derepression of transcription, 3-fold and 9-fold for Gal4-v-ErbA and Gal4-RARα(143-403), respectively. The transcription from Gal4-TR fusion proteins was not affected by the presence of VP16-C-NCoR. Gal4-v-ErbA and Gal4-RARα(143-403) in contrast to Gal4-TRα and Gal4-TRβ, do not posses an intact helix H12 in their LBDs. It has been demonstrated that absence of H12 strengthens the interaction between receptors and corepressors (Chen and Evans, 1995; Chen *et al.*, 1996; Li *et al.*, 1997; Wong and Privalsky, 1998; Hu and Lazar, 1999; Zhang *et al.*, 1999). Therefore, it is likely that differential activation by the Gal4-LBDs reflect the higher ability of Gal4-v-ErbA and Gal-RARα(143-403) to interact with corepressors as compared to Gal4-TRα and Gal4-TRβ.

Of note is that neither repression by Gal4-LBDs nor activation with VP16-C-NCoR were robust in HD3 cells. A possible explanation is that v-ErbA, present in high levels in HD3 cells, squelches endogenous corepressors and consequently transfected Gal4-LBDs failed to efficiently interact with corepressors to repress transcription. Similarly, VP16-C-NCoR might not be expressed to sufficiently high levels to interact with Gal4-LBDs and displace endogenous NCoR. To assess the interaction between v-ErbA and endogenous NCoR from HD3 cells imunnoprecipitation experiments were carried out. v-ErbA was efficiently immunoprecipitated using the monoclonal antibody 1G10 directed against the gag-moiety of the v-ErbA protein (Figure 2B). Probing the blot with a polyclonal antibody against NCoR revealed its presence in the

1G10 immunoprecipitates. An unrelated monoclonal antibody against the myc epitope did neither precipitate v-ErbA nor NCoR (Figure 2B, compare lanes 1 and 3).



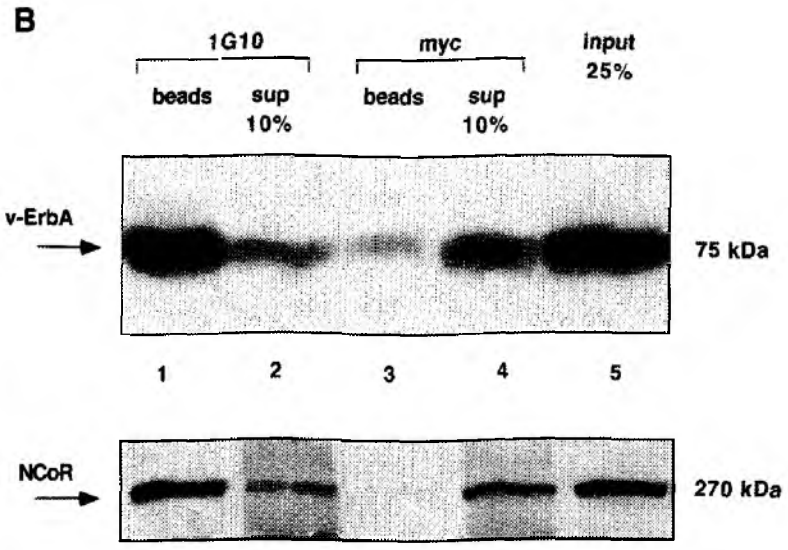


Figure 2 ➔

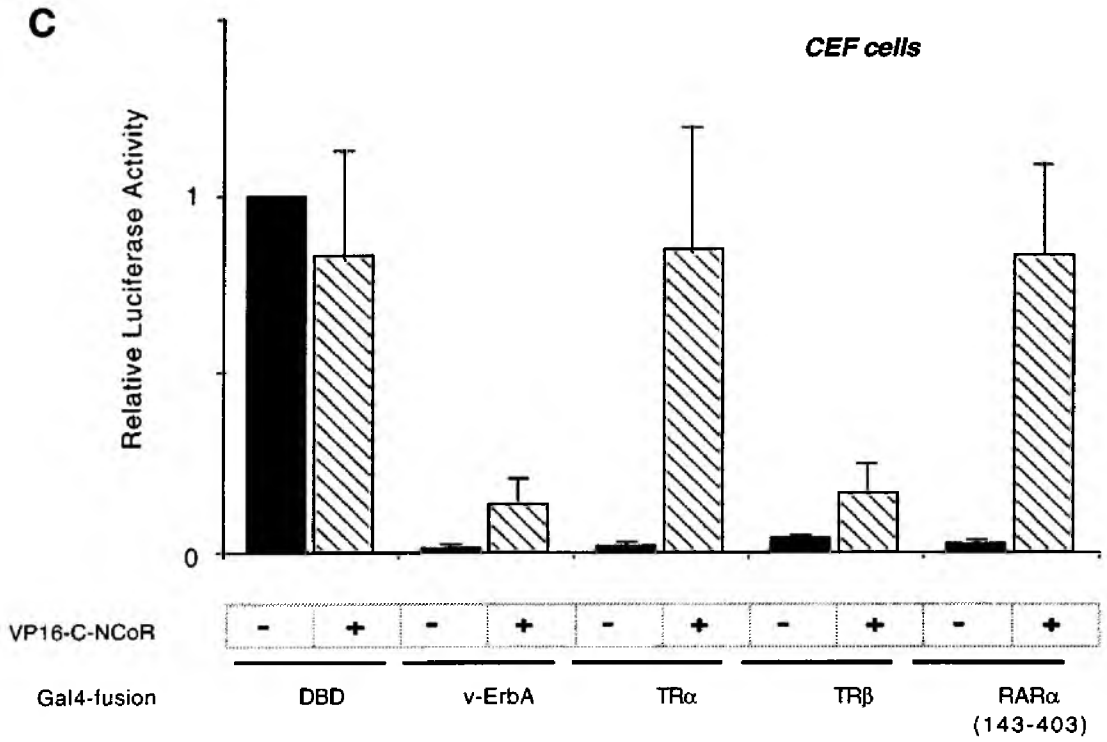


Figure 2 v-ErbA interacts with C-NCoR as well as with endogenous NCoR. (**A**) Transient transfection assays in HD3 cells with a 3xUAS-tk-luc reporter construct along with Gal4-DBD, Gal4-v-ErbA Gal4-TRα Gal4-TRβ Gal4-RARα (146-403). An expression vector for VP16-C-NCoR fusion protein was cotransfected where indicated. (**B**) Extracts from HD3 cells were subjected to immunoprecipitation using the monoclonal antibodies against v-ErbA (1G10) and myc-epitope (control). Equal amounts of precipitates were analysed by SDS-PAGE (7% or 10% polyacrylamide gels) and immunoblotted using antibodies against TRα (polyclonal) (upper panel) or NCoR (monoclonal) (lower panel). The input lanes represent 25% of the extract analysed with immunoprecipitation. (**C**) Transient transfection assays in CEF cells with plasmids as in (A).

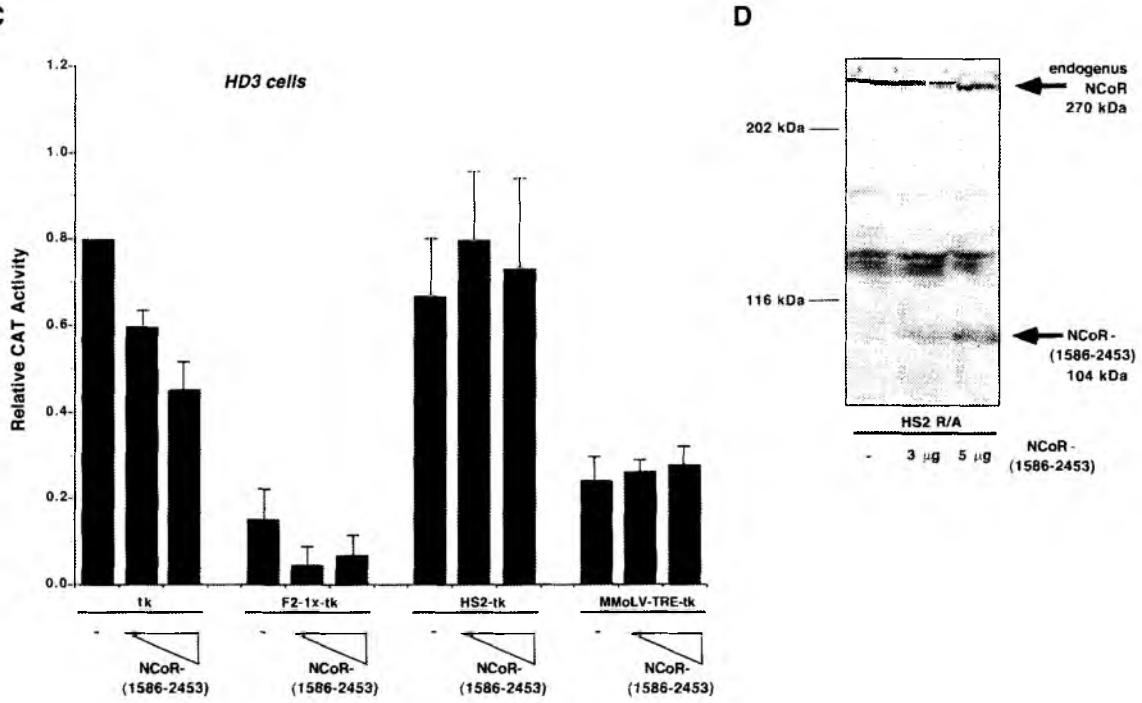
Since immunoprecipitation experiments demonstrated the interaction of v-ErbA with NCoR but two hybrid assays showed low affinity for this interaction in HD3 cells, we tested whether Gal4-v-ErbA and VP16-C-NCoR could interact in cells that lack endogenous v-ErbA. Therefore transient transfection experiments were performed in chicken embryonic fibroblasts (CEF cells). As shown in Figure 2C expression of the

various Gal4-LBDs strongly repressed (30- to 40-fold) transcription from the 3xUAS-tk reporter as compared to Gal4-DBD alone. Upon co-transfection of VP16-C-NCoR, a robust 30-fold increase in the level of transcription could be obtained with Gal4-RARα(143-403) and Gal4-TRα. A lower but significant 6-fold activation was obtained with Gal4-v-ErbA and Gal4-TRβ (Figure 2C). Note that the expression levels of the transfected Gal4-fusion proteins were similar (data not shown). Taken together, these results suggest that type II nuclear receptors including v-ErbA can interact *in vivo* with NCoR.

