Klonierung und Charakterisierung des humanen TIF1γ, eines neuen Mitglieds der Familie Transkriptioneller Intermediär-Faktoren

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A Michael, à Leonardo et son petit frère qui n'a pas encore un nom mais dont la présence est déjà très concrète dans nos cœurs et notre vie

Ai miei cari genitori

Dans le champ de l'expérimentation le hasard ne favorise que l'esprit préparé.

Louis Pasteur

...on ne voit bien qu'avec le coeur. L'essentiel est invisible pour les yeux.

Antoine de Saint-Exupéry

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I declare and certify herewith, that this work has been conducted by myself, without employing unauthorized procedures or materials, and that it has not been submitted at any other university or elsewhere in order to obtain an academic grade.

Letizia Venturini

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Key words: Transcription Intermediary Factor 1, RBCC proteins, PHD/bromodomain proteins.

Schlüsselworte: Transkriptionelle Intermediär-Faktoren 1, RBCC Proteine, PHD/bromodomain Proteine.

1. INTRODUCTION

1.1 Transcriptional regulation of eukaryotic genes

Transcriptional regulation of gene expression in eukaryotes is a complex process which is highly regulated at many levels. It requires the concerted action of several factors to integrate different transcriptional regulatory signals controlling cellular responses such as proliferation, differentiation and apoptosis.

Transcription of a gene by RNA polymerase II (pol II) requires the assembly of the general transcription factors (GTFs) at the promoter to form a preinitiation complex (PIC). At most promoters this begins with the binding of the multi-subunit TFIID GTF which is composed of the TATA-binding protein (TBP) and TBP-associated factors (TAFs), to the TATA box. The basal transcription initiation complex is then completed by the recruitment of the other GTFs (TFII A, B, F, E, and H) and the pol II which must be activated by phosphorylation of its C-terminal domain (CTD). This is sufficient to direct basal transcription of a DNA template (Buratowski, 1994).

However, eukaryotic genes are generally maintained in a repressive state through their nucleosomal organisation which blocks the assembly of the basal transcription machinery at the core promoter. It is now well established that reversible structural modifications of chromatin actively contribute to the control of gene expression. Enzymatic activities which modify chromatin structure can be recruited to promoter regions through direct interaction with DNA-binding proteins such as sequence-specific transcription factors. They are proteins with a modular architecture, which, in addition to a DNA binding domain (DBD), possess an independent, functionally separable effector domain that can either activate or repress transcription through interaction with co-activator or co-repressor multiprotein complexes. These co-regulator copmplexes remodel chromatin structure or covalently modify the core histones, thus stimulating or inhibiting the formation of the transcription initiation complex (Kornberg *et al*., 1999; Struhl, 1999; Wolffe & Haynes, 1999).

1.1.1 Transcriptional regulation at the chromatin level

Fundamental effectors of nucleosomal conformational changes include histone acetyltransferases (HATs) which acetylate specific, evolutionarily conserved lysine residues at the N-terminal tails that extend outwardly from the globular core of the histone octamer. Histone acetylation, by neutralising the positive charge and reducing the affinity

of histone-DNA interaction, enhances the accessibility of DNA by transcription factors and thus favours transcriptional activation (Grunstein, 1997; Struhl, 1998; Wade *et al*., 1997). Several transcriptional regulatory proteins which are part of multiprotein activator complexes and possess histone acetyltransferase activity have been shown to activate transcription: GCN5 (Brownell *et al*., 1996), the $TAF_{II}250$ (Mizzen *et al*., 1996), the p300/CBP transcriptional coactivator (Bannister & Kouzarides, 1996; Ogryzko *et al*., 1996) which interacts with a variety of DNA-binding activators, thus integrating multiple signalling pathways (Janknecht & Hunter, 1996), ACTR (Chen *et al*., 1997) and SRC-1 (Spencer *et al*., 1997), two transcriptional coactivators for several nuclear receptors, the p300/CBP associated factor p/CAF (Yang *et al*., 1996), forming protein complexes with multiple histone acetylases. Histone acetylation is a dynamic process and may be reversed by histone deacetylases (HDACs) which restore a repressive chromatin configuration. Currently, seven mammalian histone deacetylases have been isolated. They have been grouped into two classes on the basis of their structural organisation: Class I includes HDAC1, 2, and 3, which appear to be similar to the yeast Rpd3; Class II comprises HDAC 4, 5, 6, and 7, which resemble the yeast deacetylase Hda1 (Fischle *et al*., 1999; Grozinger *et al*., 1999; Kao *et al*., 2000; Verdel & Khochbin, 1999).

Another class of chromatin-modifiers are the chromatin-remodeling multiprotein complexes which have ATPase and helicase activity, such as SWI/SNF, NURF or RSF. These complexes use the energy from ATP hydrolysis to induce changes in nucleosomal DNA topology and mobility through alteration of DNA-histone contacts within the nucleosome (Cairns, 1998; Travers, 1999). ATP-driven chromatin remodelling complexes enhance the accessibility of DNA and may be implicated in both transcriptional activation and repression, thus facilitating the function of transcription repressors as well as activators. These activities have been demonstrated for SWI/SNF in yeast and for the NuRD/Mi-2 complex, which present both chromatin remodelling and deacetylation activity (see Knoepfler & Eisenman, 1999, and Tyler & Kadonaga, 1999, for reviews).

In several other cases, HATs and ATP-dependent remodeling factors are associated in the same complex and cooperate to overcome the transcriptional repression imposed by chromatin packaging. Some of these proteins are part of the basal transcription apparatus itself, such as the TFIID complex subunity $TAF_{II}250$ (Mizzen *et al*., 1996), while others directly interact with component of the transcription apparatus such as SWI/SNF and p300/CBP, which are tightly associated with the RNA pol II holoenzyme (Wilson *et al*., 1996).

Probably all these enzymatic activities which induce a more open and accessible chromatin structure do not function only at the transcription initiation stage, but also help RNA pol II pass through the chromatin during the transcription elongation phase (Orphanides & Reinberg, 2000).

Additionally, N-terminal tails of histones likely constitute also a target for signal-activated kinases and may function as an important link between signal transduction and gene expression. Indeed, recent results demonstrate that phosphorylation of the histone H3 is closely associated with transcription activation (Cheung *et al*., 2000, for review). Analogous to histone acetylation, histone phosphorylation would probably cause decondensation of the chromatin fiber through addition of negative charge.

Reversible covalent histone modifications, such as acetylation and phosphorylation, tougether with DNA methylation, could also act as specific binding surfaces to recruit complexes which mediate downstream processes like chromatin condensation or decondensation (reviewed by Strahl & Allis, 2000).

1.2 The nuclear receptor (NR) superfamily

The nuclear receptor (NR) superfamily comprises a multitude of ligand-dependent transcription factors which influence fundamental biological processes, such as development, homeostasis, proliferation, and differentiation. They modulate transcription through homo- or heterodimeric binding to cis-acting DNA response elements at the promoter region of their target genes, and are inducible by small hydrophobic ligands which include steroids, retinoids, vitamin D, thyroid hormones, or diverse products of lipid metabolism, such as fatty acids and prostaglandins (Beato *et al*., 1995; Chambon, 1996; Mangelsdorf & Evans, 1995). The NR superfamily also includes a large number of so-called orphan receptors for which regulatory ligands have not been identified (Mangelsdorf & Evans, 1995).

1.2.1 Domain structure of nuclear receptors

The nuclear receptors present a common modular structure with six regions (A to F) and characteristic functional domains (Fig.1)

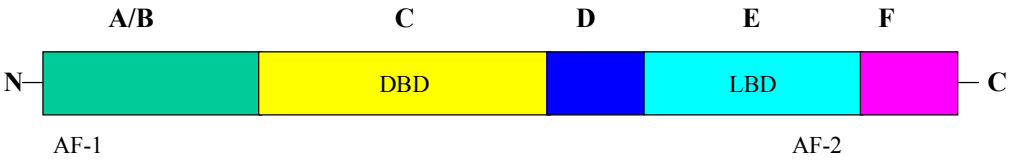


Fig. 1. Schematic representation of the NR structure.

The N-terminal A/B region, which varies in size and primary sequence among the superfamily, contains an autonomous, ligand-independent activation function (AF-1). It is followed by a DNA binding domain (DBD) in the central region (C), composed of two highly conserved type C2H2 zinc-fingers, which targets the nuclear receptors to their specific hormone response elements. The C-terminal region E comprises the ligand binding domain (LBD), and a ligand-inducible activation function (AF-2).

1.2.1 Nuclear receptor mechanism of action

The nuclear receptors regulate transcription by several mechanisms. They can activate or repress transcription in a ligand-dependent or -independent manner by recruitment of corepressor or coactivator complexes. Recently, through yeast two hybrid screening, several proteins have been isolated which interact with the nuclear receptor AF-2 domain. They mediate nuclear receptor transcriptional activity and act as corepressors or coactivators (Chambon, 1996; Horwitz *et al*., 1996; Glass *et al*., 1997 for reviews).

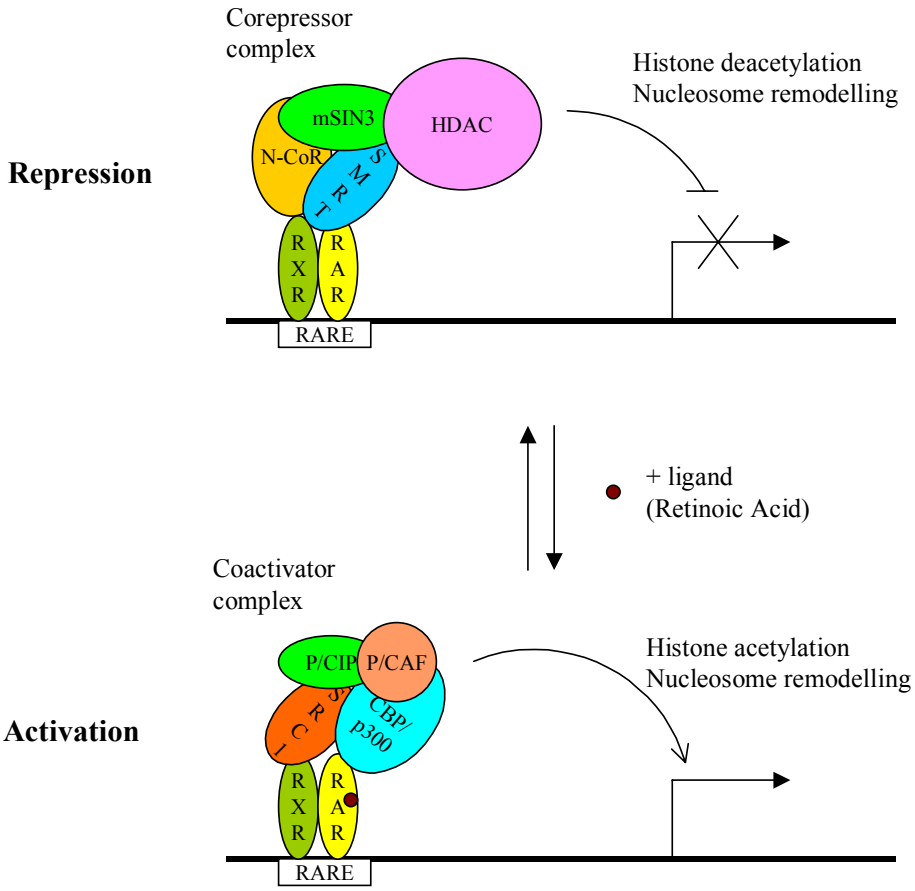


Fig. 2. Schematic illustration of mechanism of transcriptional regulation by nuclear receptors.

As demonstrated for the thyroid receptor (TR) and the retinoic acid receptor (RAR), which act as heterodimers with the retinoic receptor RXR, unbound nuclear receptors can negatively modulate transcription through binding of the silencing mediators N-CoR (Nuclear Receptor Co-Repressor) and SMRT (Silencing Mediator of Retinoid and Thyroid Receptors) (Horlein *et al*., 1995; Chen & Evans, 1995) which recruit both Class I (through direct interaction with Sin3) and Class II histone deacetylases to form a multiprotein repressor complex which also contains the chromatin remodelling factor SWI/SNF (Alland *et al*., 1997; Heinzel *et al*., 1997; Nagy *et al*., 1997; Sudarsanam & Winston, 2000;

Underhill *et al*., 2000; Kao *et al*., 2000). Ligand binding produces a conformational change which leads the corepressors to dissociate from nuclear receptors. Additionally, ligand binding induces the recruitment of a multiprotein activation complex with histone acetyl transferase (HAT) activity, including SRC-1, CBP/p300, P/CAF, and p/CIP (Fig. 2; Torchia *et al*., 1997).

The interaction of CBP/p300 with nuclear receptors is essential for their transactivation function. Competition for this nuclear cointegrator, which can be a limiting component within the cell, may explain the inhibition of the transcriptional activities of other classes of transcription factors, for example AP-1, by NRs, a process called "transrepression" (Chakravarti *et al*., 1996; Kamei *et al*., 1996).

Phosphorylation, too, appears to contribute to the modulation of the transcriptional activity of nuclear receptors. Growth factor-induced activation of several kinases, including cyclin-dependent kinases and MAP kinases (MAPK), induces phosphorylation of specific serines within the AF-1 domain of NRs, leading to stimulation or inhibition of coactivator recruitment at the AF-2 domain, independent of ligand binding (Tremblay *et al.*, 1999; Hammer *et al*., 1999; Hu *et al.*, 1996; Shao *et al*., 1998).

1.3 The RNA editing process

RNA editing is a post-transcriptional modification of the RNA sequence which produces an alteration of the gene-encoded sequence. Like RNA splicing, this process may lead to the production of functionally distinct proteins from the same gene. It is a widespread phenomenon in eukaryotes, and contributes to the generation of molecular diversity (reviewed by Niswender, 1998).

Different and unrelated molecular mechanisms may be involved in RNA editing. In the mitochondria of primitive eukaryotes, such as kinetoplastid protozoa, edited RNAs are produced through phosphodiester bond cleavage, deletions and/or additions of nucleotides (generally uridine residues) and religation. This process generates correct “readable” open reading frames, which cannot be deduced from the genomic sequence and requires a ribonucleoprotein complex, including endonuclease, uridil transferase, RNA ligase and, for deletion editing, also a U-specific 3'-exonucleas. Additionally, specific small RNAs ("guide RNAs") which present a complementarity to the sequence to be edited function as templates specifying the site of uridine insertion or deletion.

In mammals, the most common type of editing is "substitutional editing", obtained by direct nucleotide modification. It has been observed in transfer RNAs (t-RNAs), precursor messenger RNAs (pre-mRNAs) and viral RNAs which have infected mammalian

host cells. It comprises uridine-to-cytidine (U-to-C) and adenosine-to-inosine (A-to-I) conversions and is produced through nucleotide deamination reactions, catalysed by cytidine (CDARs: cytidine deaminases acting on RNA) or adenosine deaminases (ADARs: adenosine deaminases acting on RNA). CDARs are part of a large multiprotein complex, called editosome, containing additional cellular factors which contribute to the specific recognition of RNA to be edited and to the regulation of the editing process, most of which remains yet to be discovered. By contrast, ADARs can act as single proteins recognizing specific secundary structures in the RNA template.

The first RNA-editing discovered in mammals was the tissue-specific C-to-U deamination of Apolipoprotein B (Apo B) mRNA. This reaction changes a Gln codon (CAA) to a premature stop codon (UAA) producing thus a truncated Apo B protein, named Apo B48. Apo B is implicated in lipoprotein metabolism, and in humans, the expression of the Apo B48 edited form is restricted to the small intestine, while the full-length unedited Apo B100 is synthesized in liver (Chester *et al*., 2000)

1.4 The Transcriptional Intermediary Factor 1 (TIF1) family

The "Transcriptional Intermediary Factor 1" family comprises presently three nuclear proteins (TIF1α, β, and γ) believed to function as transcriptional coregulators at the chromatin level.

1.4.1 The TIF1 domain structure

Based on amino acid homologies, a typical TIF1 domain structure (Fig. 3) has been ascribed to members of this family.

Fig. 3. Schematic representation of the TIF1 protein structure

The N-terminal region contains the RBCC (Ring finger-B box-coiled coil) tripartite motif which most likely functions as a cooperative protein-protein interaction motif (Borden, 1998). RING (for "Really Interesting New Gene") fingers are cysteine domains of the form C3HC4 which chelate two molecules of zinc in a tetrahedric-coordination binding that generates a cross-braced structure (Fig. 4).

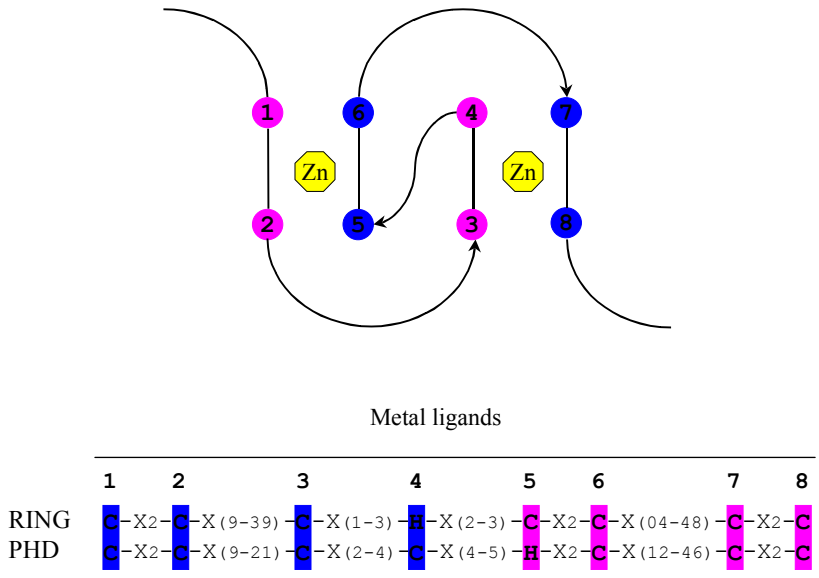


Fig.4. Schematic representation and consensus sequence of the RING and PHD finger domains (C: cysteine, H: histidine, X: any residue; ovals represent histidine residues and octagons represent zinc atoms).

Zinc binding is required for folding of the domain and, consequently, for its biological function. The affinity for zinc is higher for the first site than for the second, suggesting the possibility that, in some conditions of low zinc concentration, only the first site is folded and able to associate with its binding proteins. RING fingers have been found in many proteins of diverse functions from virus to vertebrates and likely contribute to the specificity and/or multimerisation properties of the RBCC motif to form large macromolecular complexes. (Freemont, 1993; Borden *et al.*, 1995a; Saurin *et al.*, 1996; Borden, 2000). The B-box is also a cysteine-zinc binding domain of the form CHC3H2 (Reddy & Etkin, 1991). The B-box is a globular domain which binds only one zinc atom with the other potential chelation residues unoccupied (Borden *et al.*, 1995b, Bellini *et al.*, 1995). This domain, too, is likely implicated in protein-protein interaction (Alcalay *et al.*,

1998; Cao *et al*., 1998). The third element of the RBCC motif is the coiled-coil domain which consists of α-helical regions. The helices are formed by turns of 3.5 amino acid residues where every fourth or seventh position is occupied by hydrophobic residues which together constitute a hydrophobic interaction surface. Two to five α-helices are disposed in a parallel or anti-parallel manner forming the super-helix of the coiled-coil domain (Lupas *et al*., 1996; 1997), which probably functions as an interaction surface in protein multimerisation (Cao *et al*., 1997; 1998).

The C-terminal regions of the TIF1 proteins contain a polycomb homology domain (PHD) finger (Aasland *et al.*, 1995; Koken *et al.*, 1995) and a bromodomain (Jeanmougin *et al.*, 1997), which are characteristic motifs of proteins known to function as transcriptional coregulators at the chromatin level. The PHD is a cysteine-histidine zinc finger domain of the form C4HC3 which binds two zinc atoms in a cross-brace arrangement like that of the RING finger (Capili *et al.*, 2001). Like RING fingers, the PHD domain appears to function as a protein-protein interaction motif. The bromodomain (Fig. 5) is a ~110-amino-acid module, evolutionarily highly conserved, consisting of four amphipathic α-helices (α_Z, α_A, α_B, and α_C) packed tightly against one another in an antiparallel manner, with a long loop between helices α_Z and α_A (ZA loop). The ZA and the BC loops form a hydrophobic pocket interacting with lysine-acetylated peptides derived from histones H3 and H4, suggesting a role in chromatin recognition for this domain (Dhalluin *et al.*,1999; Owen *et al*., 2000).

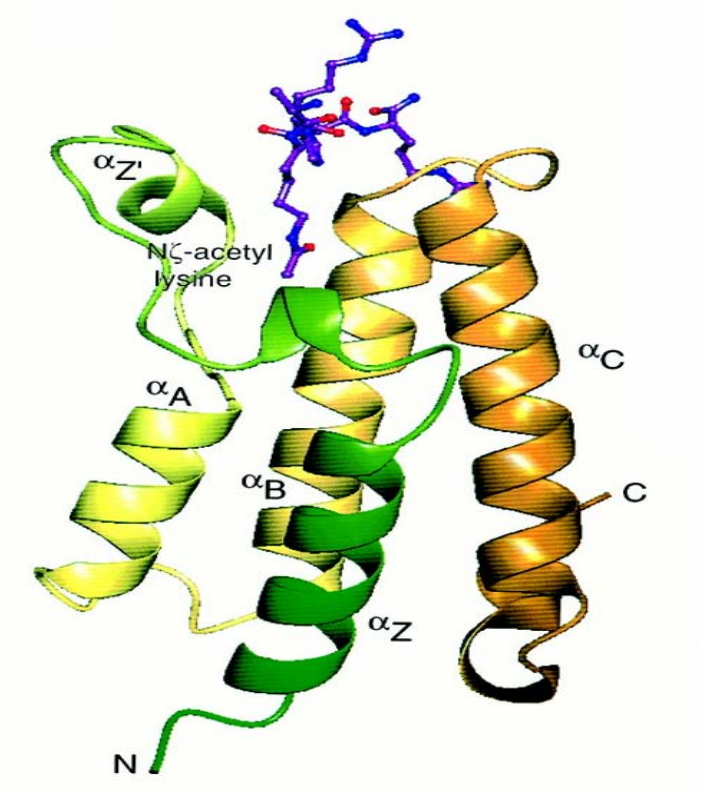


Fig. 5 Three-dimensional structure representation of the histone H4 Nζ-acetyl-lysine recognition by the bromodomain of GCN5 (Owen *et al*., EMBO J. 2000 19:6141-6149).

