

# Transcriptional repression by RING finger protein TIF1 $\beta$ that interacts with the KRAB repressor domain of KOX1

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## ABSTRACT

Many of the vertebrate zinc finger factors of the Krüppel type (C2H2 zinc fingers) contain in their N-terminus a conserved sequence referred to as the KRAB (Krüppel-associated box) domain that, when tethered to DNA, efficiently represses transcription. Using the yeast two-hybrid system, we have isolated an 835 amino acid RING finger (C3HC4 zinc finger) protein, TIF1 $\beta$  (also named KAP-1), that specifically interacts with the KRAB domain of the human zinc finger factor KOX1/ZNF10. TIF1 $\beta$ , TIF1 $\alpha$ , PML and efp belong to a characteristic subgroup of RING finger proteins that contain one or two other Cys/His-rich clusters (B boxes) and a putative coiled-coil in addition to the classical C3HC4 RING finger motif (RBCC configuration). Like TIF1 $\alpha$ , TIF1 $\beta$  also contains an additional Cys/His cluster (PHD finger) and a bromo-related domain. When tethered to DNA, TIF1 $\beta$  can repress transcription in transiently transfected mammalian cells both from promoter-proximal and remote (enhancer) positions, similarly to the KRAB domain itself. We propose that TIF1 $\beta$  is a mediator of the transcriptional repression exerted by the KRAB domain.

## INTRODUCTION

In eukaryotes, a great number of studies have analysed transcriptional activation, while much less is known about gene repression and silencing. Repressors seem to act, in principle, in three different ways: (i) by steric hindrance, such as in the simplest case of bacterial-type repression; (ii) by inducing the reorganisation of chromatin into an inactive form, e.g. by recruiting enzymes that change the acetylation state of histones (1–3) and/or proteins that are otherwise involved in the establishment and maintenance of inaccessible, genetically inert chromatin (reviewed in 4); or (iii) by interfering, through specific protein–protein contacts, with the assembly of a functional transcription initiation complex (so-called active repression; for review, see 5).

In one of the best understood examples of negative gene regulation in eukaryotes, the repression of mating type  $\alpha$ -specific genes in yeast  $\alpha$  cells, MCM1 binds together with the  $\alpha$ 2 protein specifically to DNA and represses target genes (6,7). At least two additional proteins, SSN6 and TUP1, are required as general co-repressors (8,9). The main function of the  $\alpha$ 2/MCM1 complex is to recruit mediator molecules to the DNA that in turn interact with the transcription machinery or activators and thereby repress transcription (10). Other suggested repressor–corepressor systems include the nuclear receptor superfamily (with SMRT/N-CoR) (11,12) or MAD/MAX (with homologs of yeast SIN3) (13,14). Another mechanism is responsible for *Drosophila* homeotic gene expression control. While the trithorax-group (trx-G) gene products generally activate expression, the Polycomb-group (Pc-G) proteins are required for maintenance of the repressed state, perhaps by packaging the target genes into condensed heterochromatin (15–19). Interestingly, also in the case of mating-type specific repression in yeast, remodelling of chromatin may contribute to gene repression (20).

A conserved sequence is found at the N-terminus of many human zinc finger proteins of the Krüppel type (C2H2 zinc fingers), referred to as Krüppel-associated box (KRAB) domain (21–25). The KRAB domain consists of ~75 amino acids, that can be further subdivided into an A box and a B box. The A box, but not the B box, is present in every KRAB domain and essential for transcriptional repression (21,24,26,27). Many KRAB domains have been identified in vertebrates (24,27–29), but do not occur in the yeast *Saccharomyces cerevisiae* and apparently also not in *Drosophila melanogaster*, suggesting its late appearance in evolution. Since a sequence-specific DNA-binding function has not been shown for any of the KRAB domain-containing putative transcription factors so far, the respective target promoters remain unknown. However, the differential expression of several KRAB domain-containing zinc finger factors, as it is found in T cell and myeloid differentiation (22,30), could suggest an important role in developmental processes.

In order to investigate the mechanism of KRAB-mediated transcriptional repression, we used the yeast two-hybrid system and screened for proteins that interact with the KRAB domain of the zinc finger factor KOX1/ZNF10. This factor contains 11

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C-terminal C2H2 zinc fingers and its KRAB domain consists of both an A and a B box (21). Here we report the characterization of an interacting factor, TIF1 $\beta$ , that contains several sequence features found in other nuclear regulatory or chromatin-associated proteins. TIF1 $\beta$  by itself, fused to a heterologous DNA binding domain, efficiently represses transcription and may contribute to the KRAB domain effect. After the isolation and characterisation of TIF1 $\beta$  we learned that the group of F. Rauscher has cloned the same factor as KRAB-associated protein (KAP-1) by biochemical means (31).

## MATERIALS AND METHODS

Recombinant DNA work was done according to standard protocols. Details concerning construction of plasmids, which were verified by sequencing, are available upon request.

### Plasmid construction

Appropriate fragments of the mammalian expression vectors pG-KRAB (23), pG-ZNF43, pG-KOX1-MLE and pG-KOX1-PP (kind gift of H. J. Thiesen) were cloned into pRS314 (32) and tested in bandshift assays for protein expression in yeast strain Y153 (33).

TIF1 $\beta$  full length clone TIF1 $\beta$ (1–835) was isolated by PCR amplification of the start region and combined with the 3' sequence of a two-hybrid isolate by standard cloning procedures. Deletion mutants shown originate from this parental clone and were made using appropriate restriction sites.

For expression in mammalian cells, the TIF1 $\beta$  clones were transferred to pCATCH vector as described (34). GAL fusions were expressed from the vectors described in (35).