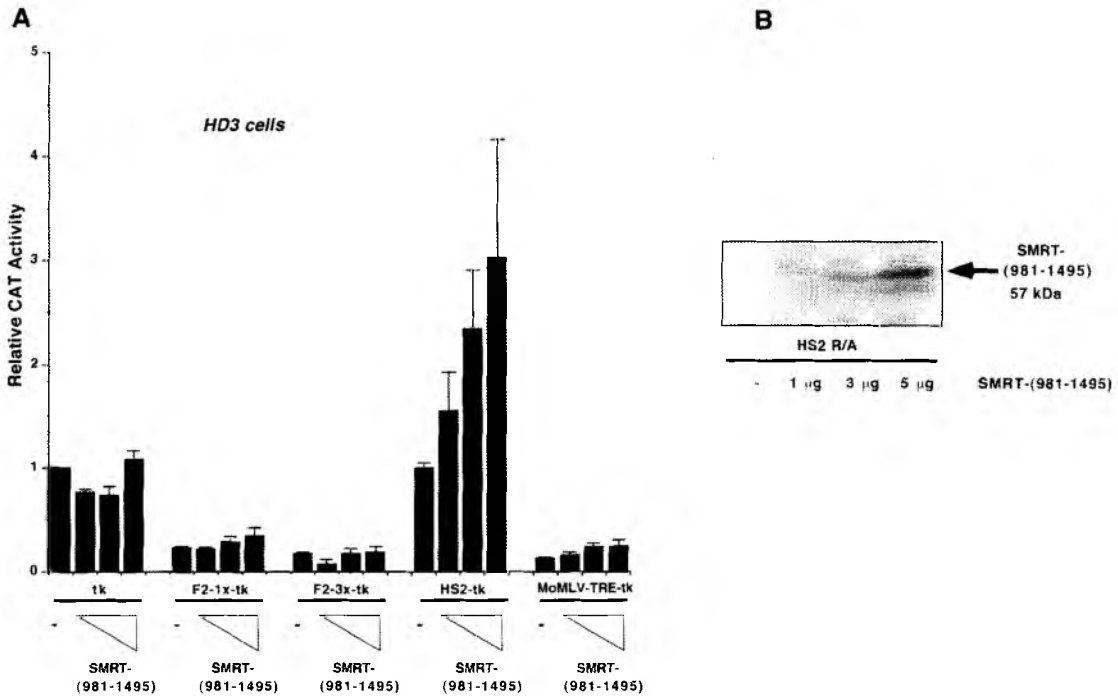
Effect of transient transfection of the C-terminus of NCoR/SMRT on transcription

To further elucidate the role of the NCoR/SMRT corepressors *in vivo*, we set out to establish a dominant negative approach. The C-terminal parts of NCoR or SMRT (from aa 970 to aa 2453 for NCoR and from aa 981 to aa 1495 for SMRT) were found to have retained the ability to bind to class II nuclear receptors but do not display repression activity in transfection assays (Chen and Evans, 1995; Horlein *et al.*, 1995). Thus, co-expression of the receptor interaction domains (RID) would be expected to displace the endogenous corepressors bound to v-ErbA, leading to abrogation of repression.

We therefore performed transient transfection assays in HD3 cells using reporters containing either a single F2 element (everted repeat 6-type TRE), a triplicate F2 element, the HS2 enhancer or the MoMLV-TRE (Sap *et al.*, 1989) along with an expression vector for C-terminal SMRT (aa 981-1495) or C-terminal N-CoR (aa 1586-2453) (Chen and Evans, 1995; Soderstrom *et al.*, 1997). Although SMRT-(981-1495) and NCoR-(1586-2453) were readily detected in transfected cells by Western blot analysis (Figure 3B and 3D), derepression of transcription of the reporter constructs appeared to be minor (2- to 4-fold only for SMRT) (Figure 3A and 3C). Taken together our results show that C-terminal fragments of NCoR and SMRT do not display the anticipated dominant negative activity in terms of transcriptional derepression in transfected HD3 cells.

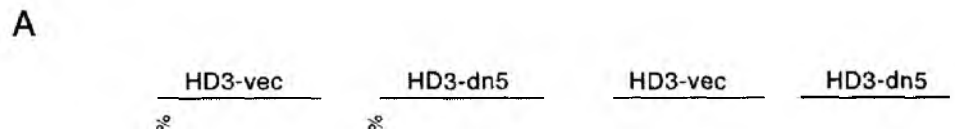


Construction of a putative dominant negative-NCoR HD3 cell line


A
HD3 cells
Relative CAT Activity
0
1
2
3
4
5
tk
F2-1x-tk
F2-3x-tk
HS2-tk
MoMLV-TRE-tk
SMRT-(981-1495)
B
SMRT-(981-1495)
57 kDa
HS2 R/A
- 1 µg 3 µg 5 µg
SMRT-(981-1495)

Assuming a one to one stoichiometry of v-ErbA/NCoR or SMRT in a complex, the apparent impairment of NCoR-(1586-2453) and SMRT-(981-1495) to act as dominant negative corepressors in HD3 cells, may be due to relatively low levels of expression as compared to the high levels of v-ErbA. Moreover, it is possible that the function of a C-terminal part of NCoR could primarily be exerted in a chromosomal context and only have minor effects in transfection assays. To overcome these potential problems we established a HD3 cell line stably expressing the C-part of NCoR comprising aa 1945-2453 (C-NCoR). This 1945-2453 region does not contain any known Sin3 or HDAC interaction domains (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Kao *et al.*, 2000; Nagy *et al.*, 1997; Wen *et al.*, 2000). Individual cell clones were analysed for continued expression of v-ErbA and *de novo* expression of C-NCoR using immunoprecipitation assays with the 1G10 (anti-gag) antibody. Apart from minor clonal variation, C-NCoR was expressed to similar levels in all individual clones analysed. Two cell clones containing either empty vector or C-NCoR (named HD3-vec and HD3-dn5, respectively) were selected and used in subsequent experiments.

Figure 3 Transiently transfected C-terminal parts of NCoR and SMRT do not significantly derepress v-ErbA mediated repression of various reporters. (**A**) Transfection assays in HD3 cells with tk, or HS2-, and F2- or MoMLV-TRE-containing reporters along with 1, 3 or 5 μg of SMRT-(981-1495). Transcriptional activity of tk-CAT construct cotransfected with empty vector is arbitrarily set to 1. (**B**) Extracts from HD3 cells transfected with HS2-tk and SMRT-(981-1495) were analysed by SDS PAGE and immunoblotted with the anti-SMRT antibody. (**C**) Transfections in HD3 cells with reporters as in (A) along with 3 and 5 μg of NCoR (1586-2453). Transcriptional activity of tk-CAT construct cotransfected with empty vector is arbitrarily set to 1. (**D**) Extracts from HD3 cells transfected with HS2-tk and NCoR-(1586-2453) were analysed by SDS PAGE and immunoblotted with the anti-NCoR antibody.



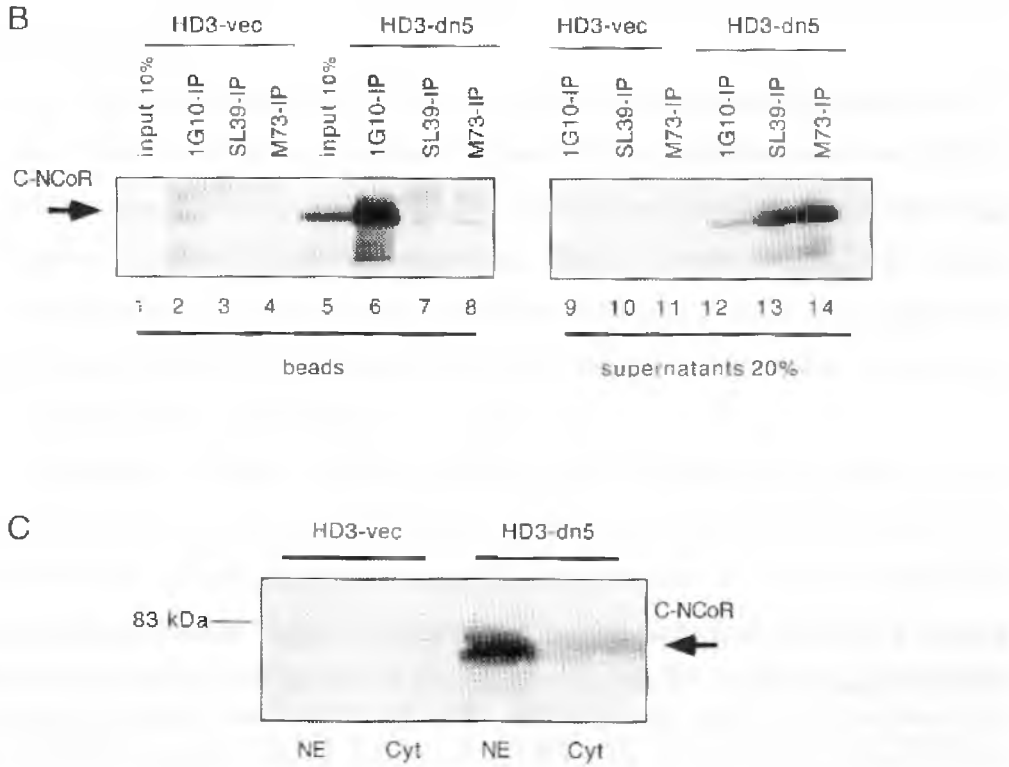
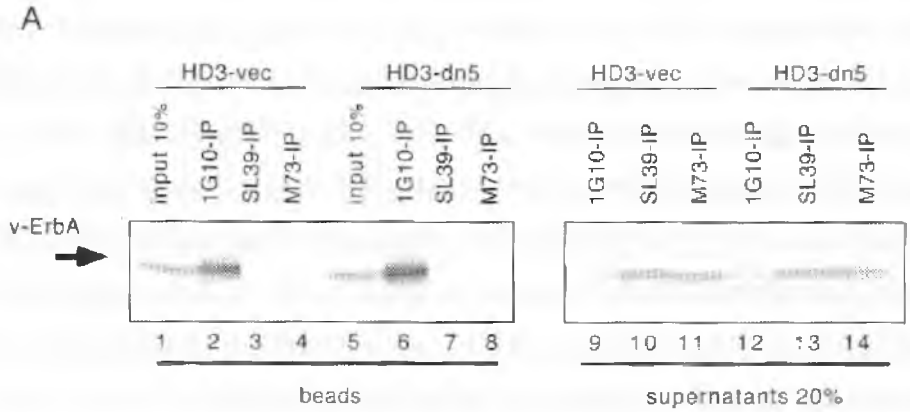

B
HD3-vec
HD3-dn5
HD3-vec
HD3-dn5
input 10%
1G10-IP
SL39-IP
M73-IP
input 10%
1G10-IP
SL39-IP
M73-IP
1G10-IP
SL39-IP
M73-IP
1G10-IP
SL39-IP
M73-IP
C-NCoR
1 2 3 4 5 6 7 8
9 10 11 12 13 14
beads
supernatants 20%
C
HD3-vec
HD3-dn5
83 kDa
C-NCoR
NE
Cyt
NE
Cyt

Figure 4 →


A
HD3-vec
HD3-dn5
HD3-vec
HD3-dn5
input 10%
1G10-IP
SL39-IP
M73-IP
input 10%
1G10-IP
SL39-IP
M73-IP
1G10-IP
SL39-IP
M73-IP
1G10-IP
SL39-IP
M73-IP
v-ErbA
1 2 3 4 5 6 7 8
9 10 11 12 13 14
beads
supernatants 20%

As shown in Figure 4A, overexpression of C-NCoR did not affect the level of v-ErbA expression (lanes 1 and 5). Immunoprecipitation using the 1G10 antibody showed that equivalent amounts of v-ErbA were precipitated from HD3, HD3-vec, and HD3-dn5 cell extracts (Figure 4A lanes 2 and 6 and data not shown). Probing the Western blot with a NCoR-specific antibody revealed that C-NCoR was efficiently co-immunoprecipitated from the HD3-dn5 cell extract (Figure 4B). The unrelated antibodies SL39 (against TBP) or M73 (against E1A) did neither precipitate v-ErbA nor C-NCoR. In addition, cell fractionation revealed that the vast majority of C-NCoR expressed in the HD3-dn5 cell line was localised in the nucleus (Figure 4C). Taken together these results showed that stably expressed C-NCoR had retained its ability to interact with v-ErbA in co-immunoprecipitation experiments.