Bromodomain containing proteins associate frequently with histone acetyltransferases or have histone acetyltransferase activity themselves. In this case, bromodomains may direct HATs to acetylated histones, where they can enhance the acetylation and activate gene transcription (Dhalluin *et al.*, 1999; Owen *et al.*, 2000; Winston & Allis, 1999). PHD and bromodomain are frequently both present in tandem in a number of proteins and they may act as a functional unit (Schultz *et al*., 2001).

1.4.2 TIF1α

Historically, the N-terminal part of TIF1α was first detected as T18, the fusion partner of bRaf in a chemically induced murine hepatic carcinoma (Miki *et al.*, 1988). The murine TIF1α was later discovered through two hybrid screening by virtue of its interaction with nuclear receptors. Indeed, it was found to interact specifically and in a

ligand-dependent manner with the ligand binding domain of several NRs, including retinoid X (RXR), retinoic acid (RAR), vitamin D3 (VDR), estrogen (ER), and progesterone (PR) receptors, and it has been proposed to be a mediator of their transactivation function AF-2 (Le Douarin *et al*., 1995; vom Baur *et al.*, 1996).

TIF1α is a phosphoprotein which undergoes a ligand-dependent hyperphosphorylation as a consequence of nuclear receptor binding (Fraser *et al*., 1998). Moreover, it possesses an intrinsic protein kinase activity and is capable of both autophosphorylation and selective phosphorylation of general transcription factors, e.g. TFIIE, TAFII28, TAFII55 (Fraser *et al*., 1998).

TIF1α was reported to silence transcription when tethered to promoters through fusion to a heterologous DNA binding domain in transiently transfected cells, and to interact *in vitro* with the heterochromatin-associated proteins HP1α, β (MOD1), and γ (MOD2) (Le Douarin *et al*., 1996), a subfamily of the chromatin organisation modifier (chromo) superfamily (Koonin *et al*., 1995) which is thought to be implicated in gene silencing, probably by induction and/or stabilisation of local heterochromatinic regions. The HP1 proteins in *Drosophila melanogaster* are involved in the position effect variegation (PEV), a well-known epigenetic mechanism of silencing euchromatic genes placed near or integrated into heterochromatin (Wakimoto, 1998, for review). This gene silencing occurs only in a subset of cells and is inherited by their progeny, leading to variegated or mosaic patterns of expression.

According to confocal and electron microscopic studies, TIF1α is a non-histone protein tightly and preferentially associated with highly accessible euchromatic regions (Remboutsika *et al.*, 1999). This supports the hypothesis that TIF1α could play a dual role in transcription, being involved in both activation and repression.

Recent data suggest that TIF1α may interact directly with the promyelocytic gene PML, and could act as a growth repressor, too (Zhong *et al*., 1999).

Oncogenic fusion proteins of TIF1α (and TIF1γ) with RET have been detected in thyroid carcinomas in children from Cernobyl (Klugbauer & Rabes, 1999).

1.4.3 TIF1β

TIF1β was isolated shortly after TIF1α by two-hybrid screening using HP1α as bait (Le Douarin *et al*., 1996). TIF1β, like TIF1α, is a phosphoprotein with intrinsic kinase activity catalysing *in vitro* autophosphorylation and phosphorylation of HP1α, β, and γ, to which they bind directly (Nielsen *et al*., 1999).

TIF1β was also referred to as KAP-1 (KRAB-associated protein 1) or KRIP-1 (KRAB-interacting protein 1), by virtute of its interaction with the transcriptional silencing

domain KRAB (krüppel-associated box; Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al*., 1996), which is a conserved motif present at the amino-termini of proteins that contain multiple C2H2 zinc fingers at their carboxy-termini. It is one of the most widely distributed trancriptional silencing domains identified in vertebrates, found in about one third of the vertebrate Krüppel-type (C2H2) zinc finger proteins (Bellefroid *et al*., 1991; Margolin *et al.*, 1994; Vissing *et al.*, 1995; Witzgall *et al.*, 1994). When fused to a heterologous DBD, this regulatory domain silences both basal and activated transcription in transfected cells, in a dose-dependent manner and over large distances (Deuschle *et al*., 1995; Moosmann *et al*., 1997; Pengue *et al*., 1994). TIF1β interacts, through its RBCC motif, with numerous KRAB domains both in yeast and *in vitro*, but not with KRAB mutants which do not repress transcription (Peng *et al*., 2000; Abrink *et al*., 2001). Overexpression of TIF1β can enhance KRAB-mediated repression (Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al*., 1996); moreover, the KRAB domain is not able to repress transcription in cells which lack TIF1β.

TIF1β was also reported to be an integral component of a histone deacetylase complex (Underhill *et al*., 2000) and to possess an autonomous silencing function which requires not only HP1 binding but also histone deacetylation (Nielsen et al., 1999).

These results support a model in which KRAB-containing zinc finger proteins (KRAB-ZFPs) repress transcription of a target gene through interaction with the TIF1β corepressor which recruits the HP1 proteins inducing histone deacetylation and targeting to heterochromatic chromosomal regions or local formation of heterochromatin-like structures with subsequent silencing of gene expression (Agata *et al*., 1999; Lechner *et al*., 2000; Nielsen *et al*., 1999; Peng *et al*, 2000; Ryan *et al.*, 1999).

Recently, it was demonstrated that the tandem PHD and bromodomain of TIF1β form a cooperative unit which is required for transcriptional repression and provides an interface for protein-protein interaction with a novel isoform of Mi-2a and other components of the NuRD histone deacetylase complex, which are downstream effectors of this repression function (Schultz *et al*., 2001).

TIF1β was also identified as an intrinsic component of an N-CoR repressor complex (Underhill *et al*., 2000).

1.4.4 TIF1γ

In this work the cloning and characterisation of TIF1γ, the third member of the TIF1 family, is reported.

2. MATERIALS AND METHODS

2.1 Reagents and biological materials

2.1.1 Reagents and solutions

0.5 M EDTA	186 g $Na_2EDTA{\cdot}2H_2O$ in 700 ml H_2O pH 8 with NaOH to 1 l with H_2O
10 X TE	100 mM Tris, pH 7.5 10 mM EDTA, pH 8
TB	10 mM PIPES acid 15 mM $CaCl_2$ 250 mM KCl pH 6.7 with KOH 55 mM $MnCl_2$
TB-DMSO Solution	7% DMSO in TB
50 X TAE	2 M Tris 0.57% acetic acid 50 mM EDTA, pH 8
6 X loading buffer	30% Glycerol 0.25% Bromophenolblue 0.25% Xylenecyanol
6 X orange G loading buffer	1 g Orange G (SIGMA) 20 g Ficoll to 100 ml with H_2O
Molecular-Weight DNA Markers	100 bp DNA Ladder (MBI Fermentas) Lambda DNA / Eco RI + Hind III Marker 3 (MBI Fermentas)

10 X TBE Buffer	108 g Tris Base 55 g boric acid 9.3 g $Na_2EDTA \cdot 2H_2O$ H_2O to 1 liter
20 X SSC	3 M NaCl 0.3 M Sodium citrate (pH 7.0)
2 X HBS	28 mM NaCl 10 mM KCl 50 mM HEPES acid 1.5 mM Na_2HPO_4 to pH 7.05 with NaOH filter sterilise (0.45 µm nitrocellulose filter) store at -20°C in 50 ml aliquots
Lambda Diluent	20 mM TrisCl, pH 7.5 20 mM $MgSO_4$
2.5 M $CaCl_2$	183.7 g $CaCl_2 \cdot 2H_2O$ (Sigma) H_2O to 500 ml filter sterilise (0.45 µm nitrocellulose filter) store at -20°C in 15 ml aliquots
10 X PBS (for 5 l)	400 g NaCl 10 g KCl 11.5 g $Na_2HPO_4 \cdot 7H_2O$ 10 g KH_2PO_4
ONPG Solution	4 mg / ml 0.1 M Na_2HPO_4 / NaH_2PO_4, pH7.5
100 X Mg-Solution	0.1 M $MgCl_2$ 4.5 M β-mercaptoethanol
Neutralisation solution	1 M TrisCl, pH 7.4 1.5 M NaCl

Denaturation solution

1.5 M NaCl
0.5 M NaOH

50 X Denhardt's solution

5 g Ficoll 400
5 g PVP
5 g BSA
to 500 ml with H2O
filter and store at -20°C in 50 ml aliquots

Hybridisation solution

3 X SSC
5 X Denhardt's solution
0.5% SDS
100 µg / ml fish sperm DNA

4.1.7 Media for bacterial culture

LB broth *per liter*:

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH

LB agar *per liter LB*:

15 g agar

LB top agar *per liter LB*:

7.5 g agar

SOB medium *per liter*:

20 g bacto tryptone
5 g yeast extract
0,5 g NaCl
10 ml 250 mM KCl
to pH 7 with 1 N NaOH
autoclave
5 ml 2 M $MgCl_2$

SOC medium

1 l SOB
20 ml 1 M glucose

NZC broth *per liter*: 10 g NZ amine A
5 g NaCl
2 g $MgCl_2.6H_2O$
autoclave
5 ml 20% casamino acids

NZC top agarose *per liter NZC*: 6 g agarose

2.1.2 Oligonucleotides and Primers

M13 Forward primer for sequencing reactions

R9: 5'- TCTGACTTTAGGCGTTTTCTG -3'
hTIF1γ 3' specific primer (nucleotides 3423-3444) , used for Reverse Transcription

G7F: 5'- ACACCACCTCTCTCAACCAACC -3'
hTIF1γ (nucleotides 2528–2548) forward primer for RT-PCR

G7R: 5'- TTGCCGACCTGTGCATGAGG -3'
hTIF1γ (nucleotides 2683-2702) reverse primer for RT-PCR

PEXT.F: 5'-CCTTGTTAATGGAAAGTCCCC-3'
hTIF1γ (nucleotides 2653–2673) specific primer for Primer Extension

TSS-P*fl*MI-BamHI oligonucleotides:

I	5'-CGGATCCAAACCAGCT-3'	(coding)
II	5'-CTGGTTTGGATCCGAAG-3'	(non-coding)

2.1.3 Plasmids and Constructs

M13mp18 (Boehringer Mannheim-Roche)
pBSII SK+ (Stratagene)
pSG5 : mammalian expression vector (Green *et al*., 1988)
pSG5M : a modified pSG5 vector with a 62 bp Bgl II - Eco RI polylinker from pIC20R cloned in the Bgl II - Eco RI restriction sites of pSG5 (Green *et al*., 1988)
pCH110 : β-galactosidase expression vector

TK-βGAL : β-galactosidase expression vector (Clontech)
TK-LUC : reporter plasmid which contains the firefly luciferase reporter gene under the control of the thymidine kinase promoter (de Thé *et al*., 1990)
RAREβ3-TK-LUC : derived from the TK-LUC plasmid by insertion of three copies of the DR5 retinoic acid response element into the SalI site (de Thé *et al*., 1990)
pG4M-polyII : encodes amino acids 1-147 of the GAL4 cDNA and used for the GAL4(1-147) chimeras (Tora *et al*., 1989)
17M2-ERE-G-LUC : derived from the 17M2-ERE-G-CAT, a reporter plasmid containing two GAL4 binding sites (17M2) and an estrogen response element (ERE) in front of a β-globin (G) promoter-chloramphenicol acetyl transferase reporter gene (Tora et al., 1989), by substitution of the CAT box by the full-length luciferase cDNA (De Wet *et al*., 1987)
ER(C)-VP16 : encodes amino acids 176-280 (the DNA binding region) of ERα and amino acids 413-490 of VP16 (Tora *et al*., 1989)
pBK-CMV (Stratagene)
pSG5-hTIF1α : mammalian expression vector containing the complete hTIF1α cDNA sequence.
pSG5M-hTIF1γ : mammalian expression vector containing the complete hTIF1γ cDNA sequence.
pG4MpolyII-hTIF1γ : mammalian expression vector containing the complete hTIF1γ cDNA sequence fused to the GAL4 DBD (1-147).
pG4MpolyII-hTIF1γΔ(466-479) : deletion mutant derived from pG4MpolyII-hTIF1γ by replacing amino acids WAKNVVNLGNLVIE of the TIF1γ-TSS domain by a single glycine residue, using the two PflMI sites at nucleotides 1473 and 1529, and a spacer containing a unique BamHI site derived from annealing of TSS-PflMI-BamHI oligonucleotides.
pG4MpolyII-hTIF1γΔPHD/Bromo : deletion mutant derived from pGMpolyII-hTIF1γ by deletion of the PHD and bromodomain, using the BglII sites present at nucleotides 2781 and 3326.

2.1.4 cDNA libraries

FOIE A3 **:** cDNA library from human liver (HepG2 cell line) constructed into the EcoRI site of the λgt10 bacteriophage (de Thé et al., 1987)

Human testis cDNA library (Stratagene) **:** constructed into Lambda ZAP Express / EcoRI / Xho I vector (Stratagene)

2.1.5 Bacterial strains

DH5αF'
C600 (host strain for the FOIE 3A human liver cDNA library)
XL1-Blue MRF1 (host strain for the human testis cDNA library)

2.1.6 Cell lines

CHO
HeLa
COS-6

2.2 Methods

2.2.1 Agarose gel electrophoresis of DNA

The separation of DNA fragments after their size was obtained through 0.8%-2% gel electrophoresis (the concentration of agarose solution was chosen depending on the expected length of the fragments to be separated), with 1X TAE as running buffer. The DNA samples were prepared with loading buffer and loaded into the wells. The migration was performed under constant voltage (between 80 and 120 V depending on the gel size). The DNA bands were visualised through a UV-light transilluminator by addition of ethidium bromide (0.5 µg/ml) to the melted agarose cooled to 55°C. The size of the DNA fragments was determined by comigration of molecular-weight DNA markers.

2.2.2 Isolation and purification of DNA fragments from agarose gels

At the end of electrophoresis, the band containing the fragment of interest was excised from the gel and DNA was purified by the geneclean II Kit (BIO 101 Inc.). This procedure utilises a 6 M NaI solution to dissolve agarose at 55°C, and a special silica matrix, called Glassmilk, which binds single and double stranded DNA without binding of contaminants.

Alternatively, the insert was electroeluted from the gel into a dialysis tube, extracted with phenol/chloroform, purified through an Elutip-d column (Schleicher & Schuell) and precipitated with ethanol.

2.2.3 Phenol-chloroform extraction and ethanol precipitation of DNA

To clear DNA solutions from protein contaminations, an equal volume of phenol/chloroform/isoamyl-alcohol (50:24:1) was added and well mixed by vortexing. After 5 min of centrifugation at 13,000 x g at room temperature, the top aqueous phase, containing the DNA, was recovered to a clean tube and another extraction was performed with an equal volume of chloroform/isoamyl-alcohol (24:1) using the same conditions. The aqueous phase was transferred to a new tube and the DNA precipitated with ethanol.

For ethanol precipitation 0,1 volumes of 3 M NaAcetate pH 5.2 and 2 volumes of 100% ethanol were added to the DNA solution, mixed well and the tube placed at -80°C for 10 min or at 20°C overnight. The DNA was pelleted through centrifugation (15-30 min, 15,000 rpm, 4°C) and the pellet was washed with 70% ethanol, air dried, and dissolved with bidistilled water or TE.

2.2.4 Digestion of DNA with restriction endonucleases

Restriction endonuclease cleavage was accomplished by incubating the DNA in 1 X appropriate buffer with 1 to 5 U/µg DNA restriction enzyme for 1 hr at the recommended temperature (generally 37°C). For multiple digestions with more than one endonuclease, which are not active at the same salt concentration, the DNA sample was first incubated with the enzyme working at the lower salt concentration. The volume of the reaction was then increased and the concentration of salts optimised for the next enzyme.

2.2.5 Repairing 3' or 5' overhanging ends to generate blunt ends

The Klenow fragment of DNA polymerase I was used to convert overhanging ends generated by restriction endonucleases to blunt ends. 1 µl of Klenow fragment (Boehringer Mannheim-Roche) was directly added to the digestion reaction (up to 5 µg DNA in 50 µl) together with 1 µl of 10 mM dNTP-mix. The incubation was continued for 15 min at 37°C. The reaction was stopped by adding 1 µl 0.5 M EDTA and heating to 75°C for 10 min.

2.2.6 Dephosphorylation of linearised plasmid vectors

After linearisation of the plasmid vector by restriction endonuclease digestion, alkaline phosphatase was used to catalyse the hydrolysis of the 5'-phosphate residues in order to prevent self-ligation of vector termini. 0.1 units of alkaline phosphatase (Boehringer Mannheim-Roche) were added directly to the digestion reaction and the incubation at 37°C was continued for 30 min. The vector DNA was then extracted with phenol/chloroform, isolated through electrophoresis gel migration, and purified by Geneclean (BIO 101 Inc.) .

2.2.7 Ligation of a DNA insert into a linearised and dephosphorylated plasmid vector

To obtain ligation of DNA sticky- or blunt-ended inserts into plasmid vectors, the Rapid Ligation Kit from Boehringer Mannheim (now Roche Molecular Biochemicals) was used: 100 ng of linearised, dephosphorylated, and purified vector were mixed with purified DNA insert in a molar ratio 1:2 in 1X DNA dilution buffer to 10 µl total volume. After addition of 10 µl T4 DNA ligation buffer (2X concentrated) and 1 µl of T4 DNA ligase, the ligation reaction was incubated for 5 min (sticky ends) or 30 min (blunt ends) at room temperature. 4 µl of the reaction were used for transformation of competent bacteria and the remainder was stored at -20°C.

2.2.8 Preparation of competent bacterial cells

A single bacterial colony was used to inoculate 3 ml LB and grown overnight at 37°C while shaking at 250 rpm. 500 µl of the overnight preculture were diluted in 100 ml SOB medium and grown further to an OD_{600} of 0,450 - 0,6. The culture was then tranferred to prechilled sterile polypropylene tubes and left on ice for 10 min. The cells were collected by centrifugastionat 4°C and 2500 rpm for 10 min. After gentle resuspension of the pellet with 30 ml TB, the tubes were placed on ice for 10 min and then centrifuged at the same conditions. 8 ml of TB-DMSO solution were used to carefully resuspend the pellet and the now competent cells which were dispensed in 200 µl aliquots into prechilled polypropylene tubes and immediately frozen at -70°C.

2.2.9 Transformation of competent bacterial cells

A 200 µl aliquot of competent bacterial cells was thawed on ice, mixed with 5-10 ng of plasmid DNA or 1/5 of the ligation reaction and the tube placed on ice for 30 min. The tube was then placed into a water bath at 42°C for 1 min and rapidly replaced on ice for at least 2 min to heat shock the cells. After addition of 800 µl of SOC medium the cells were incubated for 1 hr at 37°C while shaking at 250 rpm. Aliquots of the transformation were plated on LB agar plates containing the appropriate antibiotic for selection. The dried plates were incubated for 16 hr at 37°C.

2.2.10 Small-scale preparation of plasmid DNA

For isolation of small amounts of plasmid DNA a procedure based on the alkaline lysis method was used.

A single bacterial colony was used to inoculate 5 ml LB with the appropriate antibiotic (generally 50 µg/ml Ampicillin) and grown *overnight* at 37°C while shaking at 250 rpm. 1,5 ml of the saturated culture was transferred to a microcentrifuge tube and spun for 30 sec to pellet the cells. The supernatant was removed and the bacterial pellet resuspended by vortexing with 100 µl of Solution 1 (resuspension buffer: 50 mM Tris/HCl, 10 mM EDTA, pH 8). After addition of 200 µl of Solution 2 (lysis buffer: 0,5 M NaOH, 10% SDS), the tube was gently inverted 5-6 times and placed on ice for 5 min. 150 µl of cold Solution 3 (neutralisation buffer: 7,5 M NH_4Ac) were then added, mixed well and the tube placed on ice for another 5 min. After centrifugation for 5 min at 4°C and 13,000 x g, the supernatant was transferred to a fresh tube and mixed with 1 ml of 100% Ethanol. After 5 min of centrifugation at *room temperature* and 13,000 x g to pellet plasmid DNA, the supernatant was removed and the pellet washed with 70% ethanol, air dried and resuspended with 20 µl of bidistilled H_2O or TE buffer.
2 µl of the resuspended DNA were used for restriction enzyme digestion.

2.2.11 Large-scale preparation of plasmid DNA

For large-scale preparation of plasmid DNA, a single colony was picked from a selective plate (or directly from glycerol stock) and grown in 50-100 ml LB with the appropriate antibiotic *overnight* at 37°C while shaking at 250 rpm. Isolation of plasmid DNA was performed using the QIAGEN plasmid purification kit. The procedure is based on the alkaline lysis method followed by loading the cleared lysate onto the QIAGEN-tip

resin columns, isopropanol precipitation of the eluted DNA and washing the pellet with 70% ethanol. After briefly air drying, the purified plasmid DNA was resuspended in TE or bidistilled H_20 and the concentration determined with a spectrophotometer by measuring the OD_{260} (50 µg of double strand DNA correspond to 1 OD_{260}).

2.2.12 Radioactive labelling of a DNA probe by random priming

^{32}P radioactive labelling of DNA was performed by using the Random Primed DNA Labeling Kit from Boehringer Mannheim (Roche Molecular Biochemicals).

The DNA to be labelled (50-100 ng) was denatured for 5 min at 90°C and incubated with the hexanucleotide mix containing random oligonucleotides which anneal and function as primers for the Klenow-fragment.