TIF1 $\alpha$  constructs were obtained by PCR and cloned into the yeast expression vectors pASV3 or pASVT3 (36,37).

Lex DBD and GAL DBD fusions of VP16 are as described (38).

### cDNA library screen and transactivation assays

A peripheral blood leukocyte cDNA library was expressed from pACT vector (33) (gift of S. Elledge) and introduced by lithium acetate treatment (39) into yeast strain Y153 containing the bait construct, pRSKRAB. Transformants ( $\sim 2 \times 10^6$ ) were plated on adenine-supplemented minimal medium plates in the presence of 25 mM 3-aminotriazole. After 5 days, 26 clones that showed growth and blue staining in an X-gal filter assay were isolated. DNA was isolated and re-transformed in Y153+pRSKRAB or empty vector to control for specific interaction with the bait.

To isolate the 5'-end of TIF1 $\beta$ , a  $\lambda$ gt10 cDNA library from BJA-B cells (kind gift of M. Busslinger) was screened. The 5' end region of the longest clone was used to re-screen the library once again.

Yeast transformants were grown to a density of  $1 \times 10^6 - 1 \times 10^7$  cells/ml in minimal medium supplemented with adenine and containing 25 mM 3-aminotriazole.  $\beta$ -galactosidase activity was assessed in at least three independent experiments by a permeabilised cell assay (40). Units are expressed as  $[10^4 \times \text{OD}_{420}] / [\text{OD}_{600} \times \text{volume assayed culture (ml)} \times \text{time (min)}]$ .

### Transfection and RNA analysis

HeLa cells were grown under standard conditions and transfected by the calcium phosphate co-precipitation method (35) with 5  $\mu$ g

of reporter plasmid, 3  $\mu$ g of transactivator plasmid (or 5  $\mu$ g in the case of GAL-TIF1 $\beta$  constructs that have an  $\sim 1.5$ -fold higher molecular weight compared with GAL-KRAB or GAL-VP constructs) and 1.5  $\mu$ g reference plasmid. The total amount of DNA transfected was adjusted with empty vector plasmid to 20  $\mu$ g per 10 cm dish. In all transfections OVEC-REF was used for reference (35). After 36 h incubation, RNA was isolated according to (41) and hybridised to a radiolabelled oligonucleotide (35). Hybridisation was performed overnight at 30°C. Hybridisation products were digested with 150 U S1 nuclease for 1 h and separated on a 10% denaturing polyacrylamide gel. For quantification, dried gels were exposed to a phosphor storage screen or autoradiographs were analysed densitometrically (Molecular Dynamics, Inc.). The signals derived from the reference transcripts were used to normalise for variability in the transfection efficiency.

### FISH mapping

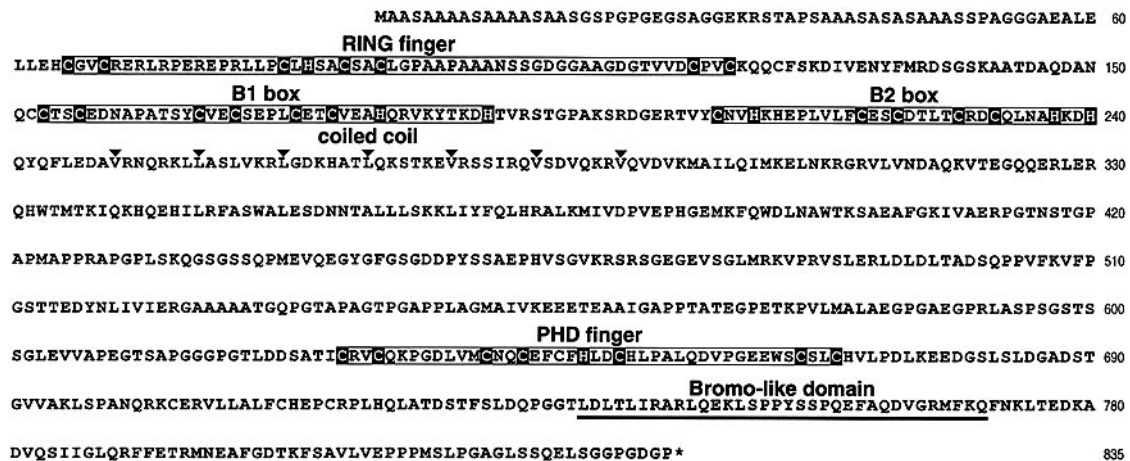
Lymphocytes isolated from human blood were cultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum and phytohemagglutinin (PHA) at 37°C for 68–72 h. The lymphocyte cultures were treated with BrdU (0.18 mg/ml, Sigma) to synchronise the cell population. Synchronised cells were washed three times with serum-free medium and recultured at 37°C for 6 h in MEM with thymidine (2.5  $\mu$ g/ml, Sigma). Cells were harvested and slides were prepared by standard procedures, including hypotonic treatment, fixation and air-drying.

The cDNA probe was biotinylated with dATP using the BRL BioNick labelling kit (15°C, 1 h) (42). FISH detection was done according to (43,44); slides were baked at 55°C for 1 h, RNase treated and denatured in 70% formamide, 2 $\times$  SSC for 2 min at 70°C, and dehydrated with ethanol. Denatured chromosomes were incubated with the probe overnight. FISH signals and DAPI banding pattern was recorded separately and assignment was done by superimposing both signal and DAPI banded chromosomes (44).