Figure 4 Biochemical characterisation of the novel stably transfected HD3-dn5 cell line expressing C-NCoR (aa1945-2453). (**A**) and (**B**) Extracts from HD3-vec and HD3-dn5 cells were subjected to immunoprecipitation using the monoclonal antibodies against v-ErbA (1G10) and TBP (SL39) or E1A (M73) as control. Equal amounts of precipitates were analysed by SDS-PAGE (10% or 7% polyacrylamide gels) and immunoblotted using a polyclonal antibody against TRα (A) or a monoclonal against NCoR (B). The input lanes represent 10% of the extracts analysed with immunoprecipitation. (**C**) Cytosol and nuclear extract were prepared from HD3-vec and HD3-dn5 cells, analysed by SDS-PAGE (8% polyacrylamide gel) and immunoblotted using the NCoR antibody.

Activity of C-NCoR

Next we attempted to evaluate the effect of stably expressed C-NCoR on the ability of the HD3-dn5 cells to proliferate and to differentiate. HD3-vec and HD3-dn5 cells were kept in standard growth S13 medium for 9 days and as revealed by measurements of cumulative cell number as well as by optical observation of cell morphology they did not display any difference in their proliferation potential (Figure 5A). When the two cell lines were kept under conditions that allow terminal differentiation into erythrocytes for 8 days, HD3-vec and HD3-dn5 cells also showed similar growth rates (Figure 5B). During all stages of maturation there was a similar progression in hemoglobin accumulation in HD3-vec and HD3-dn5 cells (Figure 5C). Morphological and histochemical analyses of cytospins confirmed the above results (data not shown). Taken together, our data suggest that stable overexpression of C-NCoR did neither affect culture requirements nor the overall physiology and growth behaviour of HD3 cells.

Since carbonic anhydrase II (CA II) is a v-ErbA target gene and given that C-NCoR interacts with v-ErbA, as revealed by immunoprecipitation and two hybrid assays, we next assessed whether C-NCoR overexpression affected the transcription of CA II. As shown in Figure 7B (lanes 1 and 2, and data not shown), the CA II mRNA levels were unaffected in HD3-dn5 cells as compared to those in HD3-vec, suggesting that under the tested conditions the function of C-NCoR and consequently of NCoR is not crucial for the v-ErbA mediated repression of CA II.

Next we tested whether C-NCoR interfered with v-ErbA-mediated silencing in transient transfection assays. In the HD3-vec cell line, transcription levels of the F2-tk reporter and the HS2 enhancer were repressed (as compared to tk alone and M1-HS2-tk, respectively) to the same extend as in the parental HD3 cell line (compare Figure 3A and Figure 6, and data not shown). Transcription of the F2 reporters was, if at all, only very moderately derepressed in HD3-dn5 cells, (hatched bars) as compared to HD3-vec (black bars). Similarly, the levels of transcription from the HS2 enhancer-containing reporter (HS2-tk) and the MoMLV-TRE-tk were not significantly affected by stable overexpression of C-NCoR (Figure 6). In summary, these data suggest that under the employed conditions C-NCoR does not function as a dominant negative NCoR with respect to v-ErbA repression via the HS2-, F2- or MoMLV-TRE.

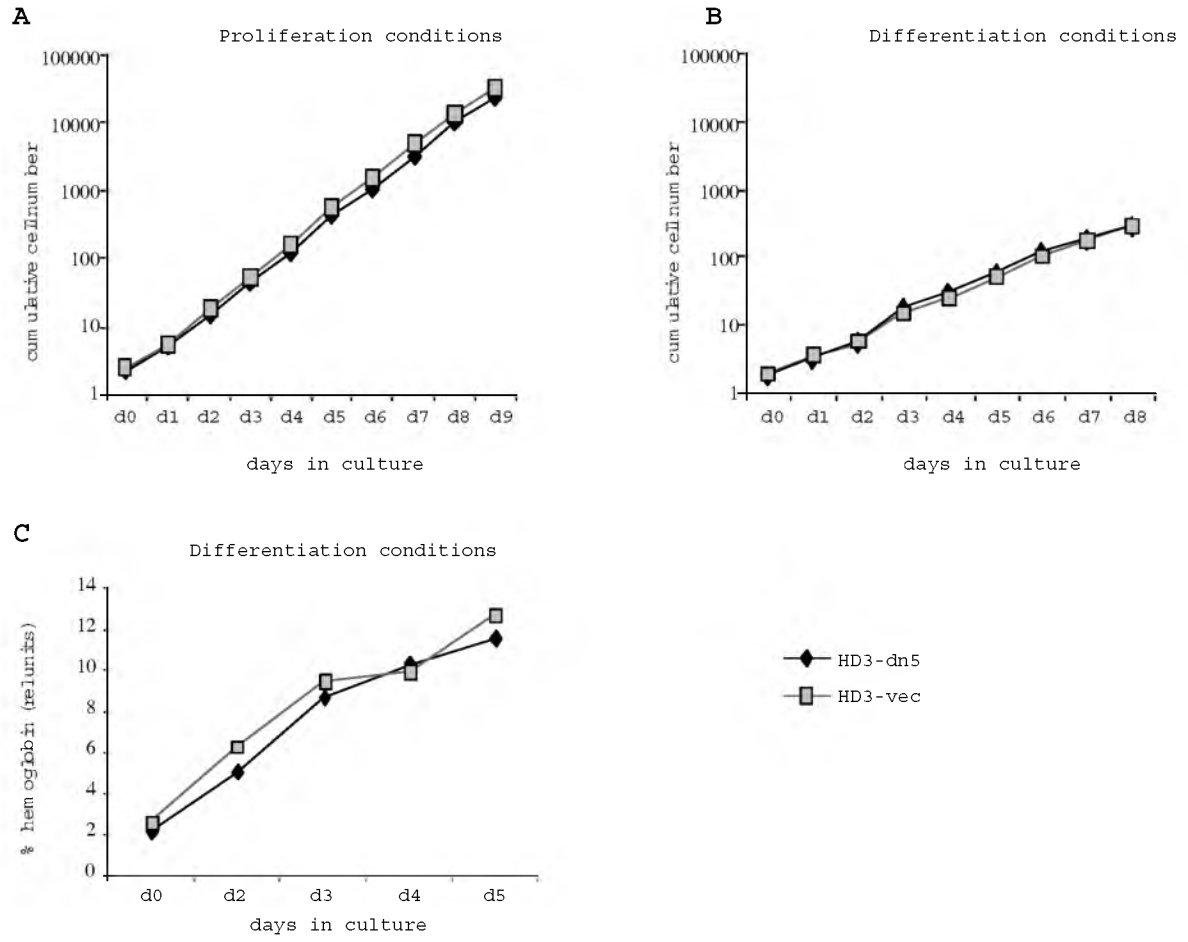


Figure 5 Biological characterisation of the novel stable transfected HD3-dn5 cell line. (**A**) HD3-vec and HD3-dn5 cells were grown in normal S13 medium and cumulative cell number measurements were taken each day. (**B**) HD3-vec and HD3-dn5 cells were grown in medium containing differentiation agents (Epo, Ins and PD153,035) and cumulative cell number measurements were taken each day. (**C**) Aliquots from HD3-vec and HD3-dn5 cells grown in conditions as in (B) were taken each day and subjected to hemoglobin assay as described in material and methods.

Does v-ErbB affect v-ErbA/NCoR interaction?

In an attempt to explain the inability of C-NCoR to function as a dominant negative NCoR, we tested whether the constitutive tyrosine kinase receptor v-ErbB, present in HD3 cells could affect the affinity of v-ErbA for NCoR. An inhibition by v-

ErbB signalling has been reported for the v-ErbA/SMRT interaction (Hong *et al.*, 1998). HD3-vec and HD3-dn5 cells were grown in the absence or presence of the tyrosine kinase inhibitor PD153,035 to inactivate v-ErbB; SCF, the ligand of c-kit, was added to prevent differentiation of the cells (Fry *et al.*, 1994; Bauer *et al.*, 1997). Immunoprecipitation experiments using the anti-gag-v-ErbA antibody and HD3-vec or HD3-dn5 cell-extracts revealed no difference in the level of coimmunoprecipitated C-NCoR (Figure 7A), suggesting that v-ErbB does not affect the v-ErbA/C-NCoR interaction.

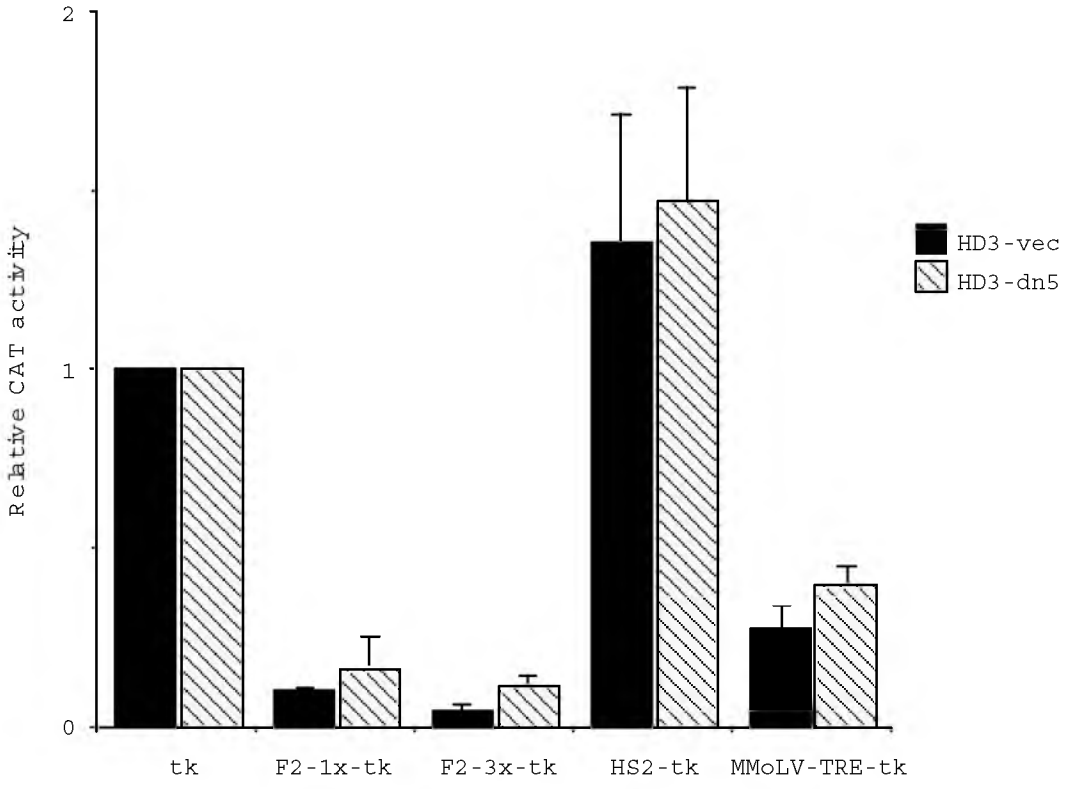


Figure 6 Stably overexpressed C-NCoR does not derepress v-ErbA mediated repression of various reporters. Transfection assays in HD3-vec (black bars) and HD3-dn5 (hatched bars) cells with tk or HS2-, F2-1x-, F2-3x- or MoMLV-TRE-containing reporters. Transcription is expressed relative to tk promoter alone for each cell line.