Reaction mixture:
- 50-100 ng template DNA
- 1 X reaction buffer (Boehringer-Roche)
- 1 X hexanucleotide mix (Boehringer-Roche)
- 50 mM dATP, dGTP, dTTP
- 50 µCi [α-^{32}P]dCTP, 3000 Ci/mmol (Amersham-Pharmacia)
- 5 U Klenow-Polymerase (Boehringer-Roche)
- in 20 µl total volume

The reaction was incubated for 30 min to 1 hr at 37°C and stopped by addition of 2 µl 0.5 M EDTA. The radiolabelled probe was then separated from unincorporated dNTPs through Sephadex G-50 column centrifugation.

2.2.13 Radioactive 5'-end labelling of oligonucleotide DNA probes by kinases

10 pmol of the oligonucleotide primer were incubated in a volume of 10 µl with 1 µl of the T4 Polynucleotide Kinase (New England BioLabs) and 5 µl [γ-^{32}P]ATP (specific activity > 6,000 Ci/mmol, Amersham) for 1 hr at 37°C.
Unincorporated nucleotides were removed using the QIAquick Nucleotide Removal Kit from QIAGEN.

2.2.14 Separation of a radioactively labelled DNA probe from unincorporated dNTPs

Column chromatography with Sephadex G-50 was used to purify labeled DNA probes from unincorporated dNTPs. The bottom of a 1-ml disposable syringe was plugged with clean cotton-wool.The syringe was filled with the resin suspension, placed in a 15 ml polypropylene tube and centrifuged for exactly 4 min at 1,400 x g to pack the column. The radioactive sample was diluted with TE to a volume of 100 µl and loaded onto the center of the column. The syringe was placed into a fresh polypropylene tube and centrifuged again for 4 min, exactly, at 1,400 x g. The liquid at the bottom of the tube, containing the purified probe, was saved and the syringe containing the unincorporated dNTPs, was discarded in radioactive waste.

Alternatively, the QIAquick Nucleotide Removal kit from QIAGEN was used to remove the unincorporated nucleotides from the radioactively labelled probe.

2.2.15 Screening of a cDNA library

2.2.15.1 Titration of the library

Tenfold serial dilutions of the recombinant bacteriophage stock were prepared in λ-diluent solution. 200 µl of a fresh culture of the λ-sensitive C600 bacteria, grown overnight in NZC broth supplemented with 0.4% maltose and 10 mM $MgCl_2$, were distributed into sterile 15 ml tubes (one for each dilution), mixed with 1 µl of the bacteriophage dilution, and incubated 20 min at room temperature. 4 ml of melted NZC top agarose, cooled to 50°C, were then added to each tube and mixed by vortexing. Bacteria and top agarose were spread over the entire surface of an NZC agar plate of 82 mm diameter, prewarmed at 37°C. The plates were inverted and incubated overnight at 37°C. The next morning, plaques were counted in non-confluently-grown plates to determine the library titer.

The 10^{-4} dilution of the FOIE 3A cDNA library produced 103 plaques, indicating a titer of about 1 x 10^{7} pfu / µl.

2.2.15.2 Plating of the library and transfer to filter membranes

20 µl (200,000 pfu) of the 10^{-4} dilution of the FOIE A3 cDNA library were incubated for 20 min at room temperature in a sterile 50 ml tube with 2.5 ml of a fresh culture of C600 bacteria, grown overnight in NZC broth supplemented with 0.4% maltose and 10

mM $MgCl_2$. 40 ml of NZC top agarose, melted and cooled to 50°C, were added and dispersed over a 245 mm x 245 mm NZC agar plate. 5 such plates were prepared to allow the screening of 1,000,000 different recombinant bacteriophages. The plates were incubated overnight at 37°C.

The next morning, before applying the filters, the plates were chilled for 1 hr at 4°C. The nitrocellulose filters were applied to cold plates and left for 5 min. To facilitate orientation of the filter on the plate, a needle was stabbed through the filter into the agar at several asymmetric points of the plate. The filter was then removed with forceps, placed face up on a Whatman paper, labelled according to the corresponding plate, and dried for at least 10 min.

The filters were placed face up successively for 1 min onto 10 ml of denaturation solution, for 1 min onto 10 ml of neutralisation solution, and for 1 min onto 2X SSC. The filters were then dried and baked for 2 hr at 80°C in a vacuum oven.

2.2.15.3 Hybridisation of the cDNA library with a radioactive probe

To detect the human TIF1 cDNA, the FOIE A3 cDNA library was hybridised with the mouse T18 cDNA (Miki *et al.*, 1988) under conditions of low stringency.
The filters were prehybridised in prehybridisation solution for 3 hr at 52°C. Prehybridisation solution was then replaced by hybridisation solution containing the radioactive T18 probe at a concentration of 2,000,000 cpm / ml. The hybridisation was performed overnight at 52°C.

The filters were washed to remove nonhybridised probe first at room temperature with washing solution 1 (2 X SSC, 0,2% SDS), then 3 times at 52°C with washing solution 2 (0,2 X SSC, 0,2% SDS). They were then wrapped with plastic wrap and exposed overnight at -80°C with Kodak X-OMAT AR films.

2.2.15.4 Identification and purification of positive bacteriophage clones

The positive bacteriophage clones were identified by placing the plates on the autoradiographic film, oriented according to the needle marks. To pick the positive clones, a circular part of the agar, corresponding to the hybridisation spot, was cut out with a 1 ml pipet tip, placed into 1 ml of Lambda diluent containing a drop of chloroform, and left for 2 hr at 4°C to allow phage to elute. A serial dilution of each of these impure bacteriophage solutions and a secondary screening by transferring to filter membranes and hybridisation

were repeated until isolation of pure positive plaques was achieved. A Lambda diluent stock of these clones was stored at 4°C.

2.2.16 DNA phage growth and isolation

1 ml of a fresh saturated culture of C600 bacteria was used to inoculate 500 ml of prewarmed NZC broth supplemented with 0.4 % maltose and 10 mM $MgCl_2$, and incubated for 30 min at 37°C while shaking. A single plaque was picked and added to the bacterial culture and the incubation at 37°C was continued under vigorous shaking until lysis occurred (usually after 6-8 hr). 2 ml of chloroform were then added to the culture to lyse any remaining bacterial cells. After 15 min of centrifugation at 10.000 rpm and 4°C to pellet the cell debris, the supernatant was recovered and incubated for 30 min at 37°C with 10µg /ml final concentration of RNase and DNase. To precipitate phage, 20 ml of 30% PEG / 3M NaCl solution was added per 100 ml of supernatant and left for 1-2 hr on ice. A centrifugation was then performed for 20 min at 10,000 rpm. The phage pellet was resuspended with 450 µl of Lambda diluent and further centrifuged for 10 min at 14,000 rpm to eliminate gross debris. The supernatant was transferred to a fresh tube and incubated at least for 1 hr at 56°C with 50 µg / ml Proteinase K, 0.5% SDS, and 20 mM EDTA. The phage DNA was then extracted with phenol/chloroform and precipitated with ethanol. The pellet was resuspended in H_2O or TE and stored at -20°C. The phage DNA concentration was established by agarose gel electrophoresis and by measurement of the A_{260}.

2.2.17 Restriction mapping of the positive phage clones by Southern blotting and hybridisation

The positive phages were digested with Eco RI, Hind III, Bam HI, and Bgl II. The digestion reactions and DANN molecular weight markers were loaded into 1% agarose gel and subjected to electrophoresis together overnight at 25 V. The next morning, the gel was photographed with a centimeter and plunged with gentle shaking twice for 15 min into denaturation solution, twice for 15 min into neutralisation solution, and for 20 min into 10X SSC for equilibration. The DNA was transferred to nitrocellulose filter through downward capillary transfer: a stack of absorbent paper about 5 cm high was mounted on a glass plate, 2 pieces of 3 MM Whatman filter paper and a nitrocellulose membrane, slightly larger than the gel, were plunged in 10 X SSC and placed on the top. The gel equilibrated in 10 X SSC was disposed on the membrane and bubbles were removed by

rolling a pipet over the gel surface. 3 pieces of 3 MM Whatman filter paper, cut to the same size as the gel and wet with 10 X SSC, and successively a sponge also wet in 10 X SSC were placed on top of the gel. To reduce evaporation all was covered with plastic wrap and left for about 6 hr. After transfer, the membrane was baked as described for the library filters, prehybridised for 3 hr and hybridised with the ^{32}P radiolabelled cDNA excised from the positive phage clone, at a concentration of 2,000,000 CPM/ml, under conditions of high stringency (overnight at 65°C, first washing at room temperature with 2 X SSC and 0.2 % SDS, successive washings with decreasing salt concentrations and increasing temperature, final washing 30 min at 65°C with 0.2 X SSC and 0.2% SDS). The filter was exposed to X-ray film (Kodak X-OMAT AR) overnight at -80°C. The restriction map of the cDNA was established by comparing the fragments produced by the different restriction enzymes.

2.2.18 Shotgun of DNA phage and cloning into M13mp18

After isolation from positive clones, phage DNA resuspended in TE solution was maintained on ice and sonicated with short burst (10 sec) at 6/10 power, using a Sonicator (Branson) with a microtip probe. After each burst, an aliquot of the suspension was removed and analysed by agarose gel electrophoresis. The sonication was continued until the optimal size of the DNA fragments was attained. The ends were first repaired by Klenow enzyme:

DNA	175 µl
Tris/$MgCl_2$ 0,1 M	20 µl
Klenow (2 U/µl)	2,5 µl
dNTP mix 10 mM	2,5 µl

The reaction was incubated overnight at room temperature.

Klenow enzyme was inactivated for 10 min at 75°C and a second repair reaction was performed with T4 polymerase:

Klenow reaction	200 µl
T4 Polymerase 5X buffer	50 µl
T4 Polymerase (Boehringer Mannheim)	5 units

The incubation was performed for 2 hrs at 16°C followed by 20 min at 37°C.

DNA fragments ranging between 200 and 400 bp were electroeluted from a 1,5% agarose gel, purified with Elutip-d columns and precipitated. They were then cloned into the Sma I restriction site of the M13mp18 vector (Boehringer Mannheim).

2.2.19 Plating bacteriophage M13 and identification of positive clones

DH5αF' competent bacteria were transformed with the M13-shotgun ligation, mixed with 200 µl of a DH5αF' confluent culture and 5 ml of NZC Top agarose and plated on NZC agar plates. After transfer onto nitrocellulose filters as described above, hybridisation was performed with the radiolabelled cDNA insert excised from the positive λ-phage with the same hybridisation conditions as for restriction cartography.

2.2.20 Preparation of single-stranded phage DNA from M13mp18 vectors

200 µl of a freshly saturated culture of DH5αF' bacteria were diluted with 2 ml of NZC broth, infected with a sterile toothpick inserted into a positive M13 plaque, and shaken for 6 hrs at 37°C. 1,5 ml of the culture was transferred to a microcentrifuge tube and centrifuged for 5 min at 15,000 x g. 1 ml of supernatant was transfered to a fresh tube and centrifuged 5 min at 15,000 x g. 0,8 ml of the supernatant was mixed in a fresh tube with a PEG/NaCl solution (3% PEG / 0,3 M NaCl final concentration). The tube was placed on ice and, after 20 min, was centrifuged for 10 min at 15,000 x g. The DNA pellet was resuspended with 200 µl TE, extracted with phenol/chloroform and precipitated with 2 volumes of absolut ethanol and one tenth volums of 3 M NaAcetate. The pellet was washed with 70% ethanol, air dried, dissolved in 25 µl TE and 1-2 µl were analysed by agarose gel electrophoresis. The remainder was stored at -20°C and used for sequencing.

2.2.21 Sequencing of double- and single-stranded DNA templates

Sequencing of DNA was performed with the chain-termination DNA sequencing method from Sanger (Sanger et al., 1977) by using the Sequenase Version 2.0 DNA Sequencing Kit from USB (Amersham/Pharmacia Life Science).

Denaturation of double-stranded template:

3-5 µg of double-stranded DNA diluted in 20 µl water was denatured by adding 2µl of 2N NaOH and incubated at 65°C for 5 min. The solution was then cooled on ice and mixed with 3 µl 3M sodium acetate pH 5.6, 7 µl H_2O, and 75 µl 100% ethanol. After 15 min at -80°C, the denatured DNA was centrifuged for 20 min at 13,000 x g at 4°C, the pellet washed with 75% ethanol, resuspended in 7 µl H_2O, and used for the annealing reaction.

Annealing reaction:

DNA	7 µl (ca.1 µg for M13, 3-5 µg for plasmid)
Reaction Buffer	2 µl
Primer	1 µl (1 pmol)
Total	10 µl

Annealing was performed by heating to 90°C, then allowing to cool down slowly to 30°C.

Labelling Reaction:

Ice-cold annealed DNA mixture	10 µl
0.1 M DTT	1 µl
Labelling Mix (5-fold diluted with H_2O)	2 µl
[^{35}S]dATP	0.5 µl
Sequenase Polymerase (7-fold diluted with Enzyme Dilution Buffer)	2 µl
Total	15.5 µl

The labelling reaction was incubated for 5 min at room temperature.

Termination reaction:

3.5 µl of the labelling reaction were transferred to each termination mix (2.5 µl of ddATP, ddCTP, ddGTP, and ddTTP) prewarmed at 37°C, and the incubation was continued for 5 min at 37°C.

The reaction was stopped by addition of 4 µl of stop solution to the reaction

The samples (2-3 µl in each lane) were denatured by heating to 75°C for 2 min immediately before loading onto a sequencing gel.

2.2.22 Denaturing gel electrophoresis of sequencing reactions

Denaturing gel (for 150 ml of 6% acrylamide / 7 M urea gel solution)

40% Acrylamide solution (Bio-Rad)	21.4 ml
2% Bisacrylamide solution (Bio-Rad)	22.5 ml
10X TBE	15 ml
Urea	63 g
TEMED	30 µl
25% APS	300 µl

The gel was prepared 2-20 hr prior to use, pre-run (50-60 W) for 15-30 min and run for 3-6 hr with 1X TBE as running buffer. After running, the gel was fixed in 5% acetic acid / 5% ethanol, transferred to Whatman paper and vacuum dried at 80°C for 1 hr. Autoradiography was performed overnight at room temperature with Kodak X-OMAT-S films.

2.2.23 Eukaryotic cell culture

CHO, HeLa, and COS-6 adherent cells were grown in DMEM medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. They were maintained at 37°C in an humidified atmosphere with 5% CO_2.

2.2.24 Preparation of RNA from eukaryotic cells

Total RNA was extracted from eukaryotic cells by using the Trizol reagent (GibcoBRL) which consists of a mono-phasic solution of phenol and guanidine-isothiocyanate. Cell pellets were lysed with 1 ml of Trizol reagent per 5-10 x 10^6 cells by repetitive pipetting. The homogenised samples were incubated for 5 min at room temperature and successively 200 µl of chloroform were added to each tube. The tubes were vigourously shaken by hand and centrifuged at 12,000 x g for 15 min at 4°C. The

upper aqueous phase containing RNA was transferred to a fresh tube and mixed with 500 µl of isopropyl alcohol. After 10 min of incubation at room temperature and 10 min of centrifugation at 12,000 x g at 4°C, the supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol, briefly air-dried, and dissolved in RNase-free water by incubating the solution for 10 min at 55°C-60°C. The RNA concentration was determined by measuring the A_{260} with a spectrophotometer.

2.2.25 Northern blot hybridisation

The hybridisation-ready Northern blots of poly A^+ mRNA from human tissues (Clontech Laboratories, Inc.) were prehybridised with 5 ml of ExpressHyb solution (Clontech Labortories, Inc.) at 68°C for 30 min. The prehybridisation solution was then removed and replaced by 5 ml of fresh ExpressHyb solution containing the radioactive probe previously denatured at 95°C-100°C for 5 min. The hybridisation at 68°C was continued for 1 hr. The hybridisation solution was discarded and the blot washed several times at room temperature with wash solution 1 (2 X SSC, 0.05% SDS). The blot was then washed 4 times (30 min, 62°C) with wash solution 2 (0.1 X SSC, 0.1% SDS), while shaking continuously and with fresh solution each wash. The blot was subsequently covered with plastic wrap and exposed to x-ray film (X-OMAT AR, Kodak) at -70°C for 2-12 hr.

The probe was removed by incubating the blot for 10 min while shaking in a sterile 0.5% SDS solution heated to 90°C-100°C ("de-hybridisation"). The blot was hybridised successively with hTIF1α cDNA, hTIF1γ cDNA, and β-actin cDNA as control.

2.2.26 Reverse Transcription-Polymerase Chain Reaction

2.2.26.1 DNase I RNase-free digestion and First Strand cDNA Synthesis

RNA was first digested with DNase I to eliminate genomic DNA contamination: 3µl of total RNA was mixed with 1µl of 10 X DNase buffer (200 mM Tris pH 8.3, 500 mM KCl, 20 mM $MgCl_2$), 1 U of amplification grade DNase I (GIBCO) in a volume of 10 µl and incubated for 15 min at room temperature. The reaction was terminated by adding 1µl of 25 mM EDTA and heated to 65°C for 10 min. This reaction was used directly for reverse transcription.

1 µl of 100 µM R9 primer, specific for hTIF1γ, in sterile H_2O was added to the mixture which was then heated to 70°C for 10 min and quickly chilled on ice. 2 µl of 5X

first strand buffer (250 mM Tris-HCl pH8,3 at room temperature, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT, 1 µl of mixed dNTP stock (10 mM each dATP, dCTP, dGTP, dTTP at neutral pH, Roche Molecular Biochemicals) were then added and the tube was placed at 37°C for 2 min to equilibrate the temperature. After addition of 1 µl (200 units) Reverse Transcriptase (SUPERSCRIPT™ RNase H⁻ Reverse Transcriptase, GIBCOBRL), the incubation at 37°C was continued for 1 hr. The reaction was terminated by adding 4 µl of 0.5 M EDTA.

2.2.26.2 Polymerase Chain Reaction

2 µl of the Reverse Transcriptase reaction were used for PCR and mixed with 5 µl 10X REDTaq PCR Buffer (SIGMA), 1 µl Deoxynucleotide Mix (10µM each dATP, dCTP, dGTP, dTTP, ROCHE Molecular Biochemicals), 1 µl 100 µM G7F forward primer, 1 µl 100 µM G7R reverse primer, 2,5 µl REDTaq DNA polymerase (Sigma), and bidistilled water to a volume of 50 µl. A master mix of all reagents except template and Taq Polymerase was prepared and then dispensed to each tube. Sterile mineral oil (Sigma) was added to prevent evaporation. The samples were denatured at 90°C before Taq Polymerase was added. The amplification (30 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 2 min, and elongation at 72°C for 2 min) was performed in a Perkin-Elmer 480 Thermocycler. The amplified DNA was evaluated by loading 5-10 µl of the PCR reaction on a 2% agarose gel, without loading buffer (Sigma's Taq DNA polymerase contains an inert red tracer which comigrates as a 125 bp fragment in a 1% agarose gel).

The RT-PCR products were purified by the PCR Purification Kit from QIAGEN.

2.2.27 Poisonned cycled primer extension

Purified RT-PCR products were mixed with 1 µl of 32-P end-labelled PEXT.F primer (ca 0,5 pmol), 100 µM (final concentration each) of dATP, ddCTP, dGTP, and dTTP (Amersham Pharmacia Biotech), 1,55 µl Thermo-sequenase reaction buffer (Amersham), 2 units of 3'-5' thermostable DNA polymerase (Thermo-Sequenase, Amersham), and bidistilled water to a final volume of 20 µl. The cycled primer extension reaction was performed in a Perkin-Elmer 480 Thermocycler. The amplification protocol consisted of 10 cycles with 10 sec of denaturation at 90°C, 30 sec annealing at 56°C, and 20 sec elongation at 72°C. After completing the amplification reaction, 20 µl of 2X gel loading buffer (90% formamide, 0.1% bromophenol blue, 178 mM Tris-borate, 5 mM EDTA) were added to each sample.

The extended primers were separated by electrophoresis in 12% denaturing polyacrylamide-8 M urea gels (30 cm long) using 1X TBE as running buffer. All samples were heated to 70°C for 5 min and cooled on ice prior to loading. Following electrophoresis, gels were fixed with 7% acetic acid / 7% ethanol, transferred to a Whatman paper and dried on a heated (80°C) vacuum dryer for 2 hrs. Gels were autoradiographed with X-ray films (X-OMAT AR, Kodak) for 30 min to 3 hrs.

2.2.28 Transient cotransfections of plasmid DNA into eukaryotic cells by the calcium phosphate precipitation method

The day before transfection, cells were plated into 6 well culture plates at a density which allowed them to grow to 40-60% confluency by the time of transfection. They were thoroughly mixed and equally separated on the well. 3 to 6 hr before transfection, the medium was replaced with 3 ml of fresh complete medium. To prepare DNA-calcium precipitates, DNA in 109.5 µl of sterile water was mixed with 15.5 µl of 2.5 M $CaCl_2$. 125 µl of 2X HBS solution were placed in sterile 5 ml tubes and a 1-ml pipet attached to a mechanical pipettor was used to bubble the HBS, while the DNA / $CaCl_2$ solution was added dropwise. The precipitate solution was immediately mixed by vortex to prevent formation of too large complexes and, after 20 min at room temperature, was distributed over the cells which were then incubated overnight under standard growth conditions (37°C, 5% CO_2). The next day, the medium containing the DNA precipitates was removed and replaced with 2 ml of fresh complete medium. The incubation was continued under identical conditions for 48 hr and then the cells were harvested for measurement of relative luciferase and β-galactosidase activities. Cells for which treatment with ATRA at 1µM or TSA at 100 nM was planned, were trypsined on the second day post transfection and split into equal aliquots (to be treated or to be left untreated), and harvested after incubation 24 hr.

2.2.29 Harvesting of transfected cells

Lysis Buffer

25 mM Na_2HPO_4 / NaH_2PO_4 pH 7.5
8 mM $MgCl_2$
1 mM DTT
1 mM EDTA
0.1% TritonX 100
15% Glycerol

The 6-well culture plates were placed on ice, the culture medium was removed and the cells were washed twice with 3 ml of cold 1X PBS. 200 µl of lysis buffer were added to each well and, after 10 min on ice, the cell lysates were transferred to 1.5 µl tubes and centrifuged for 10 min at 13.000 x g and 4°C to pellet cell debris. The supernatant was tranferred to a fresh tube and stored at -20°C.