## RESULTS

### Isolation of a cDNA clone whose product interacts with the KRAB domain of KOX1

Whereas the KRAB domain of KOX1 showed transcriptional repression in all mammalian cell lines tested, no effect was detectable in either yeast or *Drosophila* Schneider cells (P. Moosmann *et al.*, manuscript in preparation). This suggests that the proteins that mediate repression by the KRAB domain in mammals are either absent or have diverged so much that they do not recognise the mammalian KRAB domain. Therefore, the yeast two-hybrid system (45) was an appropriate tool to search for potential KRAB-interacting proteins. As a bait, we fused the KRAB domain of KOX1 and some flanking amino acids, residues 24–145 to the DNA binding domain (DBD) of GAL4 (amino acids 1–147) (23) and expressed it in yeast strain Y153 (33). Fusion protein expression was verified by bandshift assays (not shown). The human peripheral blood lymphocyte-derived cDNA library (a gift of S. Elledge) was fused to the GAL4 activation domain (46). Twenty-six clones were selected for His prototrophy and LacZ staining. Back-crosses and tests with GAL4 DBD as a control bait verified the specificity of this interaction. Restriction analysis and sequencing revealed that 23 independent clones belonged to the same type of unknown cDNA



**Figure 1.** Predicted protein sequence of TIF1 $\beta$ . The Cys/His-rich protein motifs are boxed and the conserved cysteine and histidine residues that may be involved in zinc complexing are underlined in black. Amino acids of the putative coiled-coil that are recognised by the Lupas algorithm to be at position A in the heptad repeats are marked by arrowheads. As threshold a minimal probability of 10% was set in a medium restrictive window of 21 amino acids. The core region of the bromo-like domain is underlined. Note the preponderance of alanine residues within the first 50 amino acids and of alanines and prolines in the region between amino acid 526 and the first cysteine of the PHD finger at amino acid 628. The complete nucleotide sequence has been deposited with the EMBL Data Library under the accession number X97548.

isolate. The longest insert, ~2.9 kb in length, was sequenced. Screening of a  $\lambda$ gt10 cDNA library originating from the BJA-B cell line allowed sequence extension further upstream. The cDNA contains an open reading frame for an 835 amino acid protein. Three in-frame stop codons precede the start AUG, the ORF is followed by a 166 bp 3' untranslated region that contains a putative polyadenylation signal at 23 nucleotides upstream of the poly(A) tail (see database entry).

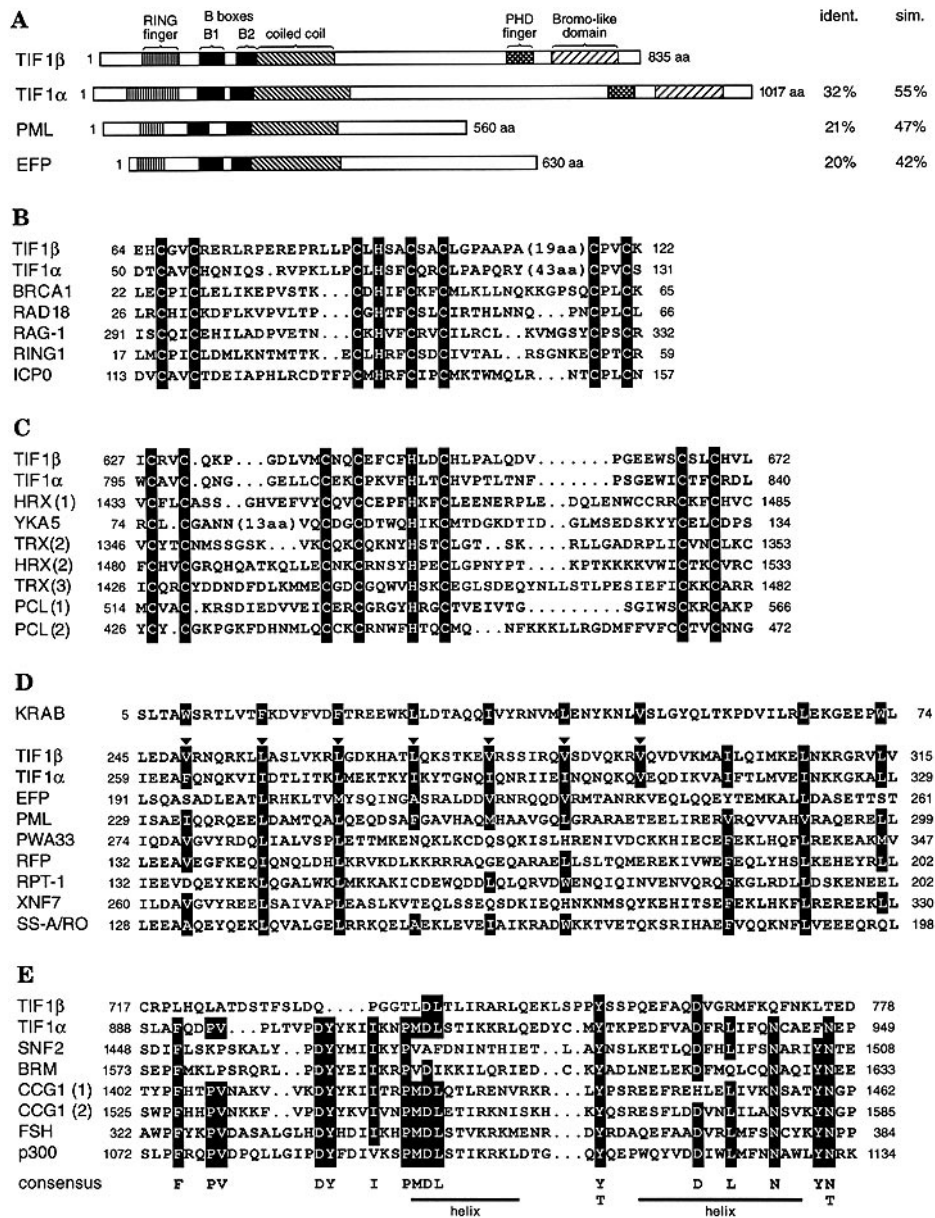
The predicted polypeptide shares its overall organisation with the mouse nuclear factor TIF1 (transcriptional intermediary factor 1) to which it is 31% identical. TIF1 was recently cloned as a putative coactivator of several nuclear receptors (37). By a different approach, the same group has also isolated a mouse factor 95% identical to ours, TIF1 $\beta$  (47). We therefore decided to adopt the pre-existing nomenclature and named our KRAB-interacting clone TIF1 $\beta$  as the human homolog. TIF1 will be henceforth referred to as TIF1 $\alpha$ , while TIF1 stands for both members of this novel family of proteins.