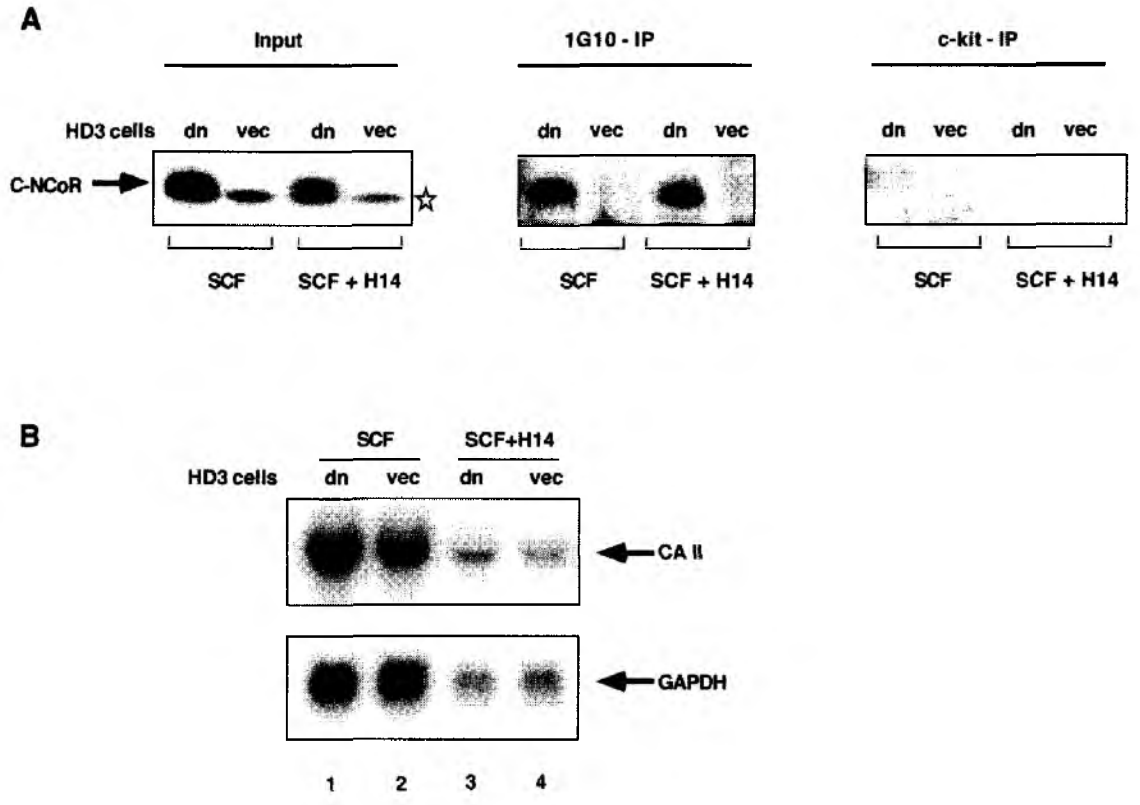


Figure 7 v-ErB does not interfere with the v-ErbA/C-NCoR physical and functional interaction. (**A**) Extracts from HD3-vec and HD3-dn5 cells grown in normal S13 medium plus SCF and in the presence or absence of the tyrosine kinase inhibitor PD153,035 were subjected to immunoprecipitation using the monoclonal anti-v-ErbA antibody 1G10 (middle panel) or an unrelated antibody against c-kit (right panel). Equal amounts of precipitates were analysed by SDS-PAGE (7% polyacrylamide gels) and immunoblotted using the antibody against NCoR. The input lanes (left panel) represent 20% of the extracts analysed with immunoprecipitation. The asterisk denotes a non-specific protein that migrates faster than C-NCoR. (**B**) Total RNA was isolated from HD3-vec and HD3-dn5 cells grown as in (A) and subjected to Northern blot analysis using probes for CA II and GAPDH.

We, finally, tested whether C-NCoR could affect the regulation of CA II by v-ErbA in the absence of v-ErbB signalling. As shown in Figure 7B addition of PD153,035 did not differentially alter CA II mRNA levels in the HD3-vec and in HD3-dn5 cells. It should be noted that in the presence of functional v-ErbB, the metabolic activity of the cells is strongly increased which is evident from increased

levels of GAPDH mRNA. Thus, it appears that v-ErbB signalling does not differentiate the CA II mRNA levels in cells expressing C-NCoR.

Discussion

The molecular mechanism that nuclear receptors utilise to regulate gene expression has recently received a lot of attention because repression appears to correlate with diseases such as thyroid hormone resistance (THR) syndrome (Tagami and Jameson, 1998), acute promyelocytic leukemia (Lin *et al.*, 1998) or AEV induced erythroleukemia (Graf and Beug, 1983). In this study we have investigated the putative role of the corepressors NCoR/SMRT in the v-ErbA-mediated transcriptional repression and transformation ability. We employed a dominant negative approach by expressing a putative dominant negative corepressor protein, C-NCoR, with the goal to relieve the ability of v-ErbA to repress transcription. We showed that although v-ErbA was able to interact with overexpressed C-NCoR in immunoprecipitation experiments, C-NCoR did neither affect transcriptional repression nor differentiation arrest of cells mediated by v-ErbA. Moreover, v-ErbB signalling did not appear to affect either the v-ErbA interaction with C-NCoR or the v-ErbA-mediated repression of CA II mRNA levels in the presence of C-NCoR.

Using transient transfection assays in the erythroid MEL cell line we have shown that v-ErbA can repress transcription of the erythroid-specific CA II-HS2 enhancer. In contrast, expression of td359-v-ErbA carrying a mutation in the helix H1, did not result in repression of transcription. Our data are consistent with findings from other groups showing that mutations in the CoR-box located at the helix 1 of LBD of TR abolish its transcriptional repressive activity and its ability to interact with corepressors (Damm *et al.*, 1987; Damm and Evans, 1993; Chen and Evans, 1995; Horlein *et al.*, 1995). However, given that CoR-box mutations also disturb interaction of nuclear receptors with RXR, it is possible that the region comprising the CoR-box serves a more architectural role (Collingwood *et al.*, 1997; Zhang *et al.*, 1997a; reviewed in Hu and Lazar, 2000). Structural studies have shown that H1 CoR-box

residues are buried in the RAR-LBD and thus might not comprise a corepressor binding site (Wurtz 1996).

Using mammalian two hybrid assays we showed that both endogenous NCoR as well as overexpressed C-NCoR fused to VP16 can interact with v-ErbA. These observations were further supported by immunoprecipitation experiments. Hence a dominant negative approach appeared a feasible way to demonstrate the involvement of NCoR/SMRT in the v-ErbA-mediated repression. However, transient co-expression of the C-terminal portions of NCoR and SMRT did not alleviate transcriptional repression of a variety of TRE/VRE-containing reporters in v-ErbA expressing cells. Our results suggest either that NCoR/SMRT are not involved in the v-ErbA-mediated repression or that NCoR-(1586-2453) or SMRT-(981-1495) were not expressed at high enough levels to squelch the endogenous corepressors from v-ErbA.

It is also possible that interference with v-ErbA transcriptional repression and transformation capacity by C-terminal parts of NCoR can only be observed on chromatin level. Therefore, we established an HD3 cell line stably expressing C-NCoR. However, HD3-dn5 cells did not display a differentiation phenotype indicative of alleviation of v-ErbA repressive function. In addition, CA II mRNA levels were not affected by overexpression of C-NCoR. These observations question the involvement of a corepressor complex containing NCoR in v-ErbA-mediated repression of transcription and oncogenicity.

Since v-ErbB signalling was reported to disturb the interaction between v-ErbA and SMRT (Hong *et al.*, 1998) we also tested whether v-ErbA/C-NCoR interaction and regulation of CA II transcription were affected by v-ErbB. Under the conditions used, it appears that the presence of an active v-ErbB does not alter C-NCoR association with v-ErbA as probed by co-immunoprecipitation assays. Moreover, transcriptional regulation of CA II was not differentially affected in HD3-dn5 cells and HD3-vec cells by the inactivation of v-ErbB. Thus, the nature of the v-ErbA/NCoR interaction might be different from that of v-ErbA/SMRT, which was shown to be phosphorylation-dependent (Hong *et al.*, 1998).

The findings that HD3-dn5 and HD3-vec cells are indistinguishable in terms of proliferation, differentiation, CA II transcription and transcriptional repression of a number of TRE/VRE-containing reporters suggest that C-NCoR does not functionally

displace the endogenous NCoR/SMRT from a putative v-ErbA/NCoR complex under the tested conditions. A number of possible mechanisms can be invoked to interpret our results. Apart from the shocking possibility that NCoR is not involved in the v-ErbA mediated repression, it seems likely that there is high redundancy between NCoR and other corepressors such as Alien (Dressel *et al.*, 1999). Cell-type determinants or fine tuned stoichiometrical requirements may also play a critical role in the corepressor usage (Soderstrom *et al.*, 1997). It is also possible that NCoR may just act as platform for other regulatory factors to facilitate the function of unliganded TR and that NCoR itself is not involved in the repression function of TR. This notion is consistent with findings involving negative TREs where transcriptional activation mediated by unliganded TR is enhanced upon interaction of TR with NCoR and recruitment of histone acetyl transferases (HATs). Upon ligand binding NCoR is displaced from TR and presence of HDACs facilitates repression through these negative TREs (Darling *et al.*, 1989; Saatcioglu *et al.*, 1993; Tagami *et al.*, 1997; Sasaki *et al.*, 1999; Tagami *et al.*, 1999). Consistent with the notion that NCoR serves only architectural roles are findings showing that NCoR (or mSin3 and HDAC) stabilise TR homodimers bound on DNA which in turn enhances the function of unliganded TR (Hollenberg *et al.*, 1996; Cohen *et al.*, 1998; Sasaki *et al.*, 1999; Satoh *et al.*, 1999). The possibility that the levels of C-NCoR in HD3-dn5 cells might simply be not high enough to saturate the highly overexpressed v-ErbA in HD3 cells cannot be ruled out either. It is also possible that NCoR requires other domains apart from the C-terminal RIDs to facilitate the stabilisation of a corepressor complex. If so, it is not unlikely that C-NCoR did not function as a dominant negative NCoR because C-NCoR may have lower affinity for v-ErbA than NCoR and thus it is not able to replace the endogenous corepressor from v-ErbA bound to chromatin. Finally we should seriously consider that in HD3 cells v-ErbA is not the only factor that regulates transcription of CA II gene.