2.2.30 Luciferase activity assay

Reaction Buffer	25 mM Na_2HPO_4 / NaH_2PO_4 pH 7.8
	8 mM $MgCl_2$
	15 mM $MgSO_4$
	0.5 mM Luciferine
	2.5 mM ATP

40 µl of the cell lysate were used to measure relative luciferase units in a luminometer (de Wet *et al.*, 1987). Results were controlled for β-galactosidase activity.

2.2.31 β-galactosidase activity measurement

Reaction Mix	66 ml 1X ONPG
	3 µl 100X Mg-Solution
	201 µl Na_2HPO_4 / NaH_2PO_4 1 M, pH 7.5
	30 µl Cell extract

After incubation at 37°C in the dark for 30 min, the reaction was stopped by addition of 500 µl of 1M Na_2CO_3, and the relative galactosidase activity was measured with a spectrophotometer at 420 nm.

3. RESULTS

3.1 Cloning and sequence analysis of human TIF1α and TIF1γ

A cDNA library from HepG2 cells (human liver) was screened using the TIF1α portion of T18, the murine TIF1α/B-*raf* fusion cDNA (Miki *et al.*, 1988) as a hybridisation probe under low stringency conditions. Restriction mapping of positive clones yielded two different restriction patterns. The longest clones of both groups were sequenced and found to contain two full-length cDNAs which were named hTIF1α and hTIF1γ. While hTIF1α was the human homologue of mouse TIF1α (Le Douarin *et al.*, 1995; Thénot *et al.*, 1997), hTIF1γ represented a novel member of the TIF1 family. The nucleotide and derived amino acid sequences of both human TIF1α and TIF1γ are shown in Fig 6 and 7, respectively.

Fig. 6. Amino acid sequences of hTIF1α

Fig. 7. Amino acid sequences of hTIF1γ

Sequence positions are indicated on the left; characteristic domains and motifs are outlined and named on the right. The putative nuclear localization signals (KRK/KKK or KK/KKK, respectively) and an LxxLL motif in hTIF1γ are underlined.

Fig. 6 **hTIF1α**

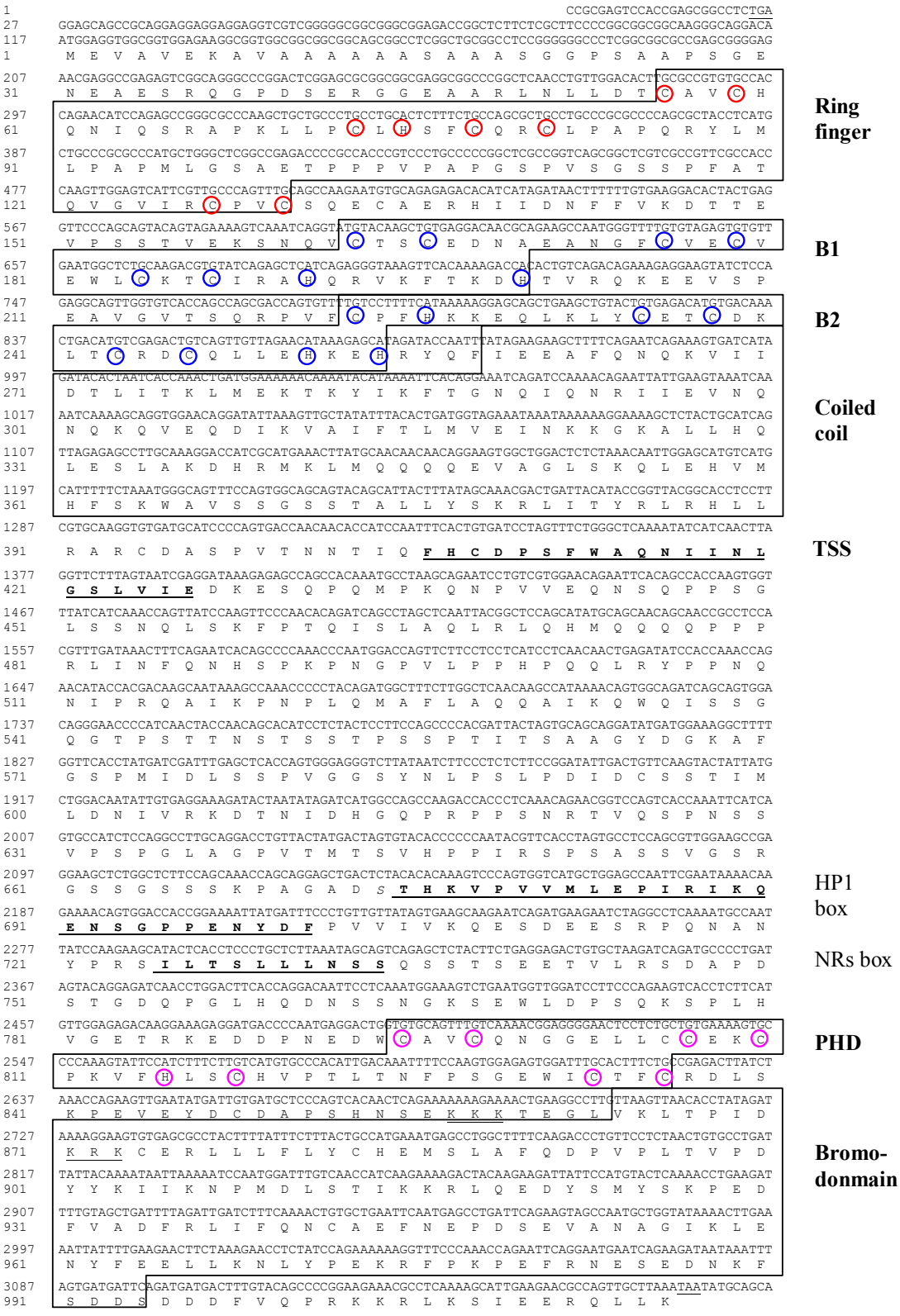
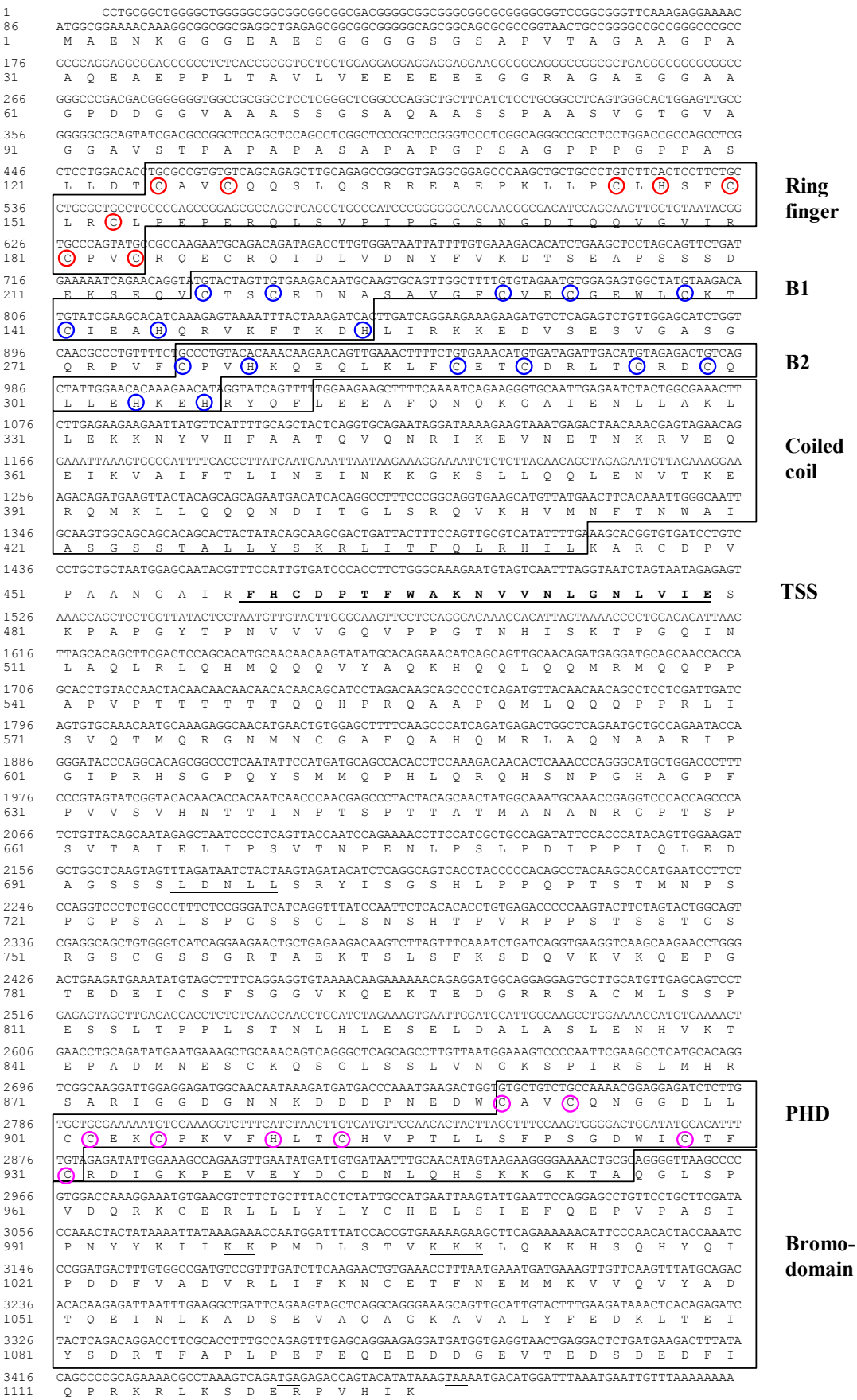

Ring finger
B1
B2
Coiled coil
TSS
HP1 box
NRs box
PHD
Bromo-donmain

Fig. 7 **hTIF1γ**



The predicted mouse and human TIF1α protein sequences are identical at more than 92% of amino acid residues. Amino acid comparison of the human TIF1γ sequence to the sequences of human TIF1α and TIF1β revealed 48% overall identity with respect to TIF1α and 32% with respect to TIF1β (Fig. 8). Among the three members of the family, TIF1α is closer to TIF1γ than to TIF1β (28% overall identity between human TIF1α and human TIF1β).

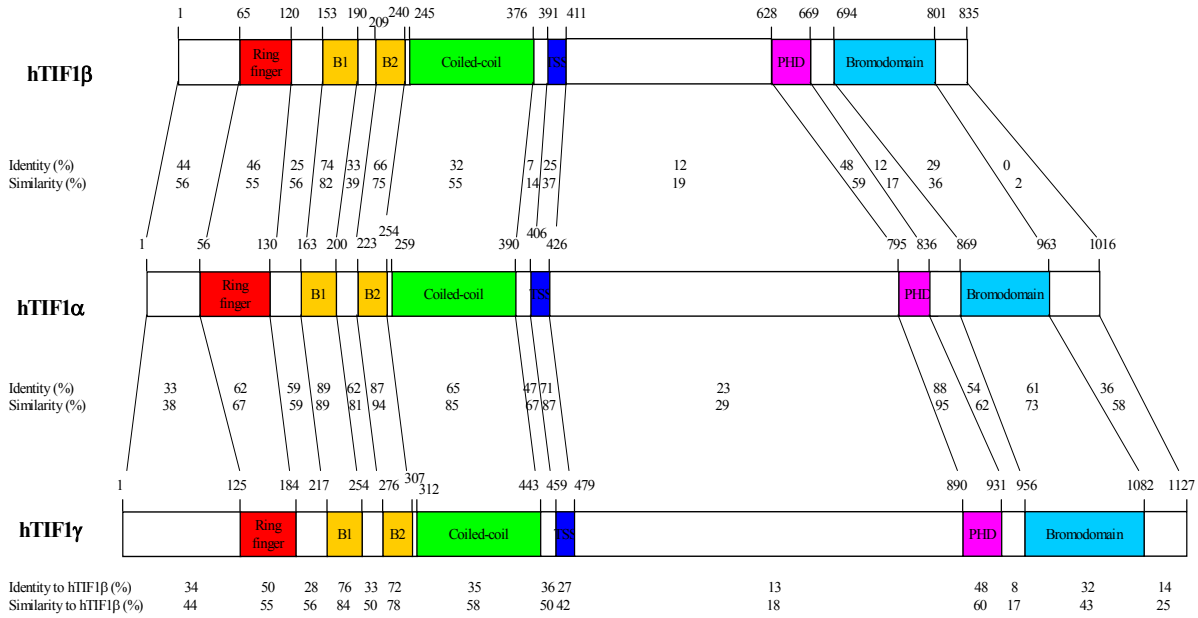


Fig. 8. TIF1γ is structurally related to TIF1α and TIF1β. Schematic representation of hTIF1α, hTIF1β, and hTIF1γ. Degrees of conservation (% identity or similarity) are indicated below protein representations, amino acid positions are shown above.

Like TIF1α and TIF1β, TIF1γ contains several evolutionarily conserved domains (Fig. 8, see Introduction). Close to the N-terminus a C_3HC_4 zinc finger motif or RING finger (Freemont *et al.*, 1993; Saurin *et al.* 1996) is followed in sequence by two B box-type fingers and a predicted α-helical coiled coil domain forming a tripartite motif, designated RBCC (Saurin et al., 1996, see Introduction).

Towards the C-terminus a bromodomain (Haynes et al., 1992; Jeanmougin et al., 1997) is preceded by a C_4HC_3 zinc finger motif or PHD finger (Aasland *et al.*, 1995; Koken *et al.*, 1995; see Introduction). The BC loop of the hTIF1γ bromodomain is longer as compared to the other bromodomains described so far (Fig. 9). An alternative splicing was identified which, through a deletion of exon 19 (see 3.3), produces an hTIF1γ isoform with a bromodomain BC loop similar to that of the other known bromodomains (Fig. 9).

Fig. 9. Sequence alignment of the bromodomain of several proteins (hGCN5, hP/CAF, hp300, hTIF1α, β, and γ). Amino acids positions are shown on the right. The BC loop of TIF1γ bromodomain which is alternatively spliced is underlined.

In addition to these conserved domains found in a number of transcriptional regulatory proteins, a 25 amino acid-long, tryptophan- and phenylalanine-rich sequence was identified downstream of the coiled coil motif (underlined in Fig. 6 and 7) which is highly conserved between the three TIF1 proteins and virtually identical between TIF1α and TIF1γ (Fig. 10). As protein sequence searches using this region of homology did not reveal any significant similarity to known proteins, this domain was designated TSS for TIF1 signature sequence.

Fig. 10. Sequence alignment of the TSS motif among members of the TIF1 family.

The central region between the TSS motif and the PHD finger, extending over ~ 370, 220, and 410 residues in TIF1α, TIF1β, and TIF1γ, respectively, is the least conserved among TIF1 proteins (Fig. 8). Interestingly, two adjacent subfragments of the TIF1α central region have been shown to mediate protein interactions (Le Douarin *et al.*, 1996): the HP1 box to which HP1α, HP1β and HP1γ bind (amino acids 675-701; underlined in Fig. 6) and the NR box in which integrity of the conserved motif LxxLL is essential for nuclear receptor interactions (Torchia *et al.*, 1997; Heery *et al.*, 1997) (amino acids 725-734; underlined in Fig. 6 and 7). While there is no motif clearly resembling the HP1 box of TIF1α in the TIF1γ sequence, an LxxLL motif has been identified in the central region of TIF1γ (amino acids 696-700; underlined in Fig. 7) which did not, however, display any direct interaction with NRs (see 3.6).

3.2 Chromosomal localisation of human TIF1α and TIF1γ

Since TIF1α and two other RBCC proteins, RFP and PML, have been implicated in oncogenic translocations (see Freemont *et al.*, 1993; Saurin *et al.*, 1996 for review, and Discussion), it was of interest to determine the chromosomal localisation of the TIF1α and TIF1γ genes. In collaboration with Marie G. Mattei (INSERM U406, Marseille, France), *in situ* hybridisations were performed with ^{3}H-labelled hTIF1α and hTIF1γ cDNA probes on chromosome preparations from PHA-stimulated human lymphocytes. As shown in Fig. 11, non-random distributions of silver grains were obtained, which allowed mapping of

hTIF1α to the q32-q34 region of the long arm of chromosome 7, and hTIF1γ to the p13 band of the short arm of chromosome 1. For the TIF1γ probe, a small secondary peak was detectable on the 1p36.1-1p34.3 region of the short arm of chromosome 1, which may indicate the existence of another, yet unknown member of the TIF1 family. Since this probe failed to label TIF1α and TIF1β (which is positionned to the 19q13.4 region, Moosmann *et al.*, 1996), this hypothetical fourth member is likely closer to TIF1γ. The locus 1p13, which contains the TIF1γ gene, is strongly associated with the non-random t(1;22)(p13;q13) chromosomal translocation of acute megakaryocytic leukemias. In contrast, the involvement of 7q32-q34 in deletions and translocations of acute lymphoblastic leukemias, and that of 1p34-p36 in acute leukemias and myelodysplastic syndromes appears weaker (Mitelman, 1993).

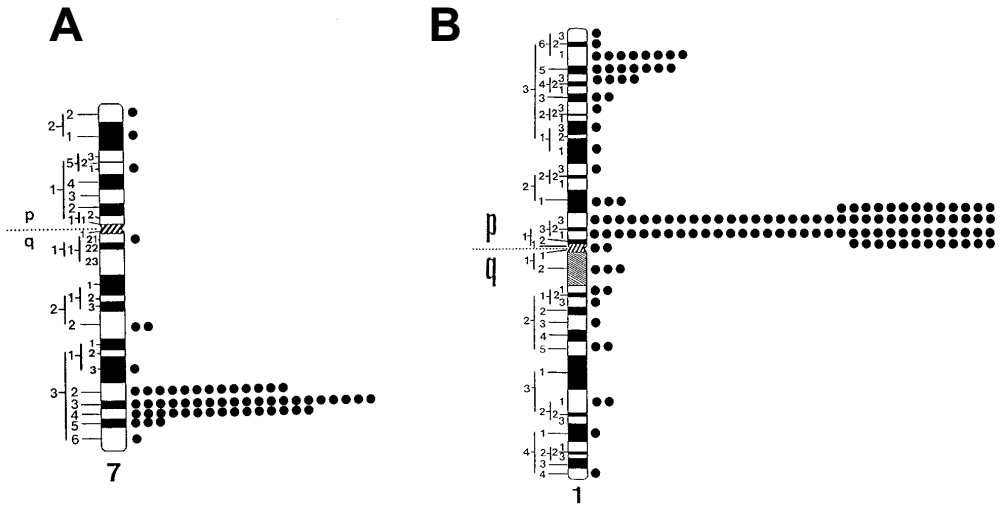
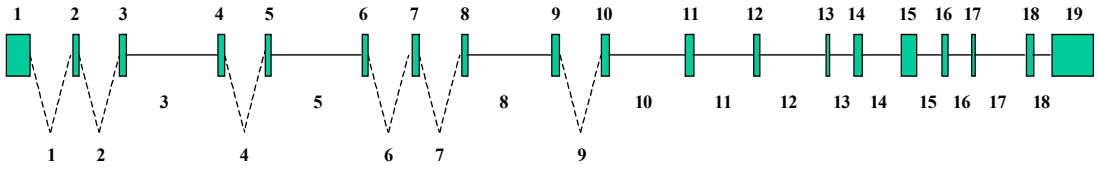


Fig. 11. Chromosomal localization of the hTIF1α and hTIF1γ genes. (**A**) Idiogram of the human G-banded chromosome 7, illustrating the distribution of labeled sites with the hTIF1α probe. (**B**) Idiogram of the human G-banded chromosome 1, illustrating the distribution of labeled sites with the hTIF1γ probe.

3.3 Genomic structure of human TIF1α and human TIF1γ

Genomic sequences of TIF1α and TIF1γ were identified and extracted from the Human Genome Project databases through comparison with the TIF1α and TIF1γ cDNAs.

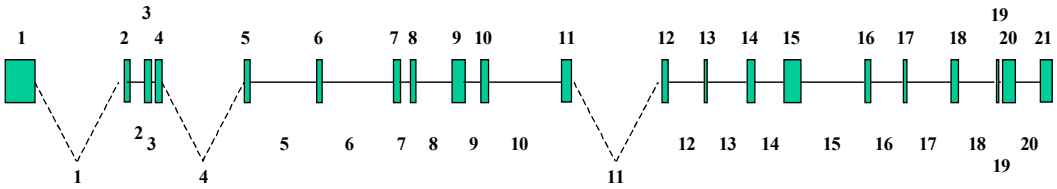


EXONS			INTRONS	
1:	480 bp	(1-480)	1:	43,379 bp
2:	119 bp	(481-599)	2:	10,804 bp
3:	48 bp	(600-747)	3:	3,831 bp
4:	133 bp	(748-880)	4:	5,921 bp
5:	117 bp	(881-997)	5:	3,760 bp
6:	115 bp	(998-1112)	6:	9,428 bp
7:	147 bp	(1113-1259)	7:	12,261 bp
8:	118 bp	(1260-1377)	8:	3,519 bp
9:	167 bp	(1378-1544)	9:	12,617 bp
10:	174 bp	(1545-1718)	10:	3,177 bp
11:	174 bp	(1719-1892)	11:	2,505 bp
12:	136 bp	(1893-2028)	12:	2,732 bp
13:	73 bp	(2029-2101)	13:	976 bp
14:	169 bp	(2102-2270)	14:	1,618 bp
15:	329 bp	(2271-2599)	15:	1,031 bp
16:	133 bp	(2600-2732)	16:	1,004 bp
17:	75 bp	(2733-2807)	17:	2,080 bp
18:	150 bp	(2808-2957)	18:	744 bp
19:	850 bp	(2958-3807)		

Fig. 12. Genomic structure of human TIF1α

The human TIF1α gene is represented in Fig. 12 and comprises 19 exons located within more than 120 kb of genomic DNA. Exons range from 73 bp (exon 13) to 850 bp (exon 19) in size. The first two introns, as well as introns 6, 7, and 9, are very large (intron 1: 43,379 bp, intron 2: 10,804 bp, intron 6: 9,428 bp, intron 7: 12,261 bp, and intron 9: 12,617 bp). The other introns range from 744 bp (intron 18) to 5,921 bp (intron 4) in size. All introns have the conserved GT and AG dinucleotides present at the donor and acceptor sites, respectively. The functional domains of the hTIF1α protein are encoded by distinct exons. The RING finger is encoded by the first two exons, interrupted by the very large

intron 1; B boxes 1 and 2 lie within individual exons 3 and 4, respectively; the coiled coil is encoded by four exons (5 to 8). The TSS motif is split into two exons, the first of which in common with the carboxy terminal extremity of the coiled coil. The PHD finger, like the RING finger, is encoded by two exons (15 and 16), the latter of which also encodes the amino terminal region of the bromodomain which is divided over four exons (16 to 19).