Northern blot analysis showed a predominant signal of ~3.3 kb in all tissues tested (i.e., spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes). *In vitro* translation and expression in cell culture yielded a polypeptide that runs at an apparent molecular weight of close to 100 kDa, whereas the predicted molecular weight is 88.5 kDa. The epitope-tagged protein was exclusively located in the nucleus of transiently transfected mammalian cells. Notably, the fine granular pattern observed with TIF1 $\beta$  is different from the association with characteristic speckles observed with PML, another member of the RING finger family with the RBCC conformation (48) (data not shown).

In the original screen TIF1 $\beta$  represented 23 of 26 clones. One of the three remaining clones is so far unknown and has similarities to a TAF (TBP-associated factor) of RNA polymerase II, while the other two do not seem to interact specifically with the KRAB domain (our unpublished results).

### Sequence motifs in TIF1 $\beta$

TIF1 $\beta$  contains several features also seen in other proteins with an established or putative regulatory function (Fig. 1). Most prominent are the four Cys/His-rich clusters that form a so-called RING finger (Fig. 2B), the two B boxes and the PHD finger (Fig. 2C). RING fingers (or C3HC4 zinc fingers) are characteristic motifs found in >50 proteins otherwise lacking homology and of apparently diverse functions (49–53). Recent studies report a function of RING fingers in RAG1 mediated recombination (54), as well as selective RNA binding for MDM2 oncoprotein (55). The classical C3HC4 zinc finger of TIF1 $\beta$  (56) is followed by two so-called B boxes (1 and 2) of unknown function and a putative coiled-coil. The arrowheads in Figures 1 and 2D indicate the amino acids predicted to be at position A (57) of the coiled-coil. With a reduced fit to the consensus, this domain could be extended towards the C-terminus. A similar coiled-coil may exist in the KRAB domain, but the respective residues are not recognised by the algorithm and the originally described leucine zipper-like structure differs from the spacing as it is represented in Figure 2D (21). The combination of a RING finger with one or two B boxes (1 and 2) and a coiled-coil (RBCC) is characteristic for a family of related proteins. Besides in TIF1 $\alpha/\beta$ , the RBCC configuration is also found in the nuclear factors PML (48,58), efp (59), RPT-1 (60), RFP (61,62), SS-A/RO (63,64), PwA33 (65) and XNF7 (66). The factors that contain two B boxes, PML, efp, TIF1 $\alpha$  and  $\beta$ , are schematically depicted in Figure 2A. The C-terminal Cys/His-rich cluster found in TIF1 $\beta$ , the so-called PHD finger, has first been described in plant homeobox domain proteins (67,68) and is shown in Figure 2C. TIF1 $\beta$  also exhibits a rather weak similarity to previously established bromodomains (69) present in several nuclear factors, such as brahma and SWI2/SNF2 (70). However, since the domain in TIF1 $\beta$  differs clearly from the consensus sequence (Fig. 2E), we refer to it as a bromo-like domain.