Concluding, our results show that a C-terminal part of NCoR, comprising only the two receptor-interaction domains, does not functionally interfere with a putative v-ErbA/NCoR repressive complex. These results are puzzling because we could show that C-NCoR interacts with v-ErbA and other class II nuclear receptors. Further

experiments will be required to unravel the mechanism of transcriptional repression by v-ErbA *in vivo*.

Materials and methods

Cell culture and transfection assays

A derivative of the AEV-transformed cell line HD, namely HD3-EpoR expressing the murine erythropoietin receptor was grown in CFU-E medium (Dolznig *et al.*, 1995). Differentiation was induced in differentiation medium containing 5 U/ml human recombinant Epo and 0.0004 U/ml insulin (Dolznig *et al.*, 1995). The tyrosine kinase inhibitor PD 153035 (5 μM) was added to inhibit signalling from the v-ErbB oncoprotein (Fry *et al.*, 1994). Chicken embryo fibroblasts (CEF) were grown in EBM+H (EAGLE (Gibco-BRL), 25 mM Hepes pH7.3, supplemented with 8% FCS, 2% chicken serum, 100 U/ml penicillin and 100 μg/ml streptomycin) (Graf and Beug, 1983). MEL cells were a kind gift from Philipsen and were grown in DMEM (Gibco-BRL) supplemented with 10% fetal calf serum, penicillin-streptomycin and non-essential amino-acids (Philipsen *et al.*, 1990).

Transient transfection assays in CEF cells or HD3 cells (wild-type or derivatives) were performed with the calcium phosphate method (Barettino *et al.*, 1993) or the DEAE-dextran method (Choi and Engel, 1988), respectively. In a typical experiment 3.10^5 CEF cells or 10^7 HD3 cells were transfected with 5 μg of reporter construct together with 1 μg of EF1α-Luc or EF1α-CAT as internal control plus the indicated amounts of expression vectors or pSG7 or pOGI to keep the same amount of transfected DNA in all samples, and harvested after 48 hours. CAT and luciferase activities were measured as described previously (Barettino *et al.*, 1993).

CEF cells stable transfected were made as described in (Damm *et al.*, 1987) with pCRNCM-LoxP1-dn-NCoR or LoxP1-pCRNCM empty-vector. Briefly, 10^6 cells in medium without serums were transfected with 7 μg of expression vector DNA and 1 μg of helper-virus DNA together with 20 μg of Dosper transfection reagent (Boehringer Manheim). After 4-6 hrs the medium was removed and fresh EBM+H

medium was added. After 2 days the cells were grown in medium containing G418 for selection. The selection stopped after all control cells were dead (5-6 days).

For HD3 cells stable transfectants, CEF (wild-type or stable transfected) cells were treated with mitomysin-C (10 μg/ml) for 2 hours washed and incubated for 4 hours in normal EBM+H medium. Washed HD3 cells were added on the CEF cells and cocultured for 2 days, ficol-purified and resuspended in the appropriate medium or selected in semi solid medium containing G418 at 2-3 μg/ml. After death of control cells, the resistant cells were collected under a microscope and resuspended in S13 medium (Beug *et al.*, 1986).

Plasmids and antibodies

Reporter plasmids HS2-tk, tk, M1-HS2-tk were described in (Ciana *et al.*, 1998). F2-1x-tk and F2-3x-tk were a kind gift from Baniahmad (Baniahmad *et al.*, 1990). Expression vector pSG-v-erbA, pSG-c-erbA and pSG-v-erbA-td-359 were described in (Barettino *et al.*, 1993; Barettino *et al.*, 1994). Plasmids 3xUAS-tk-luc and Gal4-TRβ were a kind gift from Heinzel (Heinzel *et al.*, 1997). GAL4-v-erbA, Gal4-TRα and Gal4-RARα(135-403) were a kind gift from Baniahmad (Baniahmad *et al.*, 1992). C-SMRT (aa 981-1495) and PCDNA3-NCoR-1586-2453 were kind gifts from Evans and Heinzel respectively (Chen and Evans, 1995; Soderstrom *et al.*, 1997). To generate the PCRNCM-dnNCoR construct the 1524 C-terminal bases of NCoR (including the M2 epitope) were PCR amplified from pBKS-NCoR (Soderstrom *et al.*, 1997) with primers upper (5' attt<u>GCGGCCGC</u>AGCTAACTTCATAGACGTG containing a Not I site) and lower (5'CGCATCGAT<u>GTCGAC</u>GGCCGC) containing a Sal I site). The PCR product was digested with Not I and Sal I. The Sal I excised fragment containing His-tag and H902 epitope from pTAG/CMV-neo (Baier *et al.*, 1994) was subcloned into Sal I digested pLNTLb vector that contains a loxP cassette with HSV-tk and neo resistance (gift from Horcher M). The resulting construct was digested with Cla I/Not I and together with the Not I/Sal I digested PCR fragment containing C-terminus of NCoR with the M2 epitope was ligated into Cla I/Sal I digested pCRNCM vector.

To construct the PBKS-VP16-C-NCoR plasmid the pVP16 vector (Clontech) was PCR amplified using primers upper 5'-

AGCGGCCGCGTCTACCATCGAGGGCCT (containing a Not I site) and lower 3'-CTCTGCAGCGTACTCGTCAATTCCAAG (containing a Pst I site) and digested with Not I and Pst I. Simultaneous ligation of pTag/CMV-neo digested with EcoR I/Not I, the Not I/Pst I digested PCR product and pBKS-NCoR digested with Pst I/Kpn I into EcoR I/Kpn I cleaved pBKS yielded plasmid PBKS-VP16-C-NCoR. Ligation of the EcoR I/Kpn I fragment (blunt) into blunted Bgl II/Xba I digested pOGI vector resulted pOGI-VP16-C-NCoR.

The following antibodies were used: 1G10 monoclonal anti-gag antibody, polyclonal antibody against the DBD/LBD of TRα, polyclonal antibody against 2339-2453 aa of NCoR kindly provided by Heinzel (Heinzel *et al.*, 1997), monoclonal antibody M2 against the M2 epitope of flag-tagged dn-NCoR construct (Kodac), monoclonal antibodies against the myc epitope, TBP (SL39) (Ruppert *et al.*, 1996), E1A (M73), C-SMRT and c-kit.

Photometric hemoglobin assay

Hemoglobinisation of the cells was measured as previously described (Schmidt *et al.*, 1986). Briefly, 50 µl aliquots of cultured cells were transferred onto a microtiter plate, washed with PBS, resuspended in 25 µl water and frozen down. 125 µl of a test solution (0.5 mg/ml O-phenylenediamine dihydrochloride in 0.1 M citrate phosphate buffer pH 5.0, 1 µl /ml H_2O_2) were added to each thawed sample and the reaction was stopped after two minutes with 125 µl 8N H_2SO_4. Absorbances of the samples were measured with an Elisa reader (SLT spectra image) at 492 nm with a reference wavelength at 620 nm. The results were normalised to hemoglobin content of standard erythrocyte lysate.

Immunoprecipitations and immunoblotting

Cell extracts were incubated with 1G10 antibody bound to protein A beads for 3-5 hrs at 4°C in IP buffer (120 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 8, 10% glycerol, 0.5 mM PMSF, 5 mM β-mercaptoethanol, 0.01 µM Aprotinin and 1 µM Leupeptin). After 8 washes the beads were resuspended in laemli buffer (50 mM Tris pH 6.8, 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophrnol blue, 10% glycerol) and loaded into a 7, 8 or 10% SDS-polyacrylamide gel. Electrophoresis was followed

by transfer to nitrocellulose membrane and the blots were incubated overnight with the first antibody. Subsequently the blots were incubated with alkaline phosphatase- or peroxidase- conjugated secondary antibodies for 2hrs and visualised by chromogenic substrate (Promega) or by chemiluminescence (ECL, Amersham), respectively.

Cytosol and nuclear extracts were made as follows: cells were centrifuged and washed twice with PBS. Two times the packed cell volume (PCV) of buffer A (10 mM Hepes pH 7.9, 5mM $MgCl_2$, 10mM NaCl, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF) was added and cells were left for 10' on ice and subsequently homogenised. The homogenate was centrifuged at 1440 g for 10' at 4°C and 0.1xPCV from buffer B (150 mM Hepes pH 7.9, 1.4m M NaCl, 15mM $MgCl_2$, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF) was added to the supernatant. The pellet of the centrifugation was resuspended and homogenised in 1xPCV of buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF). The homogenate and the diluted supernatant from the previous step were centrifuged at 100000 g for 1hr at 4°C. The supernatants were the nuclear extract and the cytosol, respectively.

Northern blot

Total RNA was extracted using the TRIzol™ (Gibco-BRL) method; CA II, and GAPDH mRNA levels were detected by northern blot analysis as previously described (Zenke *et al.*, 1990).

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Chapter 5

General discussion

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1. Erythroid specific transcriptional regulation of CA II gene
2. Repression by v-ErbA
   - 2.1. Quenching the activity of factors bound to GATA-sites
   - 2.2. Occlusion of TR
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GENERAL DISCUSSION

The v-ErbA oncoprotein is one of the two oncoproteins encoded by the avian erythroblastosis virus (AEV) which induces erythroleukemia in chickens. The transformation ability of v-ErbA has been correlated with its ability to repress transcription of certain erythroid stage-specific genes (reviewed in Beug *et al.*, 1996; Stunnenberg *et al.*, 1999). However, the molecular mechanisms of repression by v-ErbA have remained largely obscure. In the studies described in this thesis, we have provided evidence that carbonic anhydrase II (CA II) gene is a genuine v-ErbA target gene as previously postulated. Subsequently, we have investigated the interplay between v-ErbA and other cofactors that might be involved in its repressive function.

1. Erythroid-specific transcriptional regulation of CA II gene

Our studies have provided insight into the erythroid-specific expression of CA II gene. DNase I hypersensitivity assays revealed two regions in the CA II locus (HS1 and HS2) that undergo chromatin modifications upon induction of differentiation, thus implicating HS1 and HS2 in transcriptional regulation of CA II (chapter 2). It is widely accepted that tight packing of DNA into chromatin structure is correlated with transcriptional repression. DNase I hypersensitivity can reveal DNA regulatory elements such as enhancers, promoters and locus control regions (LCRs), occupied by transcription factors. Due to binding of transcription factors, enhancers are able to overcome the negative effects of chromatin by establishing an 'open', accessible chromatin structure (reviewed in Bagga *et al.*, 1998; Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999; Higgs, 1998; Li *et al.*, 1999; Maniatis *et al.*, 1998; Udvardy, 1999). Thus, the identification of HS2 as DNase I hypersite was the first indication that it could function as a regulatory region or enhancer. Enhancers are DNA fragments usually spanning 60 to 300 bp containing densely arranged elements for regulatory factors such as Sp1, ATF, NF-κB, NF-E2, GATA and architectural proteins like HMG I/Y or LEF-1. Their primary function is to locally increase the concentration of activating factors to form the enhanceosome. The precise

arrangement and the identity of the elements are unique for each enhancer (reviewed in Carey, 1998).