EXONS			INTRONS	
1:	609 bp	(1-610)	1:	46,163 bp
2:	119 bp	(611-729)	2:	715 bp
3:	145 bp	(730-874)	3:	177 bp
4:	133 bp	(875-1007)	4:	29,372 bp
5:	117 bp	(1008-1124)	5:	2,706 bp
6:	115 bp	(1125-1239)	6:	2,905 bp
7:	147 bp	(1240-1386)	7:	455 bp
8:	118 bp	(1387-1504)	8:	1,455 bp
9:	275 bp	(1505-1779)	9:	695 bp
10:	165 bp	(1780-1944)	10:	2,956 bp
11:	201 bp	(1945-2145)	11:	11,121 bp
12:	133 bp	(2146-2278)	12:	1,445 bp
13:	64 bp	(2279-2342)	13:	1,578 bp
14:	160 bp	(2343-2502)	14:	1,183 bp
15:	350 bp	(2503-2852)	15:	2,528 bp
16:	124 bp	(2853-2976)	16:	1,298 bp
17:	75 bp	(2977-3051)	17:	1,781 bp
18:	153 bp	(3052-3204)	18:	1,468 bp
19:	51 bp	(3205-3255)	19:	81 bp
20:	254 bp	(3256-3509)	20:	1,106 bp
21:	241 bp	(3388-)		
22:		intraexonic splicing		

Fig. 13. Genomic structure of human TIF1γ

The human TIF1γ gene (Fig. 13) contains twenty-one exons, with sizes ranging between 609 bp (exon 1) and 51 bp (exon 19), distributed across about 115 kb of genomic DNA. The intron sizes range between 46,163 bp (intron 1) and 81 bp (intron 19). All introns have the conserved GT and AG donor and acceptor sites present at their extremity. The exon organisation of the functional domains of hTIF1γ is identical to that of hTIF1α. Similarly to hTIF1α, the RING finger spans exons 1 and 2, the B boxes are contained within individual exons (exons 3 and 4), the coiled coil is divided among exons 5-8, exon 8 also encodes the N-terminal portion of the TSS motif, which is split into two exons (8 and

9). The PHD finger is contained in exons 15 and 16, the bromodomain lies within five exons (16 to 20), and exon 19 can be spliced to produce an isoform with a deletion of 17 amino acids in the BC loop of the bromodomain. Exon 21 constitutes a 3' alternative isoform which is spliced through an intraexonic AG acceptor site located in exon 20.

3.4 Tissue distribution and expression of human TIF1α and TIF1γ transcripts

Northern blot analysis was used to characterise TIF1α and TIF1γ expression in a variety of human adult tissues. Whereas TIF1α, like TIF1β (Kim *et al.*, 1966), was expressed as a single transcript in all tissues examined (Fig. 14), TIF1γ exhibited a complex expression pattern with at least five different transcripts ranging from 2.5 to 8.8 kb (Fig. 14). Some tissues (e.g., brain, lung, liver, kidney) expressed a single species, whereas others (e.g., testis, heart, skeletal muscle) contained several species (Fig. 14). Moreover, the amount of TIF1γ transcripts appeared to vary greatly among different tissues (Fig. 14). Low levels of transcript were found in lung and kidney, whereas relatively high levels of mRNA were present in the other tissues (Fig. 14).

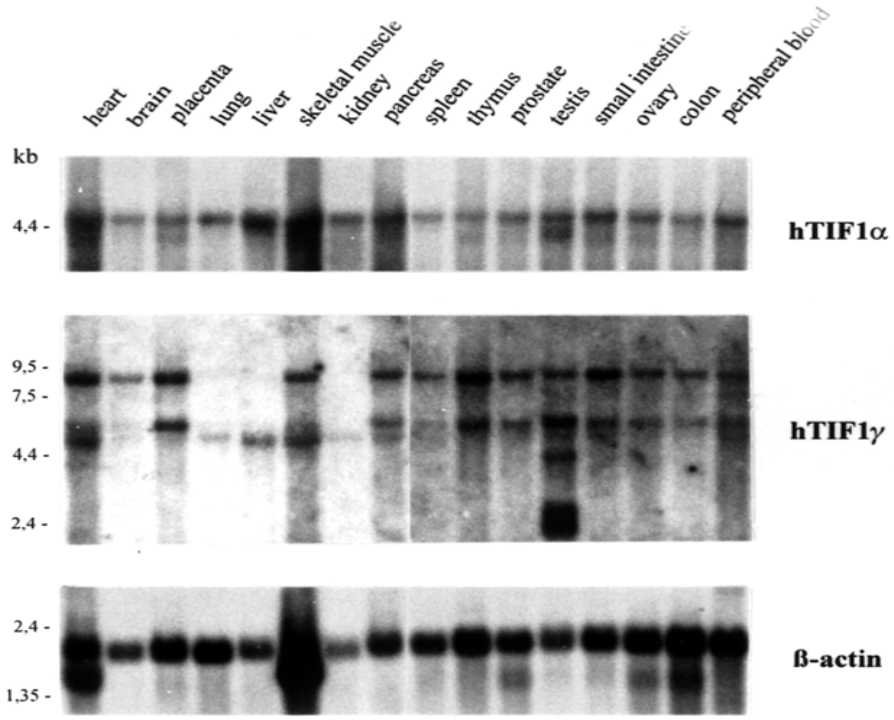


Fig. 14. Northern blot analyses of hTIF1α and hTIF1γ expression. Size markers are on the left; the β-actin control is shown below.

3.5 Modulation of nuclear receptor function by human TIF1α and TIF1γ

To investigate whether human TIF1α and TIF1γ could modulate nuclear receptor function, cotransfection assays were performed with thymidine-kinase promoter driven luciferase reporter gene under the control of multimeric DR5 retinoic acid response elements (RARE-tkLuc, de Thé *et al.*, 1990) in CHO cells. Cells were transiently transfected with 0 to 5 μg of either pSG5-hTIF1α, pSG5M-hTIF1γ, or empty pSG5M expression vector, and 1 μg of pCH110, expressing β-galactosidase. After 24 hr of retinoic acid treatment (at 1μM), cells were harvested and activities of luciferase and β-galactosidase (for normalisation) were measured. As shown in Fig. 15, the overexpression of human TIF1α inhibited the level of retinoic acid-dependent transcription in a dose-dependent manner. Conversely, overexpression of TIF1γ did not significantly affect the respective reporter gene activity. The apparent activation detected with TIF1γ was not significant because a comparable activation was found with both TIF1α and TIF1γ on a thymidine-kinase promoter driven luciferase reporter (tk-luc) construct without any response elements. Thus, in contrast to TIF1α (Le Douarin *et al.*, 1995), TIF1γ does not interfere with retinoic acid response in transfected mammalian cells.

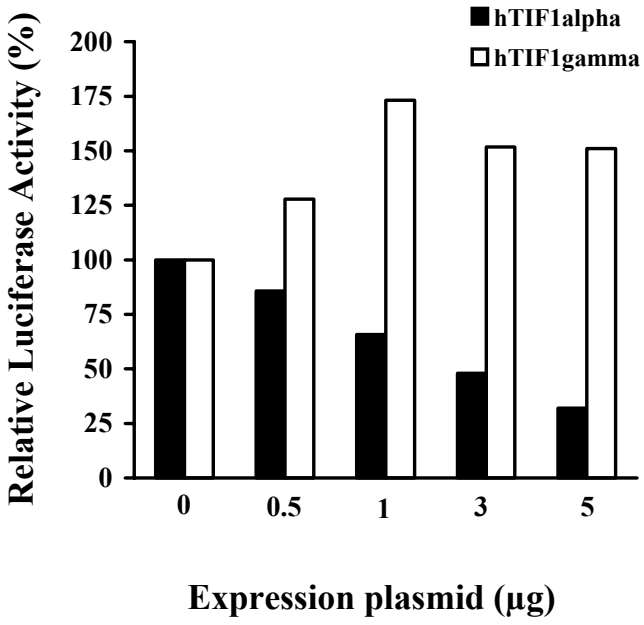


Fig. 15. Modulation of retinoic acid-dependent transcription by hTIF1α and hTIF1γ. Values (+/- 10%) are expressed as percent of those obtained using the reporter vector alone (taken as 100 %) after normalization for β-galactosidase values.

3.6 TIF1γ has no nuclear receptor binding activity

In collaboration with the group of R. Losson and P. Chambon (Institut de Génétique et de Biologie Moleculaire, CNRS / INSERM / Université Louis Pasteur, Strasbourg, France), the yeast two hybrid system was used to investigate whether TIF1γ exhibits NR binding activity, although, in contrast to TIF1α, it was unable to interfere with ligand-dependent transactivation *in vivo*.

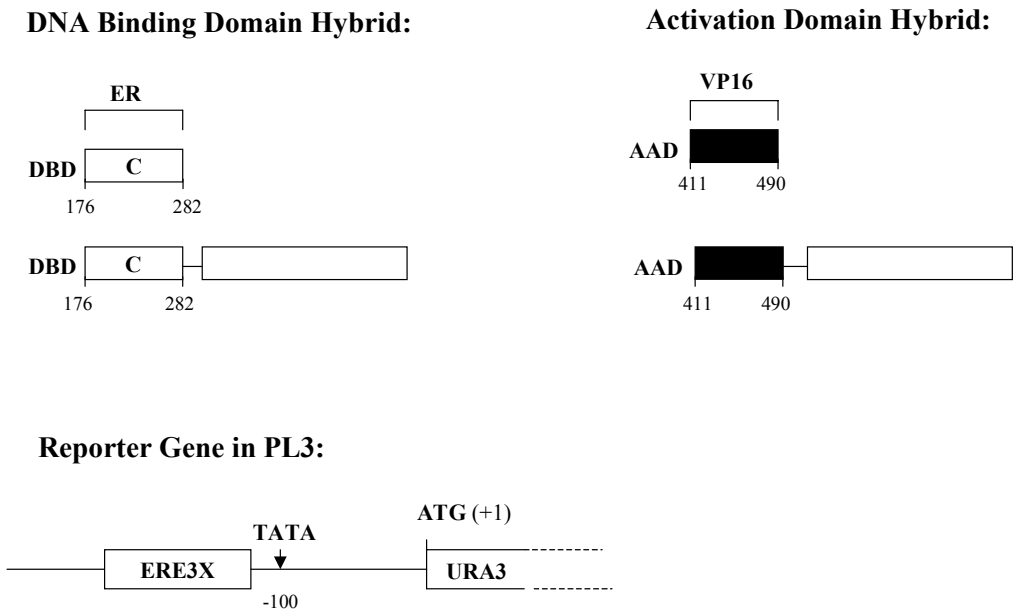


Fig. 16. Schematic representation of the yeast two hybrid system used in this study. The DNA binding domain (DBD) of the estrogen receptor (ERα; amino acids 176-282) and the acidic activation domain (AAD) of VP16 (amino acids 411-490) unfused or fused to the proteins tested for interaction (white boxes) are shown. The URA3 reporter gene which is regulated by three estrogen response elements (ERE3X) in the yeast reporter strain PL3 is represented below.

Fig. 16 shows a schematic representation of the yeast two hybrid system used in this study. The DNA binding domain (DBD) of the estrogen receptor (ERa: amino acids 176-282) and the acidic activation domain (AAD) of VP16 (amino acids 411-490) unfused (control) or fused to the proteins tested for interaction (white boxes) were co-expressed in the yeast reporter strain PL3, which contains an URA3 reporter gene controlled by three estrogen response elements (EREs). Protein interactions are revealed by the activation of the reporter, determined by measuring the specific activity of the URA3 gene product, orotidine 5'-monophosphate decarboxylase (OMPdecase) (Le Douarin *et al*., 1995b)

Full-length hTIF1γ was fused to the DBD of ER and the resulting hybrid protein (DBD-TIF1γ) was co-expressed in the yeast PL3 reporter strain with either unfused VP16 AAD (as control) or VP16 AAD fused to various members of the NR family (AAD-RAR, AAD-RXR, AAD-TR(DE), and AAD-ER(DEF) (Fig. 17). In addition, residues 694-702 of hTIF1γ which contain an LxxLL motif related to the NR box of TIF1α (Fig.17; Le Douarin *et al*., 1996) were fused to the ER DBD and tested for NR interaction (Fig. 17). No significant increase in OMPdecase activity above the unfused AAD control was detected when DBD-TIF1γ or DBD-TIF1γ(694-702) was co-expressed with AAD-NRs, either in the presence or absence of the appropriate NR ligand. In contrast, co-expression under the same conditions of DBD-TIF1α with each of the AAD-NR fusions tested resulted in a ligand dependent stimulation of the reporter gene activity (Fig. 17).

	OMPdecase Activity					
	DBD-TIF1α		**DBD-TIF1γ**		**DBD-TIF1γ (694-702)**	
Ligand	**-**	**+**	**-**	**+**	**-**	**+**
AAD	**0.3**	**nd**	**0.1**	**nd**	**1.6**	**nd**
AAD-RARα(1-462)	**0.2**	**5.0**	**0.1**	**0.2**	**1.6**	**2.1**
AAD-RXRα(1-467)	**0.2**	**7.0**	**0.2**	**0.1**	**1.9**	**2.0**
AAD-Trα(118-408)	**0.1**	**2.0**	**0.1**	**0.1**	**2.9**	**2.4**
AAD-ER(282-595)	**0.4**	**25.0**	**0.1**	**0.2**	**1.8**	**1.8**

Fig. 17. TIF1γ, in contrast to TIF1α, has no nuclear receptor binding activity in yeast. Plasmids expressing mTIF1α, hTIF1γ or residues 694-702 of hTIF1γ fused to the ERα DBD were introduced into PL3 together with VP16 AAD (as a control) or AAD

fusions as indicated. Transformants were grown in the presence (+) or absence (-) of the cognate ligand (500 nM T-RA for RARα, 500 nM 9C-RA for RXRα, 5 μM T3 for TRα, 500 nM E2 for ERα). Extracts were prepared and assayed for OMPdecase activity (expressed in nmol substrate/min/mg protein), the values (+/- 20%) are the averages of three independent experiments.

Thus, in contrast to TIF1α, TIF1γ is unable to interact with RAR, RXR, TR, and ER in yeast, either in the presence or absence of the appropriate NR ligand. Binding assays between TIF1γ and NRs were also performed in vitro. In agreement with the yeast results, no significant interaction was detected between *in vitro*-translated TIF1γ and GST-RAR(DEF), GST-RXR(DE), GST-ER(DEF) or GST-TR(DE) fusion proteins, irrespective of the presence of ligand.

3.7 Transcriptional repression by TIF1γ in transfected mammalian cells.

As TIF1α and TIF1β have previously been shown to exert a silencing activity when recruited to promoter regions (Le Douarin et al.,1996; Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al*., 1996), it was of interest to investigate whether an analogous effect on transcription could be detected for TIF1γ. The coding sequence of TIF1γ was fused to the DNA binding domain (DBD) of GAL4, amino acids 1-147. The resulting fusion protein was tested for its ability to repress transactivation by the chimeric activator ER(C)-VP16 which the DBD of estrogen receptor (ER) fused to the acidic activation domain (AAD) of the herpes simplex virus VP16 activator (amino acids 411-490).

Expression vectors for GAL4 DBD alone (1, 3, or 5 μg), GAL4-TIF1γ (1, 3, or 5 μg) and ER(C)-VP16 (100 ng) were transiently transfected into COS-6 cells, together with 1 μg of a GAL4 reporter containing two GAL4 binding sites (17M2) and an estrogen response element (ERE) in front of a β-globin (G) promoter-luciferase fusion (17M2-ERE-G-Luc) (Fig. 18), and 1 μg of pCH110 (expressing β-galactosidase) for normalisation. For comparison, assays were also performed with GAL4-TIF1α (1, 3, or 5 μg).

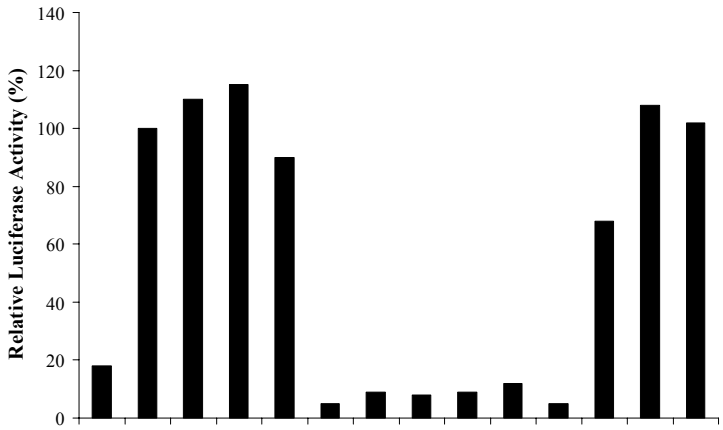


Fig. 18. Transcriptional repression by TIF1α– and TIF1γ– GAL4 fusion proteins. Luciferase activities are expressed relative to the Luciferase activity measured in the presence of ER(C)-VP16 alone (taken as 100 %). Values (+/- 10%) represent the averages

of three independent transfections after normalization for the internal control β-galactosidase activity of pCH110.

As shown in Fig. 18, GAL4-TIF1α and GAL4-TIF1γ both led to a strong repression of the reporter compared to GAL4 alone. In contrast, no reduction in luciferase activity was detected by coexpressed TIF1γ unfused to the GAL4 DBD (pSG5M-TIF1γ), indicating that repression by TIF1γ is dependent on DNA binding. Thus, likeTIF1α and TIF1β, TIF1γ has a potent transrepression activity.

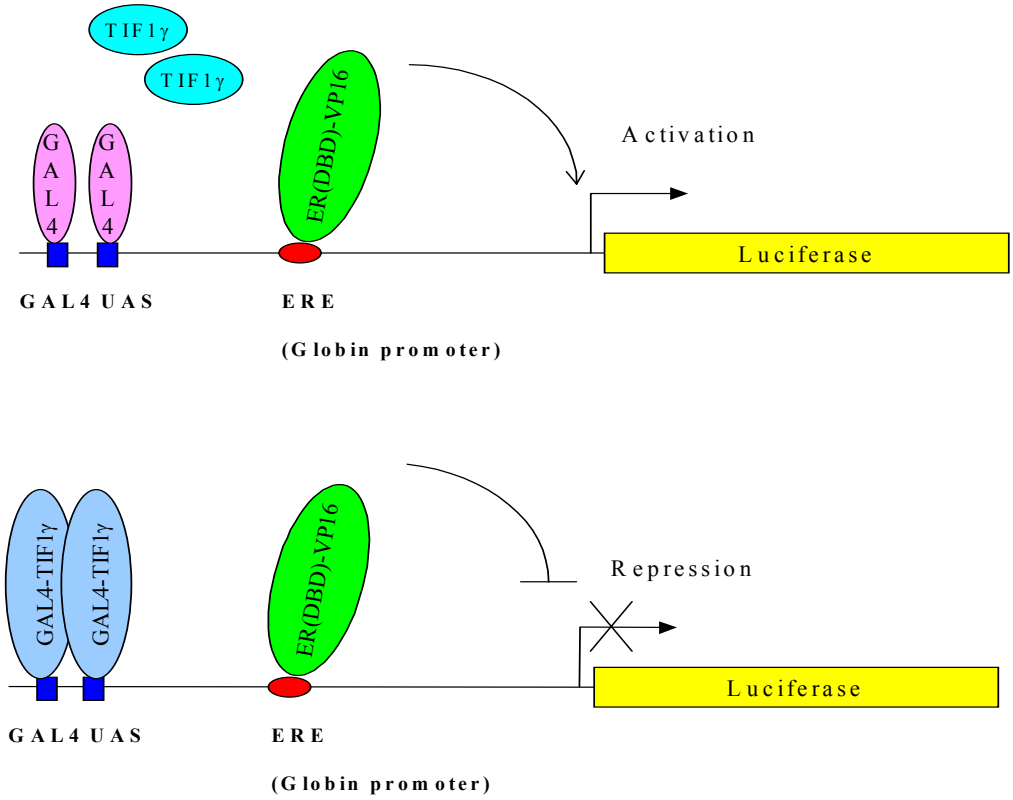


Fig. 19. Schematic representation of the TIF1γ-GAL4 transcriptional repression function as observed in COS-1 transient transfection experiment described in 3.7.

3.8 TIF1γ interacts neither with the heterochromatinic proteins HP1α, β, and γ, nor with the KRAB repression domain of KOX1

TIF1α and TIF1β have been shown to interact with the heterochromatinic proteins HP1α, β, and γ, as well as with the transcriptional repression domain KRAB of KOX1 (Le Douarin *et al*., 1996; Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al*., 1996). The transcription repression activity of TIF1α and TIF1β may thus be mediated, at least in part, by recruitment of HP1 heterochromatin proteins and induction, formation, or stabilisation of local heterochromatin-like complexes (Agata et al., 1999; Lechner *et al*., 2000; Nielsen *et al*., 1999; Peng *et al*., 1999; Ryan *et al.,* 1999).

Using the yeast two hybrid system (Fig. 16) in collaboration with the group of R. Losson and P. Chambon (Institut de Génétique et de Biologie Moleculaire, CNRS / INSERM / Université Louis Pasteur, Strasbourg, France), TIF1γ fused to the AAD of VP16 (AAD-TIF1γ; Fig) was tested for interaction with either DBD-HP1α, DBD-HP1β, DBD-HP1γ or DBD-KRAB in the yeast reporter strain PL3.