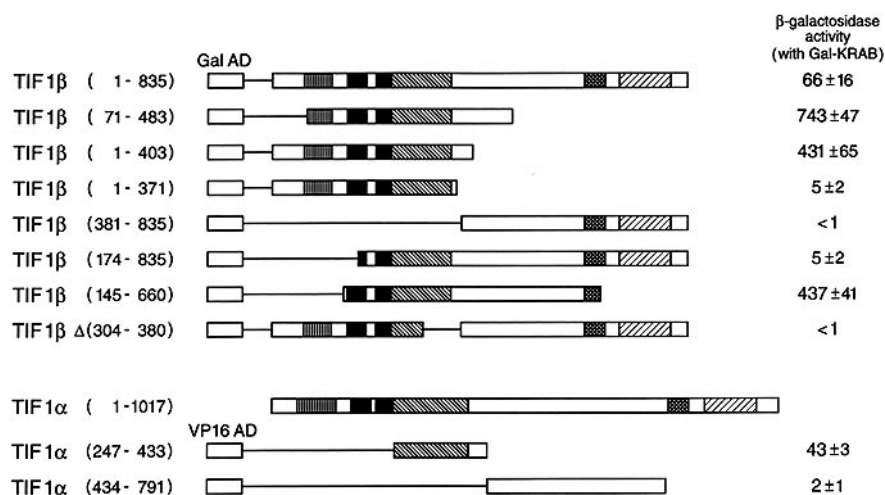


**Figure 2.** Structural motifs of TIF1β in comparison to other proteins. (A) Schematic representation of the relationship among the different members of the RING finger subfamily that contain a so-called RBCC configuration with two B boxes (i.e., RING finger, two B boxes and a coiled-coil). On the right-hand side, the overall identities and similarities of amino acids compared with TIF1β are indicated. Within the conserved sequence motifs these values are higher. (B) The RING finger of TIF1β and related proteins of the superfamily. Outside the conserved Cys and His residues there is low sequence conservation. (C) Comparison of different PHD fingers. If in a single protein more than one domain is present, the number of the respective motifs is indicated in brackets [e.g. HRX(1) is the first motif from the N-terminal end of this type in the HRX protein]. (D) Alignment of the coiled-coils of all members of the RBCC subfamily (see text) known so far. Regularly spaced hydrophobic amino acids are highlighted with black. The arrowheads correspond to those in Figure 1. A putative spacing of hydrophobic amino acids in the KRAB domain is depicted as well, even though no such coiled-coil is recognised by the computer algorithm applied and does not correspond to the spacing originally proposed (21). (E) Alignment of the bromo-like domain of TIF1β with previously established bromodomains. Conserved residues corresponding to the consensus are underlined with black. Database accession numbers are: BRCA1, U36475; BRM, M85049; efp, D21205; FSH, M23221, M23222; HRX, Q03164; ICP0, D10471; p300, U01877; PCL, L35153; PML, X63131; PwA33, L04190; RAD18, X12588; RAG-1, M29474; RING1, Z14000; RFP, J03407; RPT-1, J03776; SNF2, M55906; SS-A/Ro, U01882; TAFII250/CCG1, D90359; TIF1α, S78221; TRX, P20659; XNF7, M63705; YKA5, P36106.

In addition, the first ~50 amino acids of TIF1β are remarkably rich in alanine, and a preponderance of alanine and proline residues is found in the region between amino acid 526 and the first cysteine of the PHD finger at amino acid 628. Such an alanine/proline-rich domain has been described as an effector domain in the *Drosophila* repressor factor *even-skipped* (71).

**Delineation of the TIF1β region interacting with the KRAB domain**

In order to narrow down the domain of TIF1β that interacts with the KRAB domain, a number of N- and C-terminal deletions were constructed (Fig. 3) and tested for interaction in yeast by



**Figure 3.** Interaction of TIF1β with the KRAB repression domain in yeast. The activation domain of GAL4 was fused to TIF1β, and that of VP16 to TIF1α. Numbers in brackets correspond to present or, in the case of TIF1βΔ(304–380), to deleted amino acids. The schematic representation of the constructs is the same as in Figure 2A. On the right-hand side, β-galactosidase activity as measured in a quantitative liquid assay is shown (±SD) (for details see Materials and Methods).

qualitative and quantitative β-galactosidase assays. Expression levels as controlled by Western blot were found to be similar for all constructs (not shown). Deletion mutants TIF1β(1–403) and TIF1β(145–660) led to high β-galactosidase activity, whereas deletions beyond these boundaries, amino acids 145–403, resulted in a rapid loss of interaction. Thus, the interaction domain seems to be located within a segment of 258 amino acids that contains both B boxes and the coiled-coil region, but it clearly does not include the *bona fide* RING finger. Furthermore, the mutant TIF1βΔ(304–380) that deleted the major part of the coiled-coil was no longer able to interact. In mouse TIF1α, the residues 247–433 that comprise the coiled-coil (Fig. 3) also interact with the KRAB domain. Therefore, we think that for the KRAB–TIF1 interaction the coiled-coil is essential, though not sufficient. However, the levels of β-galactosidase activity induced by the TIF1β and TIF1α constructs are difficult to compare, since TIF1β was fused to the activation domain of GAL4 and TIF1α to the VP16 activation domain.

The interaction of the full length clone of TIF1β with the KRAB domain induced a 10-fold lower β-galactosidase activity compared with the activity induced by the strongest TIF1β deletion mutant (Fig. 3). This may mean that there is a C-terminal domain that negatively regulates TIF1β–KRAB interaction. In agreement with this, preliminary testing of the full length TIF1α showed approximately five times lower activity as compared with the truncated TIF1α(247–433).

A mutant KRAB domain (KRAB, MLE mutated to KKK) that does not repress transcription in mammalian cells was no longer able to interact with TIF1β (23). No homophilic TIF1α–TIF1α or TIF1β–TIF1β interactions were found. Furthermore, the KRAB domain did not interact with mHP1α or mMOD1, two heterochromatin-associated proteins that were shown to interact with TIF1α (47).