Sequence inspection of HS2 revealed the presence of *cis*-acting elements for the transcriptionally positive GATA-factors. GATA-sequences were first identified as *cis*-regulatory elements in the promoters of chicken globin genes (Evans *et al.*, 1988). GATA-proteins are eryhroid-specific transcription factors and key regulators for the different stages of hematopoiesis. Transcriptional activation of most erythroid genes that play a role in maturation, survival and terminal differentiation of erythroid precursors depends on GATA-factors (reviewed in Orkin, 1992; Orkin, 1996; Orkin and Weiss, 1999; Shivdasani and Orkin, 1996). GATA-binding sites are found in the different erythroid-specific DNase I hypersensitive sites (HSs) of the β-globin gene LCR and, frequently in conjunction with NF-E2-sites, are the main determinants for the establishment of hypersites (HSs). Although the exact mechanism of GATA-factor-induced chromatin remodelling is still poorly understood, it has been postulated that when bound to nucleosomal DNA GATA-factors induce extensive disruption of histone/DNA contacts (Boyes *et al.*, 1998b). Thus, GATA-sites are directly involved in chromatin remodelling (Muro-Pastor *et al.*, 1999; Pomerantz *et al.*, 1998; Stamatoyannopoulos *et al.*, 1995). In HD3 cells, HS2 gradually opens upon induction of differentiation (chapter 2). This is concomitant with the upregulation of GATA-1 expression during erythroid differentiation (Yamamoto *et al.*, 1990). A model emerges in which GATA-sites located in the HS2 enhancer play a role in the erythroid-specific expression of CA II gene. According to this model, GATA-factors are involved in the establishment of the 'open' chromatin structure of HS2. Our observations that in primary chicken erythroid cells, HS2 is already accessible by DNase I (chapter 2), and that in HD3 cells the two critical GATA-sites are occupied in both undifferentiated and differentiating cells are in line with this model.

Recently, it has been reported that acetylation of GATA-1 by p300/CBP enhances GATA-1-dependent transcription and induction of differentiation (Blobel *et al.*, 1998; Boyes *et al.*, 1998a; Hung *et al.*, 1999). Given that the HS2 activity is governed by GATA-sites (chapter 3) we can speculate that GATA-1 together with coactivators with histone acetyltransferase (HAT) activity may contribute to the formation as well as to the activity of a HS2 bound enhanceosome.

Intriguingly, the three GATA-sequences present in HS2 show differential function. While the two GATA-sites (I and II) located upstream of the VRE are critical for the HS2 enhancer activity, the contribution of GATA-motif III (downstream of the VRE) appears to be minimal. Recently, it has been reported that transcriptional activation by GATA-factors is strongly dependent on the context of the GATA-sequence and not only on the DNA binding affinity *in vitro* (Trainor *et al.*, 2000). Therefore, we can speculate that the context and the position and of a GATA-sequence with respect to the DNA helix embracing the nucleosome dictates whether a GATA-sequence contributes to transcriptional activation or may have an architectural role in the enhanceosome assembly.

Given our observation that T3-activated TR can induce chromatin changes in HS2 as well as activate CA II mRNA levels, it appears that *in vivo* CA II may be regulated by TR. It is tempting to speculate that the GATA-factors, present primarily in hematopoietic tissue, are required for initiating chromatin changes allowing TR to bind and to activate transcription in the presence of ligand. It is also possible that synergy between GATA-factors and liganded TR along with recruited coactivators is required to activate CA II expression *in vivo*. How changes in the composition of HS2 enhanceosome affect the CA II promoter activity and how promoter/enhancer communication is achieved remains to be investigated.

2. Repression by v-ErbA

CA II has been postulated to be a target gene for v-ErbA (Disela *et al.*, 1991; Fuerstenberg *et al.*, 1992). Immunoprecipitation of v-ErbA/DNA complexes showed that v-ErbA can bind to HS2 suggesting that v-ErbA may act through the HS2 to regulate CA II gene transcription (chapter 2). Using two unbiased approaches and starting with a 17 kb fragment encompassing a substantial part of the CA II locus we have identified a v-ErbA binding site (VRE). Taken together with *in vivo* footprinting experiments revealing occupancy of the site it can be concluded that v-ErbA, an unliganded class II receptor, binds to its responsive element embedded in chromatin.

In this thesis the mechanism by which v-ErbA represses transcription of CA II has been investigated. We have shown that v-ErbA neutralises the activity of factors bound to GATA-sites and confirmed that v-ErbA can occlude liganded TR from binding to its cognate DNA site *in vivo*. Finally we have investigated the involvement of corepressors in mediating repression by v-ErbA.

2.1. Quenching the activity of factors bound to GATA-sites

In chapter 3 we have demonstrated that an important role of v-ErbA in repression of CA II transcription is quenching the positive action of the GATA-factors bound to two adjacent GATA-sites within the HS2 enhancer. How v-ErbA quenches the activity of GATA-factors is yet unclear. Given that a direct interaction between v-ErbA and GATA-factors could not be detected in immunoprecipitation assays, one can envision the existence of proteins that act as bridging factors between v-ErbA and GATA-factors. Alternatively, since VRE and the two GATA-sites are adjacent, it is likely that v-ErbA, possibly in association with a corepressor complex, inhibit the interaction of GATA-factors with DNA or with coactivators such as p300/CBP by steric hindrances. If an HDAC-containing complex is associated with v-ErbA, it is also not unlikely that HDACs deacetylate GATA-factors, thus rendering them inactive. The possibility that v-ErbA associates and thus squelches coactivators of GATA-factors such as p300/CBP, is highly unlikely since v-ErbA lacks helix H12 that is crucial for coactivator interaction (Blanco *et al.*, 1998; Feng *et al.*, 1998; Masuyama *et al.*, 1997; Nolte *et al.*, 1998; Rachez *et al.*, 2000; Ren *et al.*, 2000; Westin *et al.*, 1998). Additional studies will be needed to unravel how v-ErbA neutralises GATA-factor function, and whether and how v-ErbA interferes with promoter/enhancer communication.

We have shown that the ability of v-ErbA to neutralise the activity of GATA-factors is shared with unliganded TR (chapter 3). Overexpressed unliganded TR also causes differentiation arrest and CA II down regulation (Bauer *et al.*, 1998). It has been proposed that one determinant of v-ErbA oncogenicity is its overexpression *per se* when compared to endogenous TR (Beug *et al.*, 1996). It remains to be elucidated whether endogenous unliganded TR represses CA II expression in normal primary

cells or whether repression is caused by other cellular proteins. Our observations (chapter 2) that in primary chicken erythroid cells, the HS2 enhancer is already 'open' but the locus remains silent is in line with the existence of a cellular repressor molecule other than v-ErbA that is bound to HS2 in the undifferentiated state. Apart from TR, other members of the nuclear receptor superfamily that bind to *cis*-acting motifs similar to that of v-ErbA, such as COUP-TF, LXR or Rev-ErbA, may bind to the HS2-VRE in undifferentiated cells (reviewed in Mangelsdorf and Evans, 1995; Peet *et al.*, 1998; Waxman, 1999). Thus, it is not unlikely that overexpressed v-ErbA induces transformation because it constitutively mimics the function of a cellular repressor.

2.2. Occlusion of TR

In chapter 2 we have demonstrated that liganded TR is able to induce 'full' opening of the HS2 consistent with other studies showing that ligand-activated TR instigates chromatin changes in an *in vivo* chromatin reconstitution system (Wong *et al.*, 1997). We have also shown that liganded TR can activate transcription via the HS2 region suggesting that the HS2-VRE element can function as a TRE. Taken together our data suggest a more general role of TR in transcriptional regulation of CA II gene and reinforce the notion that TR is involved in erythroid differentiation (Disela *et al.*, 1991; Gandrillon *et al.*, 1994; Schroeder *et al.*, 1992).

In cells expressing v-ErbA and gag-TRα at equimolar levels (HD3V3 cells), the T3-mediated transcriptional activation of HS2 was not as high as the transcription obtained with a HS2-containing construct carrying a mutated VRE (M1-HS2) to which neither v-ErbA nor gag-TRα can bind (chapter 2). Thus, it appears that v-ErbA occludes liganded TR from binding to the VRE resulting in ablation of the T3-induced activation. Such a feature has often been correlated with the ability of v-ErbA to sustain cell proliferation (Damm and Evans, 1993; Forrest *et al.*, 1990; Fuerstenberg *et al.*, 1992; Sap *et al.*, 1989; Schroeder *et al.*, 1990; Zenke *et al.*, 1990). Interestingly, while in transfection experiments the T3-mediated activation of HS2 was moderate (4- to 5-fold), the mRNA levels of endogenous CA II were highly enhanced (chapter 2). In interpreting the T3-responsiveness in transfection experiments versus endogenous

CA II transcription, the intrinsic differences in the experimental approaches should be taken into consideration. Northern blot analysis of CA II mRNA levels reveals the net result of the action of liganded TR that may be exerted at multiple levels. It has been reported that TR can bring about chromatin changes by interaction with histone acetyltransferases (HATs) (Wong *et al.*, 1998). The TRAP coactivator complex that does not contain HAT activity is presumably recruited at a subsequent step (reviewed in Glass and Rosenfeld, 2000). Thus, it is possible that the observed CA II mRNA levels represent the combinatorial or sequential effect of liganded TR with various coactivators. Transiently transfected DNAs do not form a normal nucleosomal structure (Archer *et al.*, 1992; Bresnick *et al.*, 1990; Cereghini and Yaniv, 1984; Innis and Scott, 1983; Jeong and Stein, 1994a; Jeong and Stein, 1994b; Pennie *et al.*, 1995; Reeves *et al.*, 1985; Schlake *et al.*, 1994; Van Lint *et al.*, 1996). Therefore, it is possible that in transfection assays the T3-induced activation of transcription from HS2 reflects only the function of coactivators without HAT activity.

From our data it is obvious that v-ErbA is able to occlude liganded TR in transfection assays. However off-rate experiments show that RXR-TR binds with higher affinity than RXR-v-ErbA to the CA II-VRE. Therefore, it is possible that additional mechanisms contribute to the ability of v-ErbA to antagonise TR *in vivo*. One can speculate that signalling from v-ErbB to v-ErbA might affect the affinity of v-ErbA for the DNA element *in vivo*. It is also conceivable that the more than 100-fold overexpression of v-ErbA as compared to TR (Disela *et al.*, 1991) in the AEV transformed HD3 cells compensates for the reduced DNA affinity of v-ErbA.

In conclusion, our data corroborate the notion that v-ErbA occludes liganded TR thus repressing T3-activated transcription. We have additionally shown that occlusion depends largely on the sequence of the DNA responsive element, because substitution of the VRE with a canonical DR4 potentiates the T3-response (chapter 3). It is not unlikely that the presence of a weak TRE within the HS2 is one of the determinants of the proper timing of CA II expression during differentiation.

