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The corepressor N-CoR and its variants RIP13a and RIP13Δ1 directly interact with the basal transcription factors TFIIB, $TAF_{II}32$ and $TAF_{II}70$

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ABSTRACT

Repression of transcription by the classical nuclear receptors (e.g. TR, RAR), the orphan nuclear receptors (e.g. Rev-erbAα/β), Mxi-1 and Mad bHLH-zip proteins and the oncoproteins PLZF and LAZ3/BCL6 is mediated by the corepressors N-CoR and SMRT. The interaction of the corepressors with the components involved in chromatin remodelling, such as the recruiting proteins Sin3A/B and the histone deacteylases HDAc-1 and RPD3, has been analysed in detail. The N-CoR/Sin3/HDAc complexes have a key role in the regulation of cellular proliferation and differentiation. However, the interaction of these corepressors with the basal transcriptional machinery has remained obscure. In this study we demonstrated that the N-terminal repression domains and the receptor interaction domains (RID) of N-CoR and its splice variants, RIP13a and RIP13Δ1, directly interact with $TAF_{II}32$ *in vivo* and *in vitro*. We show that interaction domain II within the N-CoR and RIP13a RID is required for the interaction with $TAF_{II}32$. We also observed that N-CoR directly interacts with each of the basal factors, TFIIB and $TAF_{II}70$, and can simultaneously interact with all three basal factors in a non-competitive manner. Furthermore, we provide evidence that suggests the RVR/Rev-erbβ-corepressor complex also interacts with the general transcriptional machinery, and that the physical association of TFIIB with N-CoR also occurs in the presence of Sin3B and HDAc-1. Interestingly, we observed that N-CoR expression ablated the functional interaction between TFIIB and $TAF_{II}32$ that is critical to the initiation of transcription. In conclusion, this study demonstrates that the N-terminal repressor region and the C-terminal RIDs are part of the corepressor contact interface that mediates the interaction with the general transcription factors, and demonstrates that TAFs can also directly interact with corepressors to mediate signals from repressors to the basal machinery. We also suggest that N-CoR interacts with the central components of the transcriptional initiation process (TFIIB, TAFs) and locks them into a non-functional complex or conformation that is not conducive to transcription.

INTRODUCTION

Cofactors function as bridges between DNA binding proteins and the basal transcriptional machinery. The identification of corepressors (i.e. N-CoR and SMRT) that interact with the thyroid hormone, retinoic acid receptors and vitamin D receptors (TR, RAR and VDR), has shed some light on the mechanism of repression (by classical nuclear receptors) in the absence of ligand (1–3). The C-terminal Receptor Interaction Domain (RID) of the corepressors interacts with the Ligand Binding Domain (LBD/DE) region of unliganded receptors. This interaction induces a series of protein–protein interactions that repress transcription. The corepressors contain two interaction domains that interact independently and synergistically with the nuclear receptors (4,5). These corepressors interact with Sin3 and recruit the histone deacetylases (HDAc-1 or RPD 3) that lead to hypo-acetylation of the histones. This de-acetylation leads to conformational changes that stabilise the nucleosome structure, and limit the accessibility of the chromatin to the transcriptional machinery (6–8). The N-CoR/Sin3/HDAc complex mediates transcriptional repression from a wide variety of other non-receptor mediated pathways including the Mad/Mxi mediated repression of *myc* activities and tumour suppresion, and the oncoproteins PLZF-RARα (9) and LAZ3/BCL6 (10) that are involved in non-hodgkin lymphomas and acute promyelocytic leukemia, respectively. These receptor and non-receptor mediated pathways share common attributes; they are converted in response to environmental stimuli from the repressive to the operative condition and function in the management of differentiation and cell division.

N-CoR and the splice variants (RIP13a and RIP13Δ1) mediate transcriptional repression by the orphan nuclear receptors, Rev-erbA (5,11), RVR (5,11) and COUP-TF II (12). The C-terminal regions of N-CoR and RIP13 that encode the RIDs are almost identical. There are two distinct differences between RIP13a and N-CoR (4 and Fig. 1). The first 1016 amino acids of N-CoR that encode that encode repression domains I and II are replaced by 10 unique amino acids at the N-terminal end of

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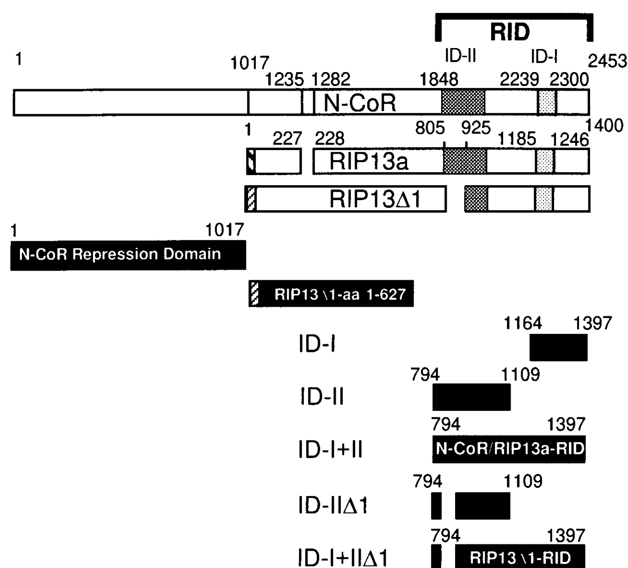

Figure 1. Schematic presentation of regions and domains in N-CoR, RIP13a and RIP13Δ1 used in the mammalian two hybrid assay. The shaded regions indicated the position of ID-I and ID-II in the C-terminal RID of N-CoR and its variants, RIP13a and RIP13Δ1. Striped areas in the N-termini of RIP13 and RIP13Δ1 represent unique N-terminal regions. There are two distinct differences between RIP13a and N-CoR. The first 1016 amino acids of N-CoR that encode that encode repression domains I and II, are replaced by 10 unique amino acids at the N-terminal end of RIP13a. However, RIP13a contains seven copies of a repeated motif, G-s-l-s/t-q-G-t-P, that associated with repressor activity in SMRT. Another difference is the replacement of 48 amino acids of N-CoR (amino acids 1235–1282) with a serine in RIP13a (amino acid 228). There are several minor amino acid changes described in detail in Seol *et al.* (4). The N-CoR and RIP13a RIDs are completely identical. Specifically, ID-I corresponded to the-region between amino acids 2218 and 2451 of N-CoR (1), and to amino acids 1164–1397 in RIP13a (ID-I in N-CoR, RIP13a and RIP13Δ1 are identical). ID-II corresponded to the region between amino acids 1848 and 2163 of N-CoR and to amino acids 794 and 1109 in RIP13a (ID-II in N-CoR and RIP13a are identical). ID-IIΔ1 from RIP13Δ1 has an internal deletion of 120 amino acids, that lacks amino acids 805–925 from the RIP13a ID-II. Regions of proteins containing the RIDs of N-CoR/RIP13a and RIP13Δ1 and the N-terminal repression domains used in the mammalian two hybrid and GST pulldown assays that were linked to GAL4, VP16 or pSG5 are shown in black. Amino acids in the ID-IIΔ1 and ID-I+IIΔ1 constructs refer to the corresponding amino acids in RIP13Δ1.

RIP13a. However, RIP13a contains seven copies of a repeated motif, G-s-l-s/t-q-G-t-P, that is associated with repressor activity in SMRT. Another difference is the replacement of 48 amino acids (1235–1282) of N-CoR with a serine (amino acid 228) in RIP13a. There are several minor amino acids changes (4).

The C-terminal N-CoR and RIP13 RIDs are composed of interaction domains (IDs) I and II that function synergistically and can also independently interact with some nuclear receptors, albeit weakly (5,11,12). Although the RIDs from N-CoR and RIP13a are absolutely identical, the RIP13Δ1-RID has an internal deletion in ID II (see Fig. 1 for details). Interaction of the orphan nuclear receptors with the corepressors requires an intact LBD (5,11,12). Physical association between the corepressors and the orphan receptors is dependent on two corepressor interaction regions, located in helices 3 and 11, that are separated by 150–200 amino acids and probably form a corepressor interface in three-dimensional space (11).

The corepressor complexes that mediate repression of transcription by the nuclear hormone receptors have been characterised in terms of the proteins that regulate chromatin architecture (6–8). Furthermore, notable progress in elucidating the central role of the corepressor complex (N-CoR/Sin3/HDAc) and protein–protein interactions involved in oncoprotein mediated transcriptional repression has been observed (9,10). However, the components of the basal transcriptional machinery involved/targeted in the repression of gene expression by the nuclear receptors and oncoproteins that interact with N-CoR and SMRT remains obscure. Clues to the involvement of the basal transcriptional machinery in N-CoR mediated inhibition may be gleaned from the fact that N-CoR is involved in the repression of GAL4VP16 mediated transactivation by the Rev-erb E-region, that involves a minimal region (~35 amino acids) that spans the ligand binding domain (LBD)-specific signature motif (**F/WAK**XXXX**F**XX**L**XX**-XDQ**XX**LL**), helix 3, loop 3–4, helix 4 and helix 5 of the Rev-erb family of orphan nuclear receptors (13–15).

VP16 mediated *trans*-activation involves the direct interaction of $TAF_{II}32$ (or its *Drosophila* homologue-$TAF_{II}40$) and TFIIB with VP16 (16–18). Concurrently there is direct protein–protein interaction between TFIIB and $TAF_{II}32$. Antibodies directed against $TAF_{II}32$ block GAL4VP16 mediated *trans*-activation, suggesting that TAFII32 is a critical coactivator