### Repression by TIF1β tethered to DNA

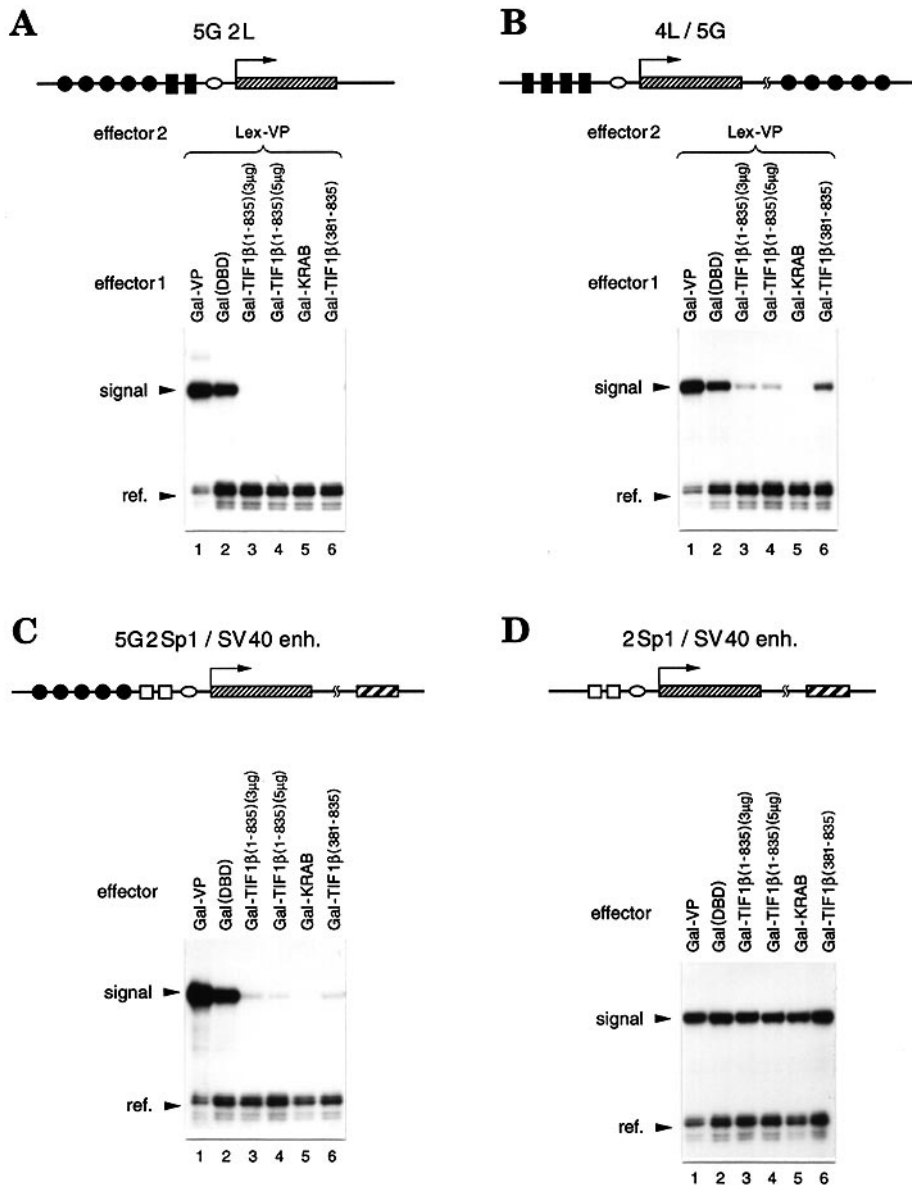
To test whether TIF1β could repress transcription by itself, different deletion mutants were fused to the GAL4 DBD(1–93) (Fig. 5) and transiently expressed in mammalian cells (HeLa and

293T) cotransfected with an activator plasmid. Expression was controlled by Western blot and bandshift assays (not shown). The reporters that were used contain GAL4 upstream activating sequences at different positions and showed transcription from the β-globin promoter, in this case only upon activation by a fusion of VP16 activation domain to Lex DBD (amino acids 2–202). TIF1β does indeed repress transcription when bound to DNA. From a promoter-proximal position, repression by GAL–TIF1β was about half as strong as the one achieved with GAL–KRAB. Nevertheless, a >10-fold reduction in transcript level was detected when equimolar amounts of activator and repressor plasmids were transfected (Fig. 4). Even under influence of the (remote) SV40 enhancer, a clear repression was seen (Fig. 4C). From a remote position, 1.5 kb downstream of the β-globin promoter, repression was ~6-fold (Fig. 4B).

To find out whether the repression by TIF1β was dependent on DNA binding, GAL–TIF1β(1–835) was tested either with a reporter without GAL4 UAS, or with an epitope-tagged TIF1β without DBD on reporters containing GAL4 UAS. No transcription repression was observed under these conditions (Fig. 4D and not shown). Therefore, we conclude that TIF1β has to be tethered to DNA in order to exert its repressing effect.

From comparison of the different GAL fusions shown in Figure 5, we conclude that the repression domain lies between the coiled-coil and the PHD finger. TIF1β(381–660) represses less efficiently than the full length clone; nevertheless, the 5–8-fold repression observed is significant. Further deletions abolished repression completely. In particular, we note that TIF1β(1–403) that strongly interacts in yeast, did not repress in mammalian cells. Conversely, the mutant that deleted the coiled-coil and does not interact with the KRAB domain can still repress transcription.

Therefore, the domain of TIF1β responsible for KRAB interaction and the domain required for the silencing effect are different, and may at most overlap by 22 amino acids. Whereas the B boxes and the putative coiled-coil seem to contact the KRAB domain, the adjacent region towards the C-terminus direction mediates repression, independent of KRAB. Interestingly, the equivalent region in mouse TIF1α interacts with the nuclear receptor RXRα (and other members of the superfamily), and with



**Figure 4.** Transcriptional repression in transiently transfected HeLa cells. Graphics above the pictures show the organisation of the reporter genes. The TATA box is represented by an open oval; Sp1 binding sites by open squares; GAL4 UAS motifs by filled circles, Lex binding sites by filled squares, the  $\beta$ -globin gene by a hatched bar, the SV40 enhancer by a bar in bold hatching and the transcription initiation site by an arrow. (A) Repression from promoter-proximal position and (B) from remote binding sites. (C) Repression of a promoter that is driven by an SV40 enhancer 1.5 kb downstream of the  $\beta$ -globin gene. (D) No repression is seen at a constitutively active promoter which is comparable with the promoter used under (C) but lacks GAL4 binding sites.

the mouse homologues of *Drosophila* heterochromatin protein 1, mHP1 $\alpha$  and mMOD1, via two overlapping domains (47). As we had previously observed with the KRAB domain alone, no repression was seen in yeast, whether TIF1 $\beta$  alone or in combination with the KRAB domain was expressed.