Comparing the repressive activities of v-ErbA and unliganded TR revealed an unexpected and intriguing observation. While unliganded TR could repress transcription from the heterologous tk reporter construct containing the VRE sequence, v-ErbA could not repress the intrinsic activity of tk promoter (chapter 3). When acting

via the everted repeat element F2, both TR and v-ErbA significantly repressed the tk promoter activity, reinforcing the critical role of the sequence and the architecture of the element for v-ErbA function (Olson *et al.*, 1998; Wahlstrom and Vennstrom, 1998). Thus it appears that the repressive function of v-ErbA is not equivalent to that of TR. It is possible that occlusion of unliganded TR by v-ErbA also contributes to the transformation activity of v-ErbA.

2.3. Involvement of corepressors in the v-ErbA mediated repression

Immunoprecipitation and two hybrid assays have clearly shown that v-ErbA can interact with the corepressor NCoR (chapter 4). Thus, it seemed feasible that overexpression of a N-terminally truncated NCoR (C-NCoR) comprising the receptor interaction domains (RIDs) and lacking silencing functions would result in interference with v-ErbA/NCoR interaction and in a relief of transcriptional repression. Surprisingly, using HD3 cells stably overexpressing C-NCoR a functional interference of the presumed dominant negative corepressor could not be observed. In addition, no differences were observed in the phenotype of the stable cell line expressing C-NCoR as compared to 'empty vector' cells, leading to the idea that NCoR is not crucial for the v-ErbA-mediated repression. It is tempting to speculate that NCoR may be a platform protein that can facilitate docking of proteins contributing to the repressive function of TR and v-ErbA. This model is corroborated by the observations that TR in the presence of ligand associates with HDACs repressing transcription via negative TREs, whereas unliganded TR-mediated activation is enhanced by NCoR and HATs (Darling *et al.*, 1989; Saatcioglu *et al.*, 1993; Sasaki *et al.*, 1999; Tagami *et al.*, 1997; Tagami *et al.*, 1999). It is also possible that the truncated C-NCoR has reduced affinity for v-ErbA as compared to full-length NCoR. Towards this end, v-ErbA may interact with full-length NCoR via other domains besides the C-terminal receptor interaction domains (RIDs), thus explaining the inability of C-NCoR to behave as a dominant negative NCoR. Additional studies are required to assess whether and under what conditions NCoR associates with v-ErbA *in vivo*.

3. How does v-ErbA regulate CA II transcription?

Based on our and other findings I propose the following model to explain how CA II is transcriptionally regulated in erythroid cells and how v-ErbA interferes with this mechanism (Figure 1). Early in hematopoietic differentiation, the CA II locus is closed. At the stage of erythroid progenitor (or earlier) the two DNase I hypersites (HS1 and HS2) are opened. While binding of GATA-1 very likely causes HS2 opening, the factors involved in HS1 opening remain unknown. However, HS2 remains transcriptionally inactive. At a next step, p300/CBP associate and acetylate GATA-factors triggering the formation of the HS2 enhanceosome and rendering GATA-factors capable of activating transcription. Endogenous liganded TR might also play a role in the enhanceosome assembly. It is also possible that at this step coactivator complexes such as TRAP/SMCC are tethered to HS2 to mediate the transcriptional activity of the HS2-enhanceosome. For transcriptional activation of CA II gene to occur, HS2 has to be brought in close proximity to the promoter by looping to locally increase the concentrations of positive transcription factors at the neighbourhood of the promoter and to initiate transcription.

In AEV-transformed cells, v-ErbA binds to the HS2 and prevents the activity of GATA-factors. Whether v-ErbA prevents their acetylation or their ability to activate transcription remains to be seen. By binding to HS2, v-ErbA also occludes liganded TR from binding. As a result of this dual activity of v-ErbA, a productive interaction between HS2 enhancer and the promoter cannot occur. It is also possible that v-ErbA interacts with and stabilises a corepressor complex, thus inhibiting the assembly of the HS2-enhanceosome. v-ErbA may also stabilise repressor complexes formed on the promoter. This hypothesis is supported by our findings that v-ErbA interacts with NCoR in immunoprecipitation assays *in vitro*. Inhibition of HDAC activity does not relieve repression by HS2 in transfection assays, whereas endogenous CA II mRNA is avidly upregulated. Supposing that NCoR is the platform for a complex tethered by v-ErbA, one can speculate that v-ErbA stabilises a NCoR/HDAC-containing complex formed at the promoter region. In the proximity of the CA II promoter there is a CpG island that may be methylated in the AEV transformed cells by the MeCP2 repressor (Antequera *et al.*, 1990; Cameron *et al.*, 1999; Issa *et al.*, 1994; reviewed in Jones and Wolffe, 1999; Razin, 1998). Thus, it is not unreasonable to speculate that v-ErbA

interacts and facilitates the function of the MeCP2 repressor protein present in the vertebrate HDAC complex (Jones *et al.*, 1998; Nan *et al.*, 1998). Our findings that v-ErbA cannot convey active repression to the VRE unless it is in the context of HS2 suggests that a combination of factors and pathways might be in place for the regulation of CA II transcription (Figure 1).

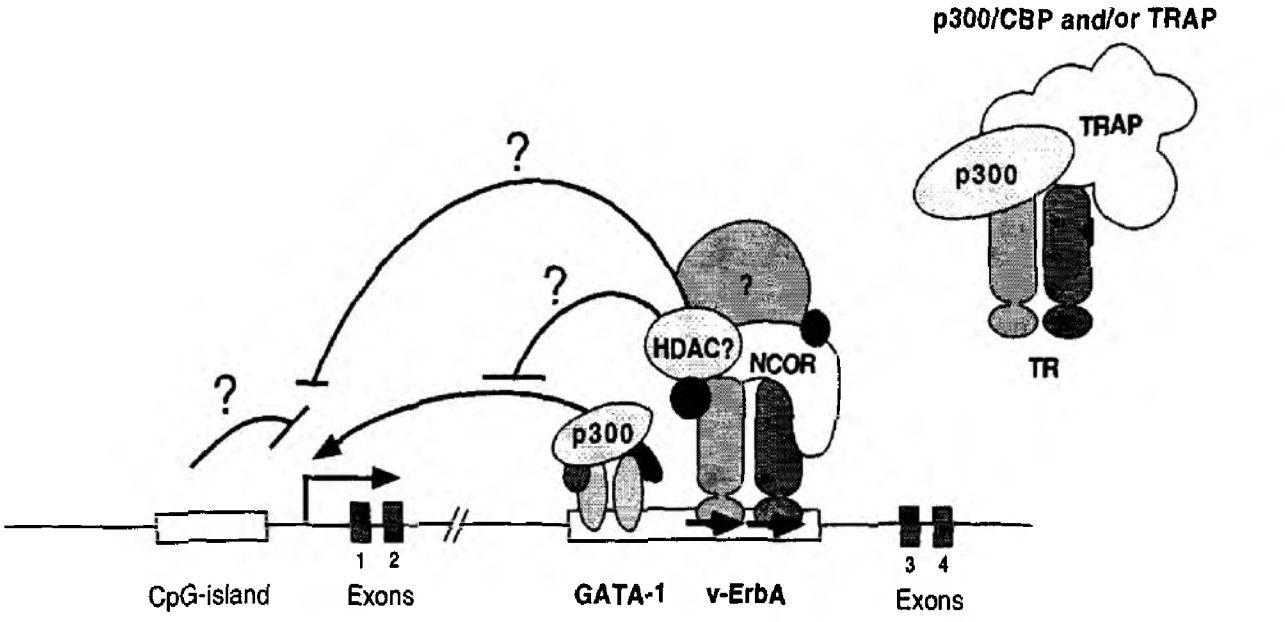

Figure 1 Model for regulation of CA II gene transcription by v-ErbA. v-ErbA quenches the activity of GATA-factors bound to the HS2 that are responsible for the erythroid-specific activation of CA II gene transcription. v-ErbA also occludes liganded TR from binding to the CA II-VRE thus preventing the assembly of the HS2 enhanceosome. In addition, v-ErbA may contribute to the activity of a corepressor complex formed on the promoter perhaps by being involved in the stabilisation/function of MeCP2 complex bound on the CpG islands of the CA II promoter. Whether a corepressor complex containing NCoR/SMRT and HDACs is involved in any of these activities of v-ErbA remains to be shown.

4. Implications

During tumor development multiple pathways appear to be deregulated implicit to the fact that cells have multiple mechanisms to regulate their growth and differentiation and that several separate changes may be required to bypass these controls. Inherent to their crucial role, class II nuclear receptors are important targets for impairment during neoplasia and their inactivation might represent a critical step in multistage processes leading to carcinogenesis. The chicken v-ErbA system has long been and still is a paradigm, and results obtained in this system have guided studies on hematopoiesis as well as on malignancies in other organisms. Undoubtedly, further in depth investigations in the v-ErbA model system will help elucidating the general mechanisms leading to oncogenic transformation, and to develop drugs to stop tumor progression, to induce apoptosis or to trigger differentiation of tumor cells.

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SUMMARY

Thyroid hormone (T3) exhibits a vast array of profound effects on homeostasis, development, amphibian metamorphosis, differentiation and neoplasia. The action of T3 is mediated via its binding to the cognate thyroid hormone receptor (TR). TR in turn binds to specific DNA sequences called thyroid responsive elements (TREs). TR in the presence of T3 activates transcription while unliganded TR leads to repression. TR is expressed in many different tissues and utilises a multitude of mechanisms to regulate transcription. The v-ErbA oncoprotein is a mutated viral variant of TRα and is encoded by the Avian Erythroblastosis virus (AEV). AEV encodes for another oncogene, v-ErbB, which is a constitutively activated EGF receptor with tyrosine kinase activity. The co-operation of the two oncogenes leads to fatal erythroleukemia as a result of enhanced proliferation and arrest of differentiation of the AEV-transformed erythroblasts. Due to mutations, v-ErbA cannot bind T3 hormone and thus it cannot activate transcription like its cellular counterpart. It is now widely accepted that the contribution of v-ErbA to leukemia is due to its ability to constitutively repress transcription of certain erythroid genes such as carbonic anhydrase II (CA II). However, the molecular mechanisms employed by v-ErbA to repress transcription have remained largely obscure. The hypothesis that v-ErbA behaves as a constitutive unliganded TR is attractive but not consistent with a number of experimental observations.

In the studies described in this thesis we set out to assess how v-ErbA regulates the transcription of the CA II gene. Using DNase I hypersensitive site mapping we have identified two hypersite regions (designated HS1 and HS2) as prime candidate regions for transcriptional regulation of the CA II gene (chapter 2). HS1 is located 7 kb upstream of the transcription start site while HS2 is located 8 kb downstream of the promoter within the second intron. Immunoprecipitation of v-ErbA/DNA complexes and *in vitro* footprinting experiments demonstrated that the intronic HS2 region has a binding site for v-ErbA. *In vivo* footprinting experiments showed that an unliganded class II nuclear receptor, v-ErbA binds to a v-ErbA responsive element (VRE) embedded into chromatin. Interestingly, the *in vivo* occupancy of the VRE by v-ErbA coincides with CA II repression while a loss of the VRE protection occurring upon

induction of differentiation correlates with CA II upregulation. Using mutagenesis analysis and transfection experiments, we showed that HS2 behaves as a potent enhancer that is repressed by v-ErbA.