OMPdecase Activity

	AAD-	**AAD-TIF1α**	**AAD-TIF1β**	**AAD-TIF1γ**
DBD	0.5	0.5	0,5	0.5
DBD/HP1α	0.3	3.0	8.3	0.4
DBD-HP1β	0.4	2.2	6.8	0.3
DBD-HP1γ	0.4	3.5	6.6	0.4
DBD-KRAB	1.2	5.1	21.9	1.3

Fig. 20. In contrast to TIF1α and TIF1β, TIF1γ does not interact with HP1α, HP1β, HP1γ or the KRAB domain of KOX1 in yeast. Plasmids expressing mTIF1α, mTIF1β, or hTIF1γ fused to the AAD of VP16 were co-expressed in the yeast reporter strain PL3 with either AAD or the indicated AAD fusions containing the coding sequences of the mouse HP1α, HP1β, HP1γ or the KRAB repression domain of the human KOX1 protein (residues 1-97). The values of OMPdecase activities determined on each cell-free extract are expressed as the averages (+/- 20%) of three independent experiments.

No increase in OMPdecase activity above the DBD control was observed with AAD-TIF1γ, whereas in all combinations tested, the presence of AAD-TIF1α or AAD-TIF1β did stimulate expression of the URA3 reporter gene (Fig. 20). Thus, TIF1γ, in contrast to TIF1α and TIF1β, has no HP1 binding activity and does not constitute a target for the KRAB repression domain.

3.9 TIF1γ possesses three independent transcriptional repression functions

To identify regions of TIF1γ which are required for its transcriptional repression function, deletion mutants of the TIF1γ protein fused to the GAL4 DBD were tested for repression activity in transient cotransfection assays. In particular, the roles of the PHD/bromodomain and the specific TIF1 signature sequence (TSS) in the transcription repression function of TIF1γ were investigated.

The C-terminal portion of TIF1γ, containing PHD and bromodomain, and/or the C-terminal two-thirds of the TSS motif were deleted in the GAL4-TIF1γ vector to create GAL4-TIF1γ(ΔPHD/bromo), GAL4-TIF1γ(ΔTSS), and GAL4-TIF1γ(ΔTSS-ΔPHD/bromo). COS-6 cells were transiently cotransfected with 1 μg of 17M2-ERE-G-Luc reporter, 1 μg of tk-β-gal, 100 ng of ER(C)-VP16 and 25 ng of the respective GAL4-fusion expression vector. As shown in Fig. 21, the transcriptional repression activity of TIF1γ was reduced by the deletion of either the PHD/bromodomain or the TSS. The simultaneous deletion of PHD/bromodomain and TSS demonstrates an additive effect in impairing the ability of TIF1γ to repress transcription. This suggests that the TSS motif and the PHD/bromodomain may both mediate in part the inhibition of transcription by TIF1γ through independent synergistic mechanisms.

To better analyse the molecular mechanism of the TIF1γ repression activity it was of interest to investigate whether histone deacetylases (HDACs) could be involved. Therefore, the effect of the specific inhibitor of HDACs, trichostatin A (TSA; Yoshida *et al*., 1995) on repression by GAL-TIF1γ fusion proteins was examined (Fig. 21). Treatment of the transfected cells with TSA only partially inhibited the silencing activity of GAL4-TIF1γ wild-type, as well as of GAL4-TIF1γ(ΔTSS) and of GAL4-TIF1γ(ΔPHD/bromo), demonstrating that HDACs are indeed implicated in TIF1γ-mediated repression, but further deacetylase-independent mechanisms are required, too. Interestingly, repression by GAL4-TIF1γ(ΔTSS-ΔPHD/bromo) was completely relieved by TSA treatment.

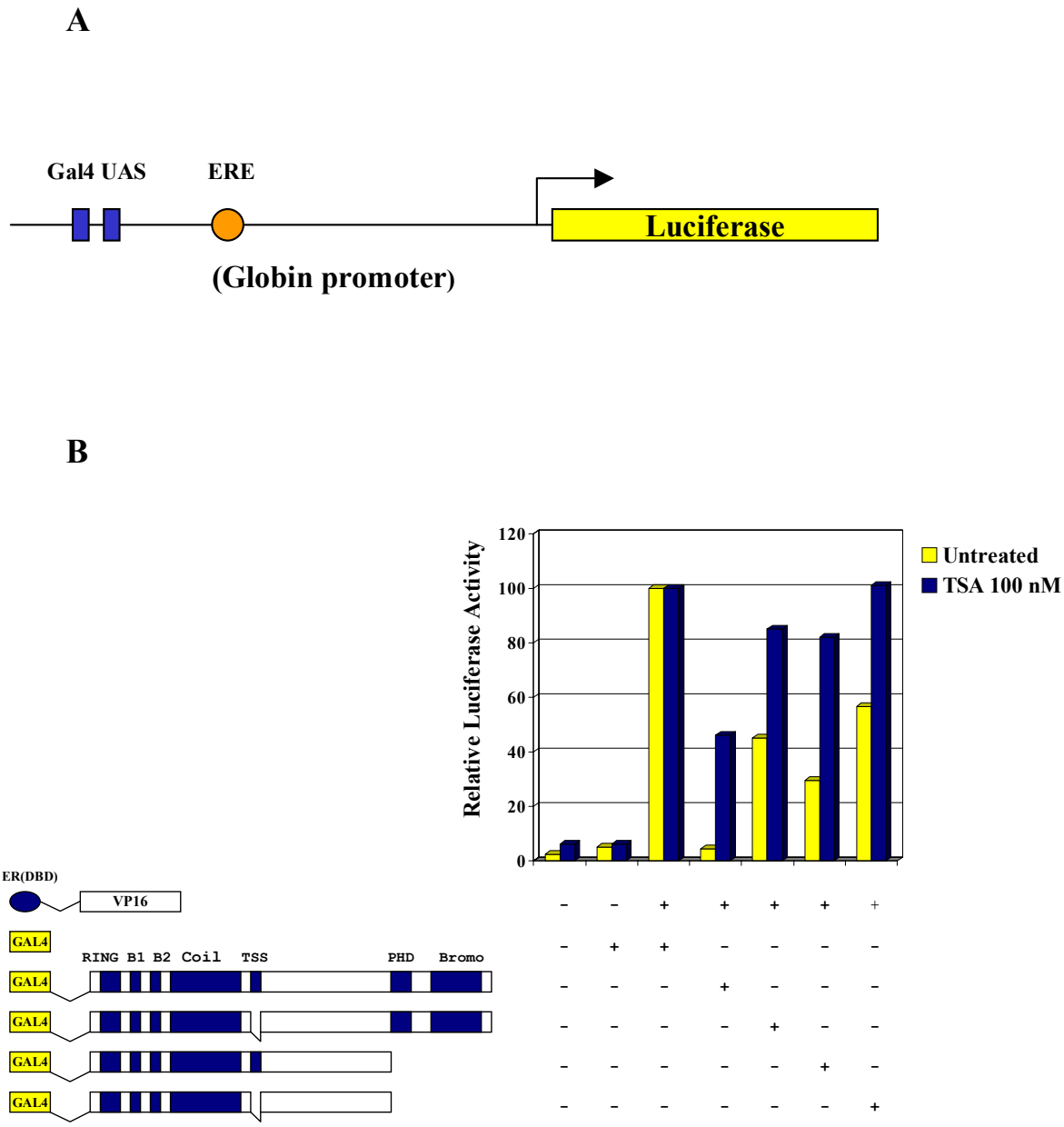


Fig. 21. (A) Schematic representation of the reporter construct used in this study. **(B)** TIF1γ possesses three independent transcriptional repression functions. Luciferase activities are expressed relative to the Luciferase activity measured in the presence of ER(C)-VP16 alone (taken as 100 %). Values (+/- 10%) represent the averages of three independent transfections after normalization for the internal control β-galactosidase activity of ptk-βgal.

These results suggest that TIF1γ may repress transcription through three different mechanisms: an HDACs-dependent repression activity and two other silencing functions which are mediated by the TSS and PHD/bromodomain, respectively.

3.10 Identification of TIF1γ mRNA C-to-U editing which produces a truncated TIF1γ protein lacking the PHD and the bromodomain

As Northern blot analysis demonstrated the existence of several TIF1γ mRNA isoforms (see 5.4), a testis cDNA library was screened in order to isolate and characterise the different TIF1γ isoforms. Among the isolates, two clones demonstrated different nucleotides (a cytidine versus a uridine) at position 2678 of the respective mRNAs (Fig. 22), where the genomic sequence presents a cytidine.

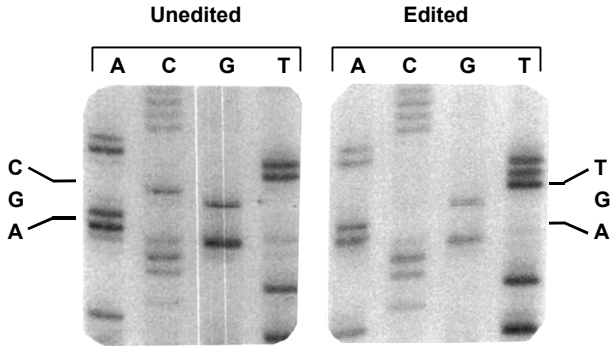


Fig. 22. Sequencing of the TIF1γ unedited and edited cDNA

This base change was interpreted as the result of a post-transcriptional C-to-U mRNA editing process, analogous to that occurring with apolipoprotein (apo) B (Chen *et al.*, 1987; Powell *et al.*, 1987). As a result of this editing event, the CGA arginin codon at amino acid position 865 of TIF1γ is converted to a premature UGA stop codon, leading to the generation of a carboxyterminally truncated TIF1γ protein which lacks the PHD and the bromodomain (Fig. 23).

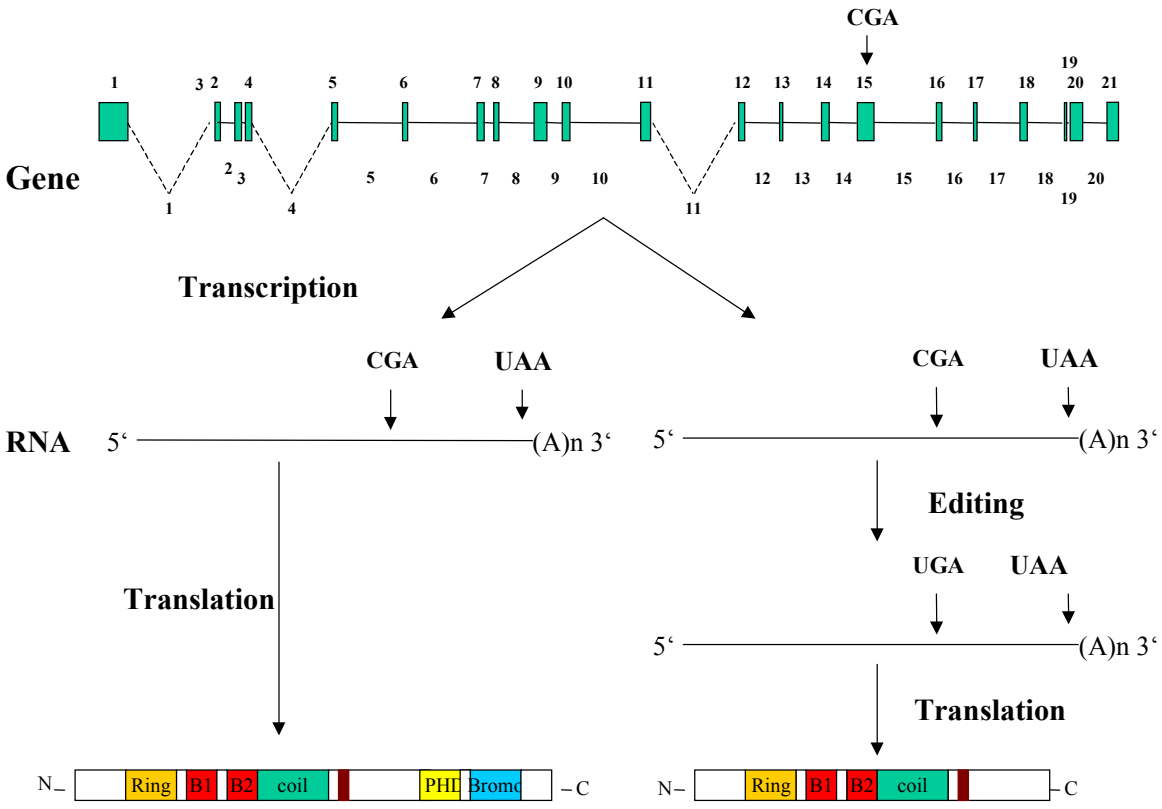


Fig. 23. Schematic illustration of the TIF1γ editing event

3.11 Expression of the edited TIF1γ isoform in normal tissues and leukaemic cells from patients

To evaluate the expression of the edited TIF1γ isoform, total RNA from a large variety of normal human tissues was examined. First strand cDNA synthesis was performed with the R9 primer specific for the TIF1γ 3'-terminus, and the sequence containing the editing site was amplified by polymerase chain reaction using G7F and G7R amplimers as described in Materials and Methods. The RT-PCR products were used as templates for a poisonned-cycled primer extension reaction with the ^{32}P-end-labelled TIF1γ PEXT.F primer and the dideoxy-termination ddCTP as illustrated in Fig. 24.

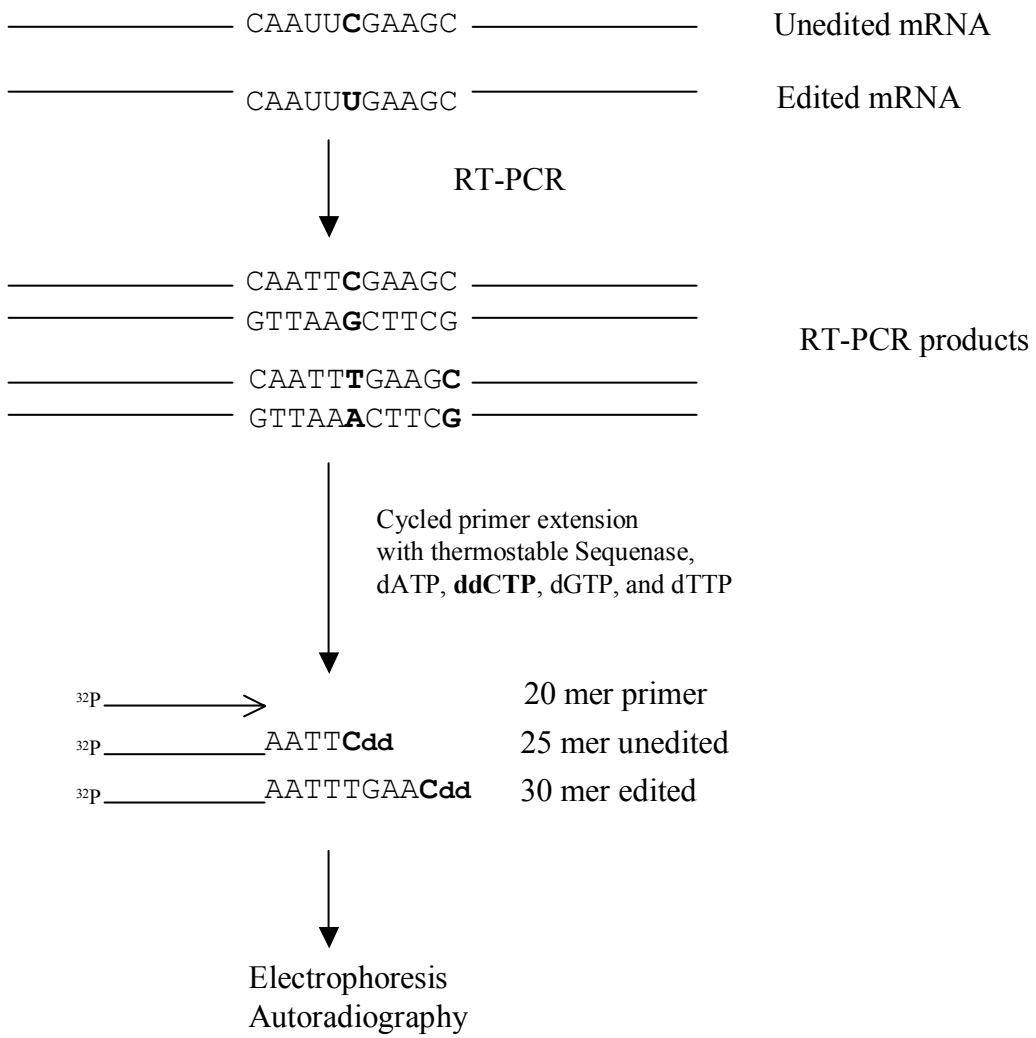


Fig. 24. Schematic representation of the RT-PCR / poisonned primer extension experiments

Reactions were analysed by 12% denaturing polyacrylamide gel elrctrophoresis and the signals were evidentiated by autoradiography (Fig. 25). Three major bands were apparent corresponding to excess primer (20 bp), the first stop primer extension product (25 bp) derived from unedited TIF1γ mRNA (CGA), and the second stop primer extension product (30 bp) resulting from the edited TIF1γ mRNA (TGA).

The results indicate that TIF1γ editing was almost undetectable in all tested human tissues including normal bone marrow (Fig.25).

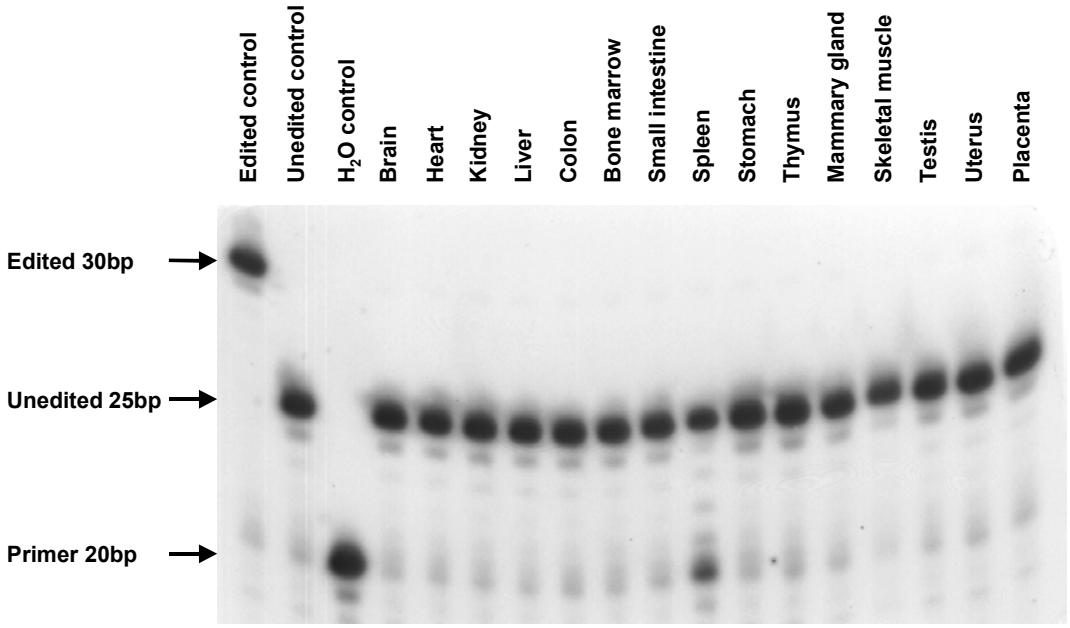


Fig.25. No detection of TIF1γ mRNA editing in normal human tissues

The same expriment was performed with total RNA isolated from bone marrow or peripheral blood cells of patients suffering from acute myelogenous leukemia. Surprisingly, a relatively high level of the edited compared to the unedited isoform was detected in about 30% of the patients analysed (Fig. 26).

Since normal bone marrow or peripheral blood cells failed to exhibit TIF1γ editing, these results suggest a possible implication of TIF1γ hyperediting in the process of leukaemogenesis.

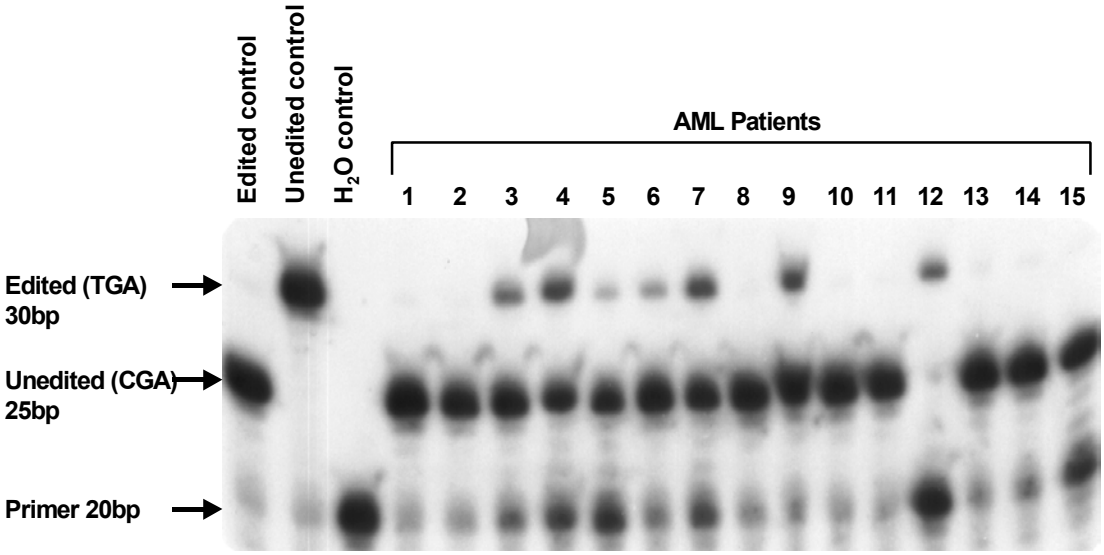


Fig. 26. Detection of TIF1γ mRNA editing in bone marrow or peripheral blood of patients suffering from acute myelogenous leukemia.