### Chromosomal position of TIF1 $\beta$

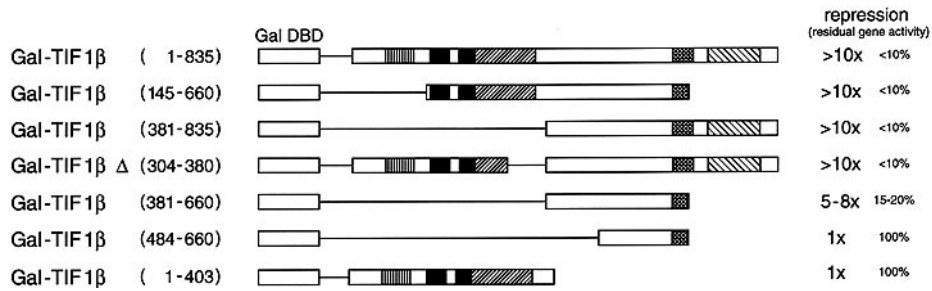
Since three out of nine members of the RBCC subgroup of RING finger proteins (RFP, PML and TIF1 $\alpha$ ) are associated with neoplastic disease, we were interested in the chromosomal position of the TIF1 $\beta$  gene. FISH mapping was performed. The hybridization efficiency was ~81% (i.e. among 100 checked mitotic figures, 81 showed signals on one pair of the chromosomes).

DAPI staining and detailed position analysis allowed for assignment of the signal to 19q13.4 (Fig. 6). No additional locus was detected under the conditions applied.

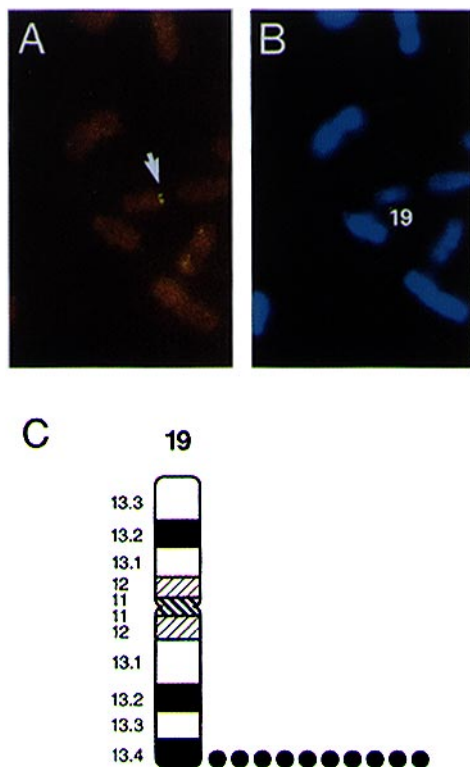
## DISCUSSION

### Sequence motifs in TIF1 $\beta$

In the yeast two-hybrid screen of cDNAs coding for proteins interacting with the KRAB repressor domain of the human zinc finger factor KOX1, one interaction partner strongly prevailed, TIF1 $\beta$ . Independently, it was isolated from mammalian cells by biochemical techniques as KRAB-associated protein KAP-1 (31), emphasizing the significance of our yeast selection. This



**Figure 5.** GAL fusion constructs used for S1 nuclease protection assay and repression ability from promoter position. For details of the signature see legend to Figure 3. Repression was quantified in at least three independent experiments. The transcription signals are compared with that of GAL(1-93) on the reporter 5G2L (Fig. 4).



**Figure 6.** Chromosomal position of TIF1β. (A) Example of FISH mapping. (B) The same mitotic figure stained with DAPI for the chromosome identification. (C) Diagram of FISH results. Each dot represents the double signals detected on human chromosome 19.

protein has several conspicuous features that make it a good candidate for a nuclear regulator, since it contains four Cys/His-rich clusters, a putative coiled-coil and a bromo-like domain. Among the Cys/His-rich clusters, the RING finger defines a superfamily of >50 proteins in organisms as diverse as plants, vertebrates and viruses which are involved in a variety of functions such as *Drosophila* development, DNA repair, immunoglobulin gene rearrangement and herpes simplex virus gene regulation (51-53,72-75). Recent reports suggest a possible role in both protein-protein interaction and specific RNA binding for this particular domain (54,55). The configuration of a RING finger, B box(es) and coiled-coil constitutes a distinct subfamily of RING finger proteins. Remarkably, three of the nine RBCC proteins, namely RFP, PML and TIF1α, were found as oncogenic

fusion genes, linked to a tyrosine kinase (*ret*), retinoic acid receptor α and B-raf (T18), respectively (37,48,58,61,62). In this light, it was of special interest to determine the chromosomal position of the TIF1β gene. Besides the breakpoint at 19q13.3-13.4 region in a thyroid tumor cell line (76) with a t(1;19), we are not aware of any association of 19q13.4 with tumoral disease. Another translocation involving this region is reported in a mesenchymal liver hamartoma (77). The putative glioma tumor suppressor rather seems to map to 19q13.2-13.3 (78). It seems worth mentioning that on the long arm of chromosome 19 (19q13.2-13.3 and 13.4, respectively) a cluster of zinc finger proteins is located (79,80).

The PHD domain is conserved in evolution and also found in a number of chromatin-associated proteins, such as Trithorax-like (Tcl), also referred to as the *Drosophila* GAGA factor (81) and Polycomb-like (Pcl) (82), which can have positive and negative effects on gene activity, respectively (68). This could indicate that the PHD domain is involved in contacts to the chromosomal structure, irrespective of whether it ultimately leads to an active or an inactive state of gene expression. Even though TIF1β, when tethered to DNA, can by itself repress transcription, it contains a bromo-like domain which is found in many transcription activators. Whether the bromodomain of TIF1α, which is more closely related to other bromodomains, and the bromo-like domain of TIF1β can also be involved in gene activation in different protein complexes, e.g., in conjunction with nuclear receptors, remains to be seen. Alternatively, one might consider the possibility that a deviant bromodomain makes a non-productive interaction with regular bromodomain partners, thus resulting in gene inactivation. However, since a deletion mutant lacking this particular domain is still able to repress in our assays (e.g., Fig. 4A, lane 6), a role for the bromodomain in repression seems unlikely at present.