Detailed mutation analyses (chapter 3) demonstrated that the HS2 region activates transcription independent of the position and the orientation relative to the heterologous tk as well as the homologous CA II promoters, thus identifying HS2 as a *bona fide* erythroid-specific enhancer. Additionally, we demonstrated that the enhancer activity of HS2 is governed by erythroid-specific factors that bind to two adjacent DNA motifs with GATA sequence located 5' to the VRE, which constitute binding sites for the GATA-factors. Thus, v-ErbA appears to neutralise the positive activity of erythroid GATA-factors bound to the HS2. Quenching of GATA-factors is observed with unliganded TR as well.

In chapter 2 we showed that the chromatin architecture of HS2 can be modulated by liganded TR in erythroid progenitor cells that express gag-TRα to levels equivalent to v-ErbA (HD3V3 cells). These findings are consistent with studies showing that TR instigates chromatin changes upon ligand activation. We show that overexpressed TR binds to the HS2-VRE as an RXR-TR heterodimer *in vitro* and conveys transcriptional activation upon addition of ligand *in vivo*. Full activation as defined by a HS2-containing construct carrying a mutation in the VRE that abolishes v-ErbA binding, could however not be accomplished. Our data corroborate the notion that v-ErbA occludes liganded TR from binding to VRE resulting in ablation of the T3-induced activation. This feature of v-ErbA correlates very well with its observed oncogenic activity (chapter 3). We show in addition that occlusion of liganded TR by v-ErbA *in vivo* depends largely on the sequence of the DNA responsive element, suggesting that v-ErbA and TR responsive elements (VREs and TREs) are not necessarily the same.

Comparison of the repressive activities of v-ErbA and unliganded TR via the VRE element (chapter 3) uncovered an unexpected and intriguing observation. The v-ErbA oncoprotein is unable to repress the intrinsic tk promoter activity while unliganded TR can efficiently repress it. Given that repression of CA II transcription is important for the v-ErbA induced leukemic phenotype, this lack of repression by v-ErbA versus unliganded TR may be part of its oncogenic activity.

In chapter 4 we started to address the question whether the presumed corepressor complex containing NCoR/SMRT and HDAC (and possibly SIN3) binds to v-ErbA enabling its repressive function *in vivo*. Using immunoprecipitation assays we showed that v-ErbA can indeed interact with endogenous NCoR. Because the C-terminus of NCoR (C-NCoR) does not comprise silencing functions but can still interact with nuclear receptors, it was anticipated that overexpression of C-NCoR would interfere with v-ErbA/NCoR interaction and ultimately would result in relief of transcriptional repression mediated by v-ErbA. Using HD3 cells stably overexpressing C-NCoR, a functional interference of the presumed dominant negative corepressor could however not be observed. In addition, the overall phenotype of the cells was not affected by overexpression of C-NCoR, suggesting that under the tested conditions NCoR is not crucial for the v-ErbA mediated repression. These data question the generally accepted but not proven model that corepressors are essential for the repressive functions of nuclear receptors.

Our studies have provided new insight into the mechanism of the v-ErbA-mediated transcriptional repression. Further *in vivo* investigations may shed light on the role of v-ErbA in leukemic transformation.

Samenvatting

Thyroid hormoon heeft een aantal duidelijke functies gedurende homeostase, ontwikkeling, amfibische metamorfose, differentiatie en tumorigenese. De thyroid hormoon receptor (TR) komt tot expressie in een groot aantal verschillende weefsels wat suggereert dat het transcriptie kan reguleren via verschillende mechanismen. Het v-ErbA oncoproteïne is een gemuteerde virale variant van TRα en wordt gecodeerd door het 'Avian Erythroblastosis Virus'(AEV). AEV codeert ook voor een ander oncogen, v-ErbB, een constitutief actieve EGF receptor met tyrosine-kinase activiteit. De samenwerking tussen deze twee oncogenen leidt tot fatale erythroleukemie als gevolg van een verhoogde proliferatie en een differentiatieblok van de AEV getransformeerde erythroblasten. Als gevolg van een aantal mutaties kan v-ErbA thyroid hormoon (T3) niet meer binden en kan het daarom transcriptie niet meer activeren, zoals zijn cellulaire tegenhanger wel kan. Het wordt nu alom geaccepteerd dat de bijdrage van v-ErbA aan leukemie het gevolg is van het vermogen van v-ErbA om constitutief de expressie van bepaalde erythroïde genen te onderdrukken, zoals carbonic anhydrase II (CA II). De mechanismen van transcriptionele repressie door v-ErbA zijn tot nu toe grotendeels onduidelijk gebleven. De hypothese dat v-ErbA zich gedraagt als een constitutieve receptor zonder hormoonbinding is aantrekkelijk maar niet consistent met een aantal experimentele observaties.

In de studies die worden beschreven in dit proefschrift hebben we geprobeerd vast te stellen hoe v-ErbA transcriptie van het CAII gen reguleert. Gebruikmakend van 'DNaseI hypersensitive site mapping' hebben we twee DNaseI hypersensitieve regios (HS1 en HS2) geïdentificeerd als belangrijkste kandidaten voor de regulatie van CAII transcriptie (Hoofdstuk 2). HS1 is 7 kb bovenstrooms van de CAII transcriptiestartplaats gelokaliseerd, HS2 8 kb benedenstrooms van de promoter in het tweede intron. Een combinatie van immunoprecipitaties van v-ErbA/DNA complexen en *in vitro* footprint experimenten toonden aan dat de HS2 regio een bindingsplaats is voor v-ErbA. *In vivo* footprint experimenten lieten zien dat een klasse II nucleaire receptor zonder hormoon, v-ErbA, bindt aan een 'v-ErbA responsive element' (VRE) in het chromatine. De *in vivo* binding van v-ErbA valt samen met repressie van het CAII gen, terwijl het verlies van protectie van de VRE na inductie van differentiatie

samenvalt met CAII activering. Gebruik makend van mutagenese- en transfectie-experimenten konden we aantonen dat de HS2 zich gedraagt als een sterke enhancer waarvan v-ErbA de activiteit onderdrukt.

Gedetailleerde mutatie analyse (Hoofdstuk 3) demonstreerde dat de HS2-regio transcriptie kan activeren onafhankelijk van de positie en de oriëntatie t.o.v. de heterologe tk promoter en de homologe CAII promoter, wat aantoont dat HS2 als een *bonafide* erythroid-specifieke enhancer kan functioneren. Daarnaast toonden we ook aan dat de enhancer activiteit van de HS2 wordt veroorzaakt door erythroid-specifieke factoren die binden aan twee GATA-factor bindingsplaatsen die aan de 5'- kant van de VRE grenzen. v-ErbA lijkt de positieve activiteit van deze erythroid-specifeke GATA-factoren te neutraliseren. Ook TR zonder hormoon is in staat tot neutralisatie van de activiteit van deze GATA factoren.

In Hoofdstuk 2 tonen we aan dat de chromatine architectuur van de HS2 gemoduleerd kan worden door hormoongebonden TR in erythroïde voorloper cellen die gag-TRα even hoog tot expressie brengen als v-ErbA (HD3-V3 cellen). Deze resultaten zijn in overeenstemming met een aantal studies die laten zien dat TR chromatine veranderingen induceert na activering door hormoon. We toonden ook aan dat TR dat tot overexpressie wordt gebracht aan de HS2-VRE bindt als een RXR-TR heterodimeer en transcriptie kan activeren na de toevoeging van hormoon. Maximale activering, zoals bij een HS2 bevattend construct met een mutatie in de VRE waardoor v-ErbA niet meer kan binden, kon echter niet worden bereikt. Onze data dragen bij aan de hypothese dat v-ErbA hormoon-geactiveerde TR van de VRE kan verdringen wat resulteert in inhibitie van thyroid hormoon geïnduceerde activering. Deze eigenschap van v-ErbA correleert goed met zijn oncogene capaciteit (Hoofdstuk 3). We toonden ook aan dat competitie van v-ErbA en TR voor een bindingsplaats *in vivo* grotendeels afhankelijk is van de nucleotidevolgorde van het bindingselement, wat suggereert dat v-ErbA en TR 'responsive elements' (VRE en TRE) niet noodzakelijkerwijs dezelfde elementen zijn.

Het vergelijken van de repressie door v-ErbA en niet-hormoongebonden TR op een VRE element (Hoofdstuk 3) toonde een onverwachte en interessante eigenschap aan. Het v-ErbA oncoproteïne is niet in staat om de tk promoteractiviteit te represseren, terwijl TR zonder hormoon dit wel kan. Gegeven het feit dat repressie van

CAII transcriptie belangrijk is voor het v-ErbA geïnduceerde fenotype, suggereert dit dat dit onvermogen tot repressie door v-ErbA ten opzichte van TR deel kan zijn van zijn oncogene activiteit.

In Hoofdstuk 4 zijn we begonnen de vraag te beantwoorden of het corepressor complex dat NCoR/SMRT en HDAC (en mogelijk SIN3) bevat, v-ErbA bindt en repressie door dit eiwit mogelijk maakt *in vivo*. In immunoprecipitaties lieten we zien dat v-ErbA inderdaad endogeen NCoR kan binden. Omdat de C-terminus van NCoR (C-NCoR) geen 'silencing'-functies heeft maar nog steeds nucleaire receptoren kan binden, werd verwacht dat overexpressie van C-NCoR zou interfereren met endogene v-ErbA/NCoR interacties wat uiteindelijk zou resulteren in derepressie door v-ErbA. Een dominant-negatieve functie voor C-NCoR in cellen die dit eiwit stabiel tot expressie brengen kon echter niet worden aangetoond. Daarnaast was het fenotype van de cellen onveranderd door overexpressie van C-NCoR, wat suggereert dat onder de geteste condities NCoR niet essentieel is voor v-ErbA geïnduceerde repressie. Deze data trekken het algemeen geaccepteerd maar niet bewezen model in twijfel dat corepressoren essentieel zijn voor repressie door nucleaire hormoonreceptoren.

Onze studies hebben nieuwe inzichten verschaft op het gebied van v-ErbA geïnduceerde transcriptionele repressie. Verder *in vivo* onderzoek kan mogelijk meer duidelijkheid brengen in de rol van v-ErbA in het ontstaan van leukemie.

Curriculum Vitae

Georgia Braliou was born on the 9th of November 1966 in Livadia, Greece. On 1984 she finished high school and the same year she entered the Faculty of Science, department of Biology of the University of Patras. In 1985 she entered the Faculty of Science, department of Chemistry of Athens University. She received her degree in Chemistry in December 1990. In 1992 she started a PhD project at the National Hellenic Research Foundation under the supervision of Nikos Tsawdaroglou, which was terminated in 1994 due to death of the supervisor. In 1995 the author joined the Gene Expression Programme at the European Molecular Biology Laboratories in Heidelberg, Germany to work on the PhD project described in this thesis, under the supervision of Prof. Dr. Ir. Henk Stunnenberg. In 1996 she joined her supervisor to his moving to the University of Nijmegen, The Netherlands. With the support of Prof. Dr. Ir. Henk Stunnenberg she will present her PhD thesis at the University of Nijmegen the 23rd of January 2001.