4. DISCUSSION

4.1 TIF1γ is a novel member of the TIF1 family which belongs to the RBCC (RING finger-B boxes-Coiled coil) subfamily of the Ring finger proteins.

TIF1γ is the third member of the TIF1 gene family to be identified. *In situ* chromosomal hybridisation suggests the existence of a fourth TIF1 gene. However, exstensive cDNA library screening failed to isolate this fourth gene which could be a pseudogene or could have a very restricted expression pattern.

The RING finger motif defines a family of proteins with diverse functions, expressed in organisms ranging from plants to viruses. Like TIF1α and TIF1β, TIF1γ possesses an N-terminal RING finger adjacent to two B boxes (Reddy *et al*., 1992). These domains together with a putative coiled coil domain form a conserved tripartite motif known as RBCC (Saurin *et al*., 1996), which identifies a subfamily of the RING finger proteins. The RBCC motif is thought to be involved in intermolecular interactions which influence the targeting to and/or assembly of subnuclear structures, as observed for several RBCC family members such as RFP, XNF7, PwA33 and PML. RFP is a nuclear matrix-associated protein (Takahashi *et al*., 1988); XNF7 associates with the spindle in mitotic cells and with the centrosomes during interphase, dependent upon the presence of an intact B-box (Li *et al*., 1994); wild type PwA33, but not PwA33 mutated in the B-box, is present in the lampbrush chromosome loops of *Pleurodeles* oocytes (Bellini *et al*., 1995); PML is normally localised, together with several other proteins, on large matrix-associated nuclear domains, called PML Nuclear Bodies (PML-NBs; Daniel *et al*., 1993; Dyck *et al*., 1994; Koken *et al*., 1994; Weiss *et al*., 1994), whose formation is prevented by point mutations in the RING finger, the B-box domains, or the coiled coil region of PML (Boddy *et al*., 1997). TIF1α and TIF1β were also found in close association with specific subnuclear structures: TIF1α is a non-histone protein tightly associated with highly accessible euchromatic regions (Remboutsika *et al*., 1999); TIF1β localisation revealed a dynamic pattern which may be correlated with the cell cycle. Particularly striking is its localisation to distinct territories within the nucleus (Ryan *et al*., 1999). Further studies of immunofluorescence and electron microscopy will attempt to analyse the subcellular localisation of TIF1γ. Reymond *et al.* (2001) investigated the homo- and heterodimerisation properties of the RBCC proteins and showed that the coiled coil region is necessary and sufficient for homo-interaction of the members of the RBCC family and may mediate the formation of high molecular weight complexes, while cooperation of the RING and B-boxes is required for proper subcellular localisation at specific cell

compartments. Preliminary studies of yeast two hybrid screens demonstrated a heterodimerisation among the three TIF1 proteins as well as a homodimerisation of TIF1γ (Venturini *et al*., 1999). With analogy to the other members of the RBCC family, we can speculate that the TIF1 coiled coil is responsible for these homo- and heterointeractions among the TIF1 proteins.

4.2 The TIF1 protein structure suggests an implication of this family in chromatin-mediated transcriptional regulation.

Towards its C-terminus, TIF1γ contains two other domains: the PHD finger and the bromodomain, which are also present in the C-terminal moiety of TIF1α and TIF1β. These domains, which are conserved from yeast to humans, have been identified in a number of transcriptional mediators/coactivators, several of which function at the chromatin level (Aasland *et al*., 1995; Koken *et al*., 1995; Jeanmougin *et al*., 1997). For instance, the *Drosophila* gene products Trithorax and Polycomb-like, which are both chromatin-associated proteins, contain multiple PHD fingers (Aasland *et al*., 1995; Koken *et al*., 1995). The CBP/p300 histone acetyltransferase, which has been demonstrated to interact functionally with components of the RNA Pol II general transcription complex and with a number of sequence-specific transcriptional activators, among them the nuclear receptors, also contains a bromodomain and a PHD finger (see Shikama *et al*., 1997 for review). Thus, in view of their amino acid sequences, members of the TIF1 family may be implicated in chromatin-mediated transcriptional regulation.

In several proteins the PHD finger was found adjacent to the bromodomain; this observation could suggest that the two domains may function together biochemically as a cooperative unit. In support of this hypothesis, Schultz *et al.* (2000) demonstrated that optimal transcriptional repression by TIF1β is dependent upon the integrity of this bipartite motif which probably provides a specific interface for protein-protein interactions with downstream effectors of transcriptional repression. Similar observations, suggesting that the function of the bromodomain may require or be influenced by adjacent motifs, are reported by Cairns *et al.* (1999) and Winston and Allis (1999). However, most PHD-containing proteins do not contain a bromodomain, suggesting that the PHD is an independent functional unit.

Bromodomains are acetyl-lysine binding domains consisting of four amphipathic α-helices (αZ, αA, αB, and αC) with two loops (ZA loop and BC loop) which form the hydrophobic pocket costituting the acetyl-lysine-binding site (Dhalluin *et al*., 1999; Owen *et al*., 2000). The bromodomain of TIF1γ presents a longer BC loop as compared to the other bromodomains described so far. An alternative splicing generates a TIF1γ isoform

with a BC loop similar to that of the other known bromodomains. The longer BC loop probably corresponds to a different conformation of the hydrophobic pocket which forms the bromodomain acetyl-lysine-binding site. As a consequence, the TIF1γ isoform containing this type of bromodomain could have a different affinity for acetyl-lysines, leading to a modified activity on transcriptional repression.

Like other modular domains, such as Src homology-2 (SH-2) and phosphotyrosine-binding (PTB) domains, which specifically interact with phosphotyrosine-containing proteins, the recognition of acetyl-lysines by the bromodomain could regulate protein-protein interaction by lysine acetylation.

4.3 The complexity of TIF1γ expression pattern and its molecular diversity are enhanced by an mRNA C-to-U editing event, which produces a truncated TIF1γ protein lacking the PHD/bromodomain.

Studies of the expression of hTIF1α and hTIF1γ by Northern blot analysis demonstrate a ubiquitous tissue distribution for TIF1α, but a complex expression pattern for TIF1γ with at least five different transcripts ranging from 2.5 to 8.8 kb. Whereas some tissues (e.g. brain, lung, liver, kidney) contain a unique isoform, others (e.g. testis, heart, skeletal muscle) express several different TIF1γ mRNA types. As these isoforms exhibit a heterogeneous expression level and vary considerably in size in the different tissues, they might contain additional domains and confer a tissue-specific functionality to the TIF1γ protein.

The complexity of this expression pattern and the molecular diversity of the TIF1γ protein are enhanced by a C-to-U mRNA editing event which converts the CGA arginine codon at amino acid position 865 to a premature UGA stop codon, leading to the generation of a carboxyterminally truncated TIF1γ protein which lacks the PHD and the bromodomain.This process probabily interferes with the transcription repression function of TIF1γ as the deletion of PHD and bromodomain considerably reduces its ability to repress transcription in cotransfection experiments.

Similarly to splicing, RNA editing is a postranscriptional, highly regulated process which can have tissue-, developmental, and species specificity. For example, in humans, hepatic ApoB is found exclusively in the unedited form, while in the small intestine the edited ApoB48 isoform is predominantly expressed; both edited and unedited forms are found in the colon, stomach, and kidney (Teng *et al*., 1990). In contrast to humans, in rats and mice approximately 65% of hepatic ApoB mRNA is edited (Chan *et al*., 1993). Moreover, in humans, ApoB mRNA editing progressively increases during development:

the unedited ApoB100 is the only isoform produced in the early human foetus, while ApoB48 is the major protein in the adult tissues (Wu et al., 1990; Glickman et al., 1986).

TIF1γ mRNA editing was almost undetectable in all normal adult human tissues studied by an RT-PCR/poisonned-cycled primer extension experimental procedure. In order to establish whether TIF1γ editing is also modulated during development, it would be interesting to quantitatively investigate this process in human foetal tissues.

4.4 Members of the TIF1 family are potent transcriptional repressors which appear functionally distinct in their silencing mechanism.

TIF1γ, similarly to TIF1α and TIF1β, is a potent transcriptional repressor when tethered to the promotor region of a reporter gene. In order to characterise the molecular mechanisms involved in the transcription repression activity of TIF1γ, its possible physical and functional interaction with the HP1 proteins was investigated through yeast two hybrid experiments. In contrast to TIF1α and TIF1β, TIF1γ did not interact with HP1 proteins in yeast or in vitro.

Trichostatin A (TSA), a specific inhibitor of HDACs, can interfere with TIF1γ-mediated repression, suggesting that histone deacetylation is involved in this process. However, repression by wild-type TIF1γ can only partially be relieved by treating cells with TSA indicating that other mechanisms independent of histone deacetylation are also required. Deletion of the TSS motif or the PHD/bromodomain region of TIF1γ also reduced its ability to repress transcription. Deletion of both TSS and PHD/bromodomain demonstrated an additive effect in impairing the TIF1γ silencing activity which was, in this case, completely relieved by TSA treatment. These results suggest that TIF1γ may repress transcription through three different mechanisms: it presents a HDAC-dependent repression activity and two other silencing functions which are mediated by TSS and PHD/bromodomain, respectively. It is probable that these two regions of TIF1γ constitute a target for other co-repressor proteins yet to be identified, which may contribute to the TIF1γ transcription silencing function.

Although these results indicate that the silencing effect of TIF1γ, as well as the other two members of the family TIF1α and β, is exerted at the chromatin level, it is not clear how they could be recruited to chromatin because they present no DNA binding domain and apparently, as reported by Remboutsika *et al*. (1999), do not have sequence-specific DNA binding activity. Therefore, their recruitment to chromatin may result from the association with DNA-bound proteins. In the case of TIF1β, Ryan *et al*. (1999) propose a model illustrated in Fig. 27. TIF1β may be recruited to specific chromatin regions through interaction between its RBCC motif and the KRAB domain of DNA-bound

KRAB-zinc finger proteins (KRAB-ZFPs). It mediates transcriptional repression by recruitment of the HP1 proteins (through its HP1 box) and HDACs (through its PHD/bromodomain, Schultz *et al*., 2001), inducing the local formation of compact heterochromatin-like structures.

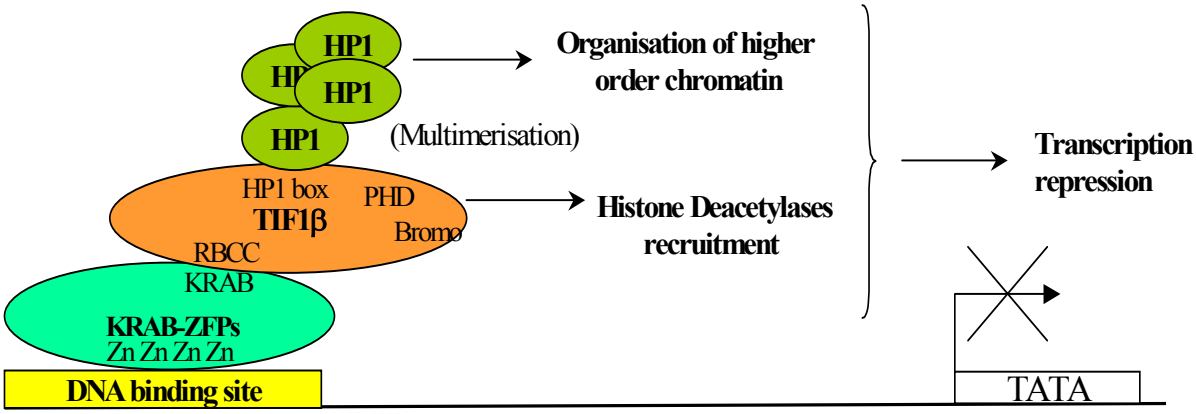


Fig. 27. Transcriptional repression of KRAB-ZFPs mediated by TIF1β: model proposed by Ryan *et al*. (1999).

Similar to TIF1β, TIF1α has also been reported to interact with the KRAB domain of KOX1 (Moosmann *et al*., 1996); however, in contrast to TIF1β, this interaction has not been reproduced for other KRAB-ZFPs (Abrink *et al*., 2001). Thus, TIF1α cannot be considered a general mediator for the KRAB-ZFPs' repression function. Le Douarin *et al*. (1995) demonstrated that TIF1α interacts with transcriptionally active nuclear receptors in a ligand-dependent manner. Thus, TIF1α could be recruited to DNA through interaction with DNA-bound nuclear receptors, and be involved in ligand-dependent repression. However, as TIF1α is preferentially associated with euchromatin (Remboutsika *et al*., 1999) in nuclear structures where RNA polymerase II and nascent RNA have been shown to colocalise (Grande et al., 1997; Lamond & Earnshaw, 1998), Nielsen *et al*. (1999) favour another model in which TIF1α functions in euchromatin as an anchor protein to which nuclear receptors bind in the presence of their ligands. As a consequence of this binding, TIF1α becomes hyperphosphorylated (Fraser *et al*., 1998) and this modification would decrease its silencing activity, thus resulting in an activation of transcription.

As concerns TIF1γ, it interacts neither with the KRAB domain nor with nuclear receptors, suggesting that it may be recruited to chromatin by other DNA binding proteins which remain to be identified.

These results support the view that the three members of the TIF1 family, although structurally related, are functionally distinct in their mechanisms of action. Additional support for this specificity of action of the TIF1 proteins comes from the recent finding that mice lacking TIF1β are defective in early postimplantation development (Camas *et al*., 2000), implying that, at least during early embryogenesis, the members of the TIF1 family exert distinct, nonredundant functions.

4.5 Chromatin-mediated transcriptional regulation and leukaemogenesis

The advances in transcriptional research have revolutionised our understanding of transcriptional mechanisms involved in haematopoiesis and leukaemogenesis (for reviews, see Redner *et al*., 1999; Golub, 1999).

The pathogenesis of acute myeloid leukaemia (AML) is associated with the presence of specific chromosome translocations which generate oncogenic fusion proteins. Of the two fusion partners, one is generally a transcription factor, whereas the other is more variable in function, but often involved in the control of cell survival and apoptosis. As a consequence, AML-associated fusion proteins function as aberrant transcriptional regulators which interfere with the process of myeloid differentiation, determining enhanced survival and proliferation of cells arrested at a specific stage of maturation.

The abnormal regulation of transcriptional networks occurs through mechanisms which include recruitment of aberrant co-repressor complexes to DNA regions which are otherwise actively transcribed and alterations in chromatin acetylation and remodelling.

4.5.1 Aberrant recruitment of corepressor complexes to DNA

About 10% of acute myelocytic leukaemias are characterised by the t(8;21)(q22;q22) translocation which fuses the myeloid transcription factor AML1 to ETO. AML1 binds DNA through its N-terminal *runt* homology domain and activates myeloid genes (IL-3, GM-CSF, M-CSF, myeloperoxydase, neutrophil elastase), at least partially by interaction with the p300 coactivator complex, local histone acetylation and nucleosome modification (Kitabayashi *et al*., 1998). In the AML1-ETO fusion protein, the p300 binding C-terminal part of AML1 is replaced by ETO which recruits, through its *nervy*

homology domain, the corepressor complex N-CoR / Sin3 / HDAC1 (Gelmetti *et al*., 1998). Thus, repression instead of activation of AML1-dependent differentiation genes ensues.

The RARα part of PML-RARα, the t(15;17)(q22;q21) fusion product of acute promyelocytic leukaemia, has been shown to bind to the N-CoR and SMRT corepressor complexes in the absence of ligand (retinoic acid), leading to HDAC1-mediated transcriptional repression. Physiological levels of retinoic acid, which are sufficient to release the corepressor complex from wild-type RARα and induce the recruitment of a coactivator complex with histone acetyltransferase activity, are unable to abrogate the repression function of the PML-RARα fusion protein which, however, can be relieved by pharmacological doses of retinoic acid used as differentiation therapy for acute promyelocytic leukemias (Grignani *et al*., 1998; He *et al*., 1998). In contrast to PML-RARα, the fusion protein PLZF-RARα of the variant translocation t(11;17)(q23;q21), also found in acute promyelocytic leukaemias, can recruit a second constitutive HDAC complex through the POZ domain of PLZF. This second repressor complex is insensitive to retinoic acid and, accordingly, acute leukaemias presenting the t(11;17) are refractory to this treatment which must be integrated with HDAC inhibitors to successful overcome the repressive function of the RARα-PLZF fusion protein and induce differentiation of blast cells.

Mechanisms of transcriptional regulation involved in leukaemogenesis are being evaluated as targets of new therapeutic principles, e.g. by competitive (butyrate), reversible (trichostatin A), or irreversible (trapoxin) inhibition of histone deacetylases. While there have been impressive results in single cases (Warrell *et al*., 1998), further research on specificity, pharmacokinetics and pharmacodynamics is required, before these new approaches can safely be established as clinical routine.

4.5.2 Aberrant chromatin acetylation and alteration in chromatin remodelling: chromosomal translocations involving PHD- and bromodomain-containing proteins

The recurrent translocation t(8;16), which is associated with a subtype of acute myeloid leukaemias, has been demonstrated to recombine two distinct PHD finger proteins, CBP/p300 and MOZ (Borrow *et al*., 1996). MOZ-CBP and MOZ-TIF2, the fusion proteins created by the translocation t(8;16)(p11;p13) and the inversion inv(8)(p11;q13), respectively, of acute myelomonocytic and monocytic leukaemias, are examples of fusion proteins containing each two partners which both possess histone acetyltransferase activity.

The so-called 11q23-leukaemias of myeloid or lymphatic lineage are characterised by duplications or translocations, with a variety of different fusion partners, of the human *trithorax* homologue MLL (for "mixed lineage leukemia", also called ALL-1 or HTRX; see Cimino *et al*., 1998, for review). MLL contains N-terminal DNA binding domains and may recruit, through its C-terminal SET domain, a chromatin modifying / ATPase complex homologous to SWI/SNF (Rozenblatt-Rosen *et al*., 1998). In fusion proteins with MLL, this transcription regulatory capacity can be lost or, depending on the respective fusion partner, may result in constitutive activation through chromatin modifying complexes or histone acetyltransferases (e.g. MLL-AF9, MLL-ENL, MLL-CBP, MLL-p300; Cairns *et al*., 1996; Taki *et al*., 1997; Ida *et al*., 1997).

4.6 Distruption of specific subnuclear compartments: oncogenic translocations involving the RBCC motif

Four of the presently known RBCC proteins (PML, RFP, TIF1α and TIF1γ) were found in the context of fusion oncoproteins, in which the RBCC motif is fused to truncated products of other genes. The majority of acute promyelocytic leukaemias results from the t(15;17) translocation which combines the RBCC motif of PML and the CDEF region of RARα (see Warrell *et al*., 1993; Grignani *et al*., 1994; Grrimwade & Solomon, 1997 for reviews). The RBCC motif of the nuclear matrix protein RFP is fused to the tyrosine kinase RET in the RFP-RET chimeric protein (Takahashi *et al*., 1988). TIF1α was detected as fusion partner of the serine/threonine kinase B-Raf in a chemically induced murine hepatic carcinoma (Miki et al., 1988). Moreover, oncogenic fusion proteins of TIF1α and TIF1γ with RET have been detected in thyroid carcinomas in children from Cernobyl (Klugbauer & Rabes, 1999).

These observations suggest that the RBCC motif may play a crucial role in cell transformation. A possible mechanism contributing to oncogenesis could be the formation of heterodimers between the wild-type protein and the fusion protein through the coiled coil region, which may produce a dominant negative effect by sequestration of the wild-type and its associated proteins in an inappropriate subcellular location. This is for example the case of the dominant negative effect of the PML-RARα/PML heterodimerisation, mediated by the PML coiled coil, on the wild-type PML protein. PML-RARα expression in acute promyelocytic leukaemia dominantly delocalises PML to microspeckles and distrupts NBs (Koken *et al*., 1994; Dyck *et al*., 1994; Weis *et al*., 1994). Exposure to therapeutic agents, such as retinoic acid or arsenic trioxide, results in the degradation of PML-RARα and accordingly restores NB structure.

TIF1γ has been mapped to the 1p13 chromosomal locus. Interestingly, a non-random t(1;22)(p13;q13) chromosomal translocation involving this region has been reported in acute megakaryocytic leukaemias (Mitelman, 1993). Although preliminary studies of Southern blot in two megakaryocytic cell lines were not able to demonstrate the presence of TIF1γ rearrangements, it is of interest to further investigate its possible implication in chromosomal translocations of acute leukaemias.

4.7 Aberrant editing and oncogenesis.

In recent years, abnormalities in post-transcriptional processes, such as RNA splicing and editing, are emerging as possible oncogenic events.

Although Greeve *et al*. (1999) failed to identify abnormal ApoB editing in any of the tumour specimens they studied, transgenic mice and rabbits overexpressing APOBEC-1, the apolipoprotein B editing catalysing enzyme, exhibited liver dysplasias and carcinomas. Moreover, the expression of an alternatively spliced form of APOBEC-1 is significantly increased in human colon carcinomas (Lee *et al*., 1998). Overexpression of APOBEC-1 probabily causes aberrant editing of genes implicated in growth and/or differentiation, supporting the hypothesis that alterations in the modulation of editing processes may be involved in tumourigenesis (Yamanaka *et al*., 1997).

Recent results have shown that malignant neurofibromas of patients with neurofibromatosis type 1 (NF1) edit NF1 mRNA more efficiently than benign tumours, suggesting an implication of upregulated NF1 editing in tumourigenesis (Cappione *et al*., 1997).

Further evidence that disorders in RNA editing may be involved in oncogenesis derives from studies on the editing of the Glutamate Receptor (GluR). This process is regulated during brain development: very little GluR editing occurs during early embryonic life, with a significant progressive increase during development and adulthood (Bernard & Khrestchatisky, 1994; Paschen *et al*., 1994, 1995). Lai *et al*. (1997) observed that in the human teratocarcinoma cell line NT2, a neuronal progenitor cell line which can be induced to fully differentiate in vitro by retinoic acid, GluR RNA editing is inactive or remains very low in undifferentiated NT2 cells, but becomes progressively activated during NT2 cell differentiation to postmitotic neurons.

RNA hyperediting was also observed for the hematopoietic tyrosine phosphatase PTPN6 in acute myeloid leukaemia. Beghini *et al*. (2000) found that the level of PTNP6 editing was higher in bone marrow cells of leukaemic patients at diagnosis than at remission, suggesting an involvement of this postranscriptional process in leukaemogenesis.

The identification of a C-terminally truncated hTIF1γ isoform generated by C-to-U mRNA-editing, which is preferentially expressed in primary blasts of patients suffering from acute myeloid leukaemia, as compared to normal tissues, rises the question whether a disorder in the control of the TIF1γ-editing process may be relevant for leukaemogenesis. The overexpression of the edited truncated TIF1γ isoform which lacks the PHD and the bromodomain and, as a consequence, is less efficient in transcriptional repression as the unedited form, could exert a dominant negative effect on the control of transcription by the TIF1γ protein. Overexpression of the TIF1γ edited isoform in a transgenic mouse model could be useful to study the possible implication of TIF1γ hyperediting in leukaemogenesis.

Different expression levels of the truncated protein, in proportion to the unedited form, are observed between the patients examined. It would be of interest to investigate whether this finding correlates with diverse clinical features and could have a value as prognostic indicator.

5. SUMMARY

Transcriptional regulation of gene expression in eukaryotes is a complex process which is highly regulated at many levels. It requires the concerted action of several factors to integrate different transcriptional regulatory signals controlling cellular responses such as proliferation, differentiation and apoptosis.

In recent years, reversible structural modifications of chromatin have been established as important mechanisms for the control of gene expression. Enzymatic activities which modify chromatin structure, such as histone acetyl transferases (HATs) and deacetylases (HDACs) and nucleosome-remodelling complexes which have ATPase and helicase activities, can be recruited to promoter regions through direct interaction with DNA-binding proteins, such as sequence-specific transcription factors. They can facilitate or inhibit the assembly of the basal transcription machinery at the core promoter, thereby activating or repressing the expression of their target genes, through interaction with such co-activator or co-repressor complexes, whose function is to induce a more open and accessible or a more compact and repressive chromatin configuration.

The "Transcriptional Intermediary Factor 1" family presently consists of three nuclear proteins (TIF1α, β, and γ) believed to function as transcriptional coregulators at the chromatin level.

Based on amino acid homologies, a typical TIF1 domain structure has been ascribed to members of this family. The N-terminal region contains the RBCC (Ring finger-B box-coiled coil) tripartite motif which most likely functions as a cooperative protein-protein interaction motif (Borden, 1998). RING (for "Really Interesting New Gene") fingers are cysteine domains of the form C3HC4 which have been found in many proteins of diverse functions from virus to vertebrates and likely contribute to the specificity and/or multimerisation properties of the RBCC motif to form large macromolecular complexes. (Freemont, 1993; Borden *et al.*, 1995a; Saurin *et al.*, 1996; Borden, 2000). The B-box is also a cysteine-zinc binding domain of the form CHC3H2 (Reddy & Etkin, 1991). This domain, too, is likely implicated in protein-protein interactions (Alcalay *et al.*, 1998; Cao *et al*., 1998). The third element of the RBCC motif is the coiled-coil domain which consists of α-helical regions which constitute a hydrophobic interaction surface for protein multimerisation (Cao *et al*., 1997; 1998). The C-terminal regions of the TIF1 proteins contain a polycomb homology domain (PHD; Aasland *et al.*, 1995; Koken *et al.*, 1995) and a bromodomain (Jeanmougin *et al.*, 1997). The PHD is a cysteine-histidine zinc finger domain of the form C4HC3 which, like RING fingers, appears to function as a protein-protein interaction motif. The bromodomain is a ~110-amino-acid module consisting of four amphipathic α-helices which interacts with lysine-acetylated peptides derived from

histones H3 and H4, suggesting a role in chromatin recognition for this highly conserved domain (Dhalluin *et al.*,1999; Owen *et al*., 2000). Both PHD and bromodomain are characteristic motifs of proteins known to function as transcriptional coregulators at the chromatin level.

Historically, the N-terminal part of TIF1α was first detected as T18, the fusion partner of bRaf in a chemically induced murine hepatic carcinoma (Miki *et al.*, 1988). The murine TIF1α was later discovered through two hybrid screening by virtute of its ligand-dependent interaction with the ligand binding domain of several nuclear receptor transcription factors, including retinoid X (RXR), retinoic acid (RAR), vitamin D3 (VDR), estrogen (ER), and progesterone (PR) receptors, and it has been proposed to be a mediator of their transactivation function AF-2 (Le Douarin *et al*., 1995; vom Baur *et al.*, 1996).

TIF1β, also referred to as KAP-1 (KRAB-associated protein 1) or KRIP-1 (KRAB-interacting protein 1), was isolated shortly after TIF1α by virtute of its interaction with the HP1 heterochromatin proteins (Le Douarin *et al*., 1996) and the transcriptional silencing domain KRAB (krüppel-associated box; Friedman *et al.*, 1996; Kim *et al.*, 1996; Moosmann *et al*., 1996) identified at the N-terminus of several DNA-binding zinc-finger proteins (ZFPs) (Bellefroid *et al*., 1991).

TIF1α and TIF1β are nuclear phosphoproteins, with intrinsic kinase activity (Fraser *et al*., 1998; Nielsen *et al*., 1999), which act as potent transcriptional repressors when tethered to the promoter region of a reporter gene. TIF1β functions as universal corepressor for the KRAB domain. The repression activity of TIF1α and TIF1β appears mediated by several mechanisms including histone deacetylation and local induction of heterochromatin-like structure (Agata *et al*., 1999; Lechner *et al*., 2000; Nielsen *et al*., 1999; Peng *et al*, 2000; Ryan *et al.*, 1999).

In this work the cloning and characterisation of TIF1γ, the third member of the TIF1 family, is reported.

The human TIF1γ was cloned by screening of a cDNA libray from human liver with the murine T18 probe under low stringency conditions. The predicted TIF1γ protein sequence revealed the characteristic domain structure of TIF1α and TIF1β, with a very high degree of identity, especially between the evolutionarily conserved domains. In addition, a 25 amino acid sequence highly conserved between the three members of the family and virtually identical in TIF1α and TIF1γ, was identified downstream of the coiled coil motif. As this new detected domain was restricted to the TIF1 proteins, it was designated TSS for TIF1 signature sequence.

The TIF1γ gene was mapped to the 1p13 locus which is involved in a non-random t(1;22)(p13;q13) chromosomal translocation associated with acute megakaryocytic leukemias (Mitelman, 1993). This leads to speculate about a possible implication of TIF1γ in rearrangements of acute leukemias.

Northern blot analysis demonstrated a complex expression pattern with at least five diverse transcripts ranging from 2.5 to 8.8 kb. As these isoforms exhybit a heterogeneous expression level and vary considerably in size in the different tissues, they might contain additional domains and confer a tissue-specific functionality to the TIF1γ protein. Two alternatively spliced isoforms of TIF1γ, harbouring bromodomains which differ in the length of their BC loop, were identified. A differentially regulated expression of these two isoforms, which likely have diverse affinities for acetyl-lysines, may influence the functionality of the TIF1γ protein.

The complexity of this expression pattern is augmented by the existence of a C-to-U editing event of TIF1γ mRNA which produces a truncated protein lacking the PHD and the bromodomain. Poisonned-primer extension experiments performed on RT-PCR products demonstrated that the expression of the edited TIF1γ form was almost undetectable in several normal human tissues, while it was considerably upregulated in primary blasts of about 30% of the examined patients suffering from acute myelogenous leukemia. These results suggest a possible implication of TIF1γ hyperediting in the process of leukemogenesis.

Transient cotransfection experiments demonstrated that TIF1γ, similarly to TIF1α and TIF1β, is a potent transcriptional repressor when tethered to the promoter region of a reporter gene. Trichostatin A (TSA), a specific inhibitor of HDACs, can partially relieve TIF1γ-mediated silencing activity suggesting that histone deacetylation is, at least in part, implicated in this process but other mechanisms are also required. Yeast two hybrid assays showed that TIF1γ does not interact with the HP1 proteins. Thus, contrarily to that occur for TIF1α and TIF1β, they cannot act as downstream effectors of the TIF1γ repression functon. Deletion of the PHD/bromodomain region or the TSS motif of TIF1γ also reduced its ability to repress transcription. The PHD/bromodomain and the TSS probably constitute interaction surfaces for TIF1γ-interacting co-repressor factors which remain to be identified. Deletion of both TSS and PHD/bromodomain demonstrates an additive effect in impairing the TIF1γ silencing activity which, in this case, was completely relieved by TSA treatment. These results suggest that TIF1γ may repress transcription through three different mechanisms: a TSS-, a PHD/bromodomain-mediated, and a histone deacetylase-dependent mechanism.

In conclusion, the three TIF1 proteins, although structurally related, have different interaction partners and appear functionally distinct.

6. ZUSAMMENFASSUNG

Transkriptionelle Regulation der Genexpression in Eukaryonten ist ein komplexer, auf mehreren Ebenen genau gesteuerter Prozeß, welcher die konzertierte Aktion mehrerer Faktoren erfordert, um verschiedene Signale für die Kontrolle zellulärer Vorgänge wie Proliferation, Differenzierung und Apoptose zu integrieren.

In den letzten Jahren wurden reversible Strukturänderungen des Chromatins als wichtige Mechanismen für die Kontrolle der Genexpression erkannt. Enzyme, welche die Chromatinstruktur modifizieren, wie Histon-Acetyltransferasen (HATs) und -Deacetylasen (HDACs) sowie Nucleosomen-remodellierende Komplexe mit ATPase- und Helicase-Aktivität, können durch direkte Interaktion mit DNA-bindenden Proteinen, wie z.B. sequenzspezifischen Transkriptionsfaktoren, an Promotoren rekrutiert werden. Durch die Interaktion mit solchen Ko-Aktivator- bzw. Ko-Repressor-Komplexen, die eine offene und zugängliche bzw. eine kompakte und repressive Chromatinkonfiguration induzieren, kann der Aufbau der basalen Transkriptionsmaschinerie erleichtert bzw. inhibiert und so die Expression von Zielgenen aktiviert bzw. reprimiert werden.

Als transkriptionelle Ko-Regulatoren auf Chromatin-Ebene sollen beispielsweise die "Transkriptionellen Intermediär-Faktoren 1" wirken, deren Familie gegenwärtig drei Kernproteine (TIF1α, β und γ) umfaßt.

Aufgrund von Aminosäure-Homologien läßt sich Mitgliedern dieser Familie ein TIF1-typischer Domänen-Aufbau zuschreiben. Die N-terminale Region enthält das dreiteilige RBCC-Motiv (RING-Finger - B-Box - "Coiled Coil"), das höchstwahrscheinlich als kooperatives Protein-Protein-Interaktionsmotiv fungiert (Borden, 1998). RING-Finger (benannt nach dem "Really Interesting New Gene 1") sind Cystein-Histidin-Domänen der Form C3HC4, identifiziert in vielen Proteinen unterschiedlicher Funktion, in verschiedenen Organismen von Viren bis zu Vertebraten, die vermutlich zur Spezifität bzw. Multimerisations-Fähigkeit des RBCC-Motivs beitragen, das große makromolekulare Komplexe bilden kann (Freemont, 1993; Borden *et al*., 1995a; Saurin *et al*., 1996; Borden, 2000). Auch die B-Box ist eine Zink-bindende Cystein-Histidin-Domäne der Form CHC3H2 (Reddy & Etkin, 1991) und wahrscheinlich ebenfalls an Protein-Protein-Interaktionen beteiligt (Alcalay *et al*., 1998; Cao *et al*., 1998). Das dritte Element des RBCC-Motivs ist die "Coiled-Coil"-Domäne, deren α-Helices eine hydrobhobe Interaktions-Oberfläche für die Protein-Multimerisierung darstellen (Cao *et al*., 1997; 1998). Die C-terminale Region der TIF1-Proteine enthält eine Polycomb-Homologie-Domäne (PHD) (Aasland *et al*., 1995; Koken *et al*., 1995) und eine Bromodomäne (Jeanmougin *et al*., 1997). Die PHD ist eine Cystidin-Histidin-Zinkfinger-Domäne der Form C4HC3 und vermutlich ebenso wie die RING-Finger ein Protein-Protein-

Interaktionsmotiv. Die Bromodomäne, ein ca. 110 Aminosäuren umfassendes, hoch konserviertes Modul aus vier amphipathischen α-Helices, interagiert mit acetylierten Lysinresten von Histon H3-/H4-Peptiden, was eine Rolle in der Chromatin-Erkennung nahelegt (Dhalluin *et al*., 1999; Owen *et al*., 2000). Sowohl PHD als auch Bromodomäne sind charakteristische Motive von Proteinen, die als transkriptionelle Ko-Regulatoren auf Chromatinebene fungieren.

Erstmals beschrieben wurde der N-terminale Anteil von TIF1α als "T18", der Fusionspartner von bRaf in einem chemisch induzierten murinen Leberzellkarzinom (Miki *et al*., 1988). TIF1α der Maus wurde später per Doppel-Hybrid-Screening entdeckt aufgrund seiner Liganden-abhängigen Interaktion mit den Liganden-Bindungsstellen mehrerer Kernrezeptoren, darunter Retinoid- (RXR), Retinsäure- (RAR), Vitamin D3- (VDR), Östrogen- (ER) und Progesteron-Rezeptoren (PR). TIF1α soll deren Trans-aktivierungsfunktion AF-2 vermitteln (Le Douarin *et al*., 1995; vom Baur *et al*., 1996).

TIF1β, auch als KAP-1 (KRAB-assoziiertes Protein 1) oder KRIP-1 (KRAB-interagierendes Protein 1) bezeichnet, wurde kurz nach TIF1α isoliert aufgrund seiner Interaktion mit den Heterochromatin-Proteinen HP1 (LeDouarin *et al*., 1996) und der transkriptionellen Repressionsdomäne KRAB (krüppel-assoziierte Box; Friedman *et al*., 1996; Kim *et al*., 1996; Moosmann *et al*., 1996) am N-Terminus mehrerer DNA-bindender Zinkfingerproteine (ZFP) (Bellefroid *et al*., 1991).

TIF1α und TIF1β sind Kern-Phosphoproteine mit intrinsischer Kinaseaktivität (Fraser *et al*., 1998; Nielsen *et al*., 1999); in Verbindung mit dem Promotor eines Reportergens wirken sie als starke transkriptionelle Repressoren. TIF1β fungiert als universaler Ko-Repressor für die KRAB-Domäne. Die Repression durch TIF1α und TIF1β scheint über mehrere Mechanismen vermittelt zu werden, z.B. Histon-Deacetylierung und lokale Induktion einer Heterochromatin-ähnlichen Struktur (Agata *et al*., 1999; Lechner *et al*., 2000, Nielsen *et al*., 1999; Peng *et al*., 2000; Ryan *et al*., 1999).

In der vorliegenden Arbeit wird die Klonierung und Charakterisierung von TIF1γ, dem dritten Mitglied der TIF1-Familie beschrieben.

Die Klonierung des humanen TIF1γ erfolgte durch Screening einer cDNA-Bank aus menschlichen Leberzellen mit einer murinen T18-Sonde unter Bedingungen niedriger Stringenz. Die abgeleitete Proteinsequenz von TIF1γ zeigte die charakteristische TIF1-Domänen-Struktur mit sehr hoher Identität besonders im Bereich der evolutionär konservierten Domänen. Zusätzlich konnte eine Sequenz von 25 Aminosäuren im variablen Teil, C-terminal der "Coiled Coil", identifiziert werden, die unter den drei TIF1-Proteinen hoch konserviert und zwischen TIF1α und TIF1γ nahezu identisch war. Da diese neuentdeckte Domäne sich für TIF1-Proteine spezifisch erwies, wurde sie als TSS ("TIF1 Signal-Sequenz") bezeichnet.

Die chromosomale Lokalisation des TIF1γ-Gens wurde auf dem Locus 1p13 ermittelt, für den eine nicht-zufällige Chromosomentranslokation t(1;22)(p13;q13) bei akuten megakaryozytären Leukämien beschrieben worden ist (Mitelman 1993); dies gibt Anlaß zu Spekulationen über eine mögliche Beteiligung von TIF1γ an Rearrangements in akuten Leukämien.

Northern Blot-Untersuchungen zeigten ein komplexes Expressionsmuster mit mindestens fünf verschiedenen Transkripten in der Größe von 2,5 bis 8,8 kb, die in verschiedenen Geweben differentiell exprimiert werden. Da diese Isoformen beträchtliche Größenunterschiede aufweisen, ist es denkbar, daß sie weitere Domänen enthalten, was ihnen in unterschiedlichen Geweben unterschiedliche Funktionen verleihen könnte. Ferner wurden zwei alternativ gespleißte Isoformen von TIF1γ mit unterschiedlich langer BC-Schleife der Bromodomäne identifiziert. Da diese vermutlich unterschiedliche Affinitäten gegenüber acetylierten Lysinresten aufweisen, könnte eine differentiell regulierte Expression ihre Funktion beeinflussen.

Die Vielfalt dieses Expressionsmusters wird noch erhöht durch den Nachweis eines C-nach-U-Editing-Ereignisses der TIF1γ-RNA, das ein trunkiertes Protein ohne PHD und Bromodomäne erzeugt. Poisonned-primer extension-Experimente zeigten, daß die Expression des editierten TIF1γ in fast allen normalen menschlichen Geweben unter der Nachweisgrenze lag, aber in der Blastenpopulation bei ca. 30% der untersuchten Patienten mit akuten myeloischen Leukämien deutlich hochreguliert war. Diese Ergebnisse legen einen möglichen Beitrag von TIF1γ-Hyperediting zur Leukämogenese nahe.

In transienten Kotransfektionen erwies sich TIF1γ in Verbindung mit dem Promotor eines Reportergens als starker transkriptioneller Repressor, ähnlich wie TIF1α und TIF1β. Der spezifische HDAC-Inhibitor Trichostatin A (TSA) konnte die TIF1γ-vermittelte Repression partiell wieder aufheben; Histon-Deacetylation ist also wohl zumindest teilweise am Mechanismus der Repression beteiligt. Nach Doppel-Hybrid-Untersuchngen in Hefezellen interagiert TIF1γ nicht mit den Heterochromatin-Proteinen HP1; diese können also, anders als bei TIF1α und TIF1β, nicht als Effektoren der TIF1γ-abhängigen Repression fungieren. Auch die Deletion der PHD/Bromodomäne oder des TSS-Motivs reduzierte die transkriptionelle Repression durch TIF1γ. PHD/Bromodomäne wie auch TSS stellen wahrscheinlich Interaktionsoberflächen noch zu identifizierender Ko-Repressoren von TIF1γ dar. Die Deletion sowohl der PHD/Bromodomäne als auch der TSS inhibierte die TIF1γ-Repression additiv, ein durch TSA vollständig umkehrbarer Effekt. Diese Ergebnisse legen drei unterschiedliche Mechanismen der TIF1γ-abhängigen transkriptionellen Repression nahe, jeweils vermittelt durch TSS, PHD/Bromodomäne sowie Histon-Deacetylasen.

Zusammengefaßt haben alle drei TIF1-Proteine, trotz struktureller Ähnlichkeiten, verschiedene Interaktionspartner und erscheinen funktionell eigenständig.

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8. ABBREVIATIONS

A	Adenine
ADAR	Adenosine deaminase(s) acting on RNA
AF-1	Activation function 1
AF-2	Activation function 2
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine 5'-triphosphatase
ATRA	*All-trans* retinoic acid
bp	Base pair(s)
BSA	Bovine serum albumine
C	Cytosine
CDAR	Cytidine deaminase(s) acting on RNA
cDNA:	Complementary DNA
cpm:	Counts per minute
C-terminal	Carboxy-terminal
CTD	Carboxy-terminal domain
dATP	Deoxy-adenosine 5'-triphosphate
dCTP	Deoxy-cytidine 5'-triphosphate
ddATP	Dideoxy-adenosine 5'-triphosphate
ddCTP	Dideoxy-cytidine 5'-triphosphate
ddGTP	Dideoxy-guanosine 5'-triphosphate
ddTTP	Dideoxy-thymidine 5'-triphosphate
dGTP	Deoxy-guanosine 5'-triphosphate
dTTP	Deoxy-thymidine 5'-triphosphate
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
Dnase	Desoxyribonuclease
DNTPs	Deoxy-nucleotide 5'-triphosphate
DTT	Dithiotreitol
EDTA	Ethylene Diamine Tetraacetic Acid
ER	Estrogen receptor
FCS	Fetal calf serum
G	Guanine
g	Gravitational force
GTF	General transcription factors
HAT	Histone acetyltransferase
HBS	Hank's buffered saline
HDAC	Histone deacetylase
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
hr	Hour
LB medium	Luria-Bertani medium
LBD	Ligand-binding domain
M	Molarity

min	Minute
mRNA	Messenger RNA
ONPG	O-Nitrophenyl-β-D-galactopyranoside
NR	Nuclear receptor(s)
N-terminal	Amino-terminal
OMPdecase	Orotidine 5'-monophosphate decarboxylase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEV	Position effect variegation
pfu	Plaque-forming unit(s)
PIC	Preinitiation complex
PIPES	Piperazine-*N*, *N'*-bis(2-ethanesulfonic acid)
PR	Progesteron receptor
PVP	Polyvinylpyrrolidone
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
RT-PCR	Reverse-transcription polymerase chain reaction
RXR	Retinoid receptor
SDS	Sodium lauryl sulfate
T	Thymine
TAE	Tris-acetate/EDTA electrophoresis buffer
TAF	TATA-binding protein (TBP) associated factor
TBE	Tris-borate/EDTA electrophoresis buffer
TBP	TATA binding protein
TE	Tris/EDTA
TEMED	*N*, *N*, *N'*, *N'*-tetramethylethylenediamine
TR	Thyroid hormone receptor
tRNA	Transfer RNA
TSA	Trichostatin A
U	Uracil
UV	Ultraviolett
VDR	Vitamin D receptor
ZFP	Zink finger proteins

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