**Protein-protein interaction**

We have narrowed the interaction domain of TIF1β with KRAB to a segment of 258 amino acids that includes the putative coiled-coil and the B boxes but not the RING finger. In a deletion experiment the coiled-coil was shown to be essential [Fig. 3; TIF1βΔ(304-380)]. These findings are in agreement with the interaction between KRAB and mouse TIF1α (Fig. 3). It is therefore conceivable that the KRAB domain heterodimerizes via the coiled-coil with TIF1β, whereas no homodimer TIF1β-TIF1β is formed. Such a specificity of interactions between coiled-coils would not be unexpected, e.g., in the light of the Jun-Fos interaction specificity where Jun can dimerize with Fos and with

itself, but no Fos homodimers are formed. However, one should bear in mind that the assay we have used constitutes a genetic selection and therefore does not demonstrate a direct interaction. It may well be that a functional TIF1–KRAB interaction is only possible in a multiprotein complex. Large complexes found in bandshift assays with mammalian nuclear extracts (83) are compatible with this idea, and could also explain why so far we have not observed any influence of TIF1 $\beta$  overexpression on KRAB-mediated repression in transiently transfected mammalian cells, which others have seen (31). Most importantly, in this context we note that the region responsible for repression by TIF1 $\beta$  is different from the KRAB-interacting domain. Instead, the repression domain of TIF1 $\beta$  is a domain which, in the related factor TIF1 $\alpha$ , interacts with nuclear receptors and also with the chromatin-associated proteins mHP1 $\alpha$  and mMOD1 (47) (see also below).

The full length TIF1 $\beta$  seems to interact to a lesser extent with the KRAB domain than several TIF1 $\beta$  mutants which are truncated, notably at the C-terminus. This could mean that a C-terminal domain is involved in negative intramolecular regulation of TIF1 $\beta$ –KRAB interaction. A similar situation holds for many positively acting transcription factors, where activity is increased upon introducing terminal deletions (e.g. 84).

### How could TIF1 $\beta$ function?

Our data seem incompatible with a mechanism of repression via direct steric hindrance in its simplest form, because repression is exerted over a large distance, even from a position 1.5 kb downstream of the  $\beta$ -globin promoter (Fig. 4B). At first sight, chromatin reorganisation appears most appealing as TIF1 $\alpha$  interacts with heterochromatin proteins mHP1 $\alpha$  and mMOD1. When mHP1 $\alpha$  was used in a two-hybrid screen, the mouse homologue of the TIF1 $\beta$  described here was selected. KOX1 might bind to DNA (and/or chromatin-associated RNA) via its zinc fingers and, via its KRAB domain, specifically to TIF1 $\alpha/\beta$  which in turn form a complex with heterochromatin proteins, thus resulting in inactivation of a chromosomal domain. TIF1 $\beta$  could be part of a repressing multiprotein complex, where the KOX1 factor with the KRAB domain might provide the sequence-specificity to nucleate the repressive complex on DNA. However, the domain that is necessary for the interaction with the heterochromatin protein mHP1 $\alpha$  is dispensable for the repression function of TIF1 $\alpha$  (47). This means that the TIF1 $\beta$ -mediated repression observed in our transient assays could also be independent of an interaction with this heterochromatin component and thus may not depend on chromatin rearrangement altogether.

The fact that repression is very efficient even in a short-term transient transfection assay, where one would not expect to find *bona fide* heterochromatin formation, would rather favour a model of 'active repression' (5). In the framework of such a model, KRAB or TIF1 $\beta$  bind in a non-productive manner to specific proteins of the basal transcription apparatus that are required for assembly of the transcriptional initiation complex. Active repression could either act on a component of the basal transcription machinery, such as a member(s) of the TAF, the SWI/SNF or the SRB complex and, of course, RNA polymerase II itself, or possibly on a specific upstream activating factor. Since in our hands KRAB silences transcription from a number of different Pol II-dependent promoters, we would favour a model of direct repression of a basal component in the transcription

apparatus rather than blocking of an upstream activator. The *in vitro* repression by KRAB reported by (85) would also support direct repression. So far, no clues exist as to the possible transcriptional partners of KRAB and TIF1 $\beta$ . Unlike KRAB that has a unique sequence with a charged domain, TIF1 $\beta$  fulfils the criteria of other 'portable' repression domains with a preponderance of alanines in combination with prolines (5). Nevertheless, fusions to the GAL4 DBD of either the KRAB domain or the TIF1 $\beta$  protein have resulted in a qualitatively similar repression of transcription.

Finally, we would like to point out that the two mechanisms of direct repression and chromatin reorganisation do not have to be mutually exclusive. As mentioned, an involvement of chromatin is suggested by the specific interaction of both TIF1 $\alpha$  and  $\beta$  with the homologues of *Drosophila* HP1 protein, mHP1 $\alpha$  and mMOD1 (47). Furthermore, repression by the two corepressors SSN6/TUP1 was found in a chromatin free *in vitro* system and, by a different experimental approach, a redistribution of nucleosomes in the promoter region was seen (10,20,86). From this, one might expect that KRAB (and TIF1 $\beta$ ) similarly act via both mechanisms. In such a case, there would be a short-term direct repression exerted via contacts to the transcription apparatus, followed by a long-term repression involving chromatin reorganisation and possibly DNA methylation at CpG sites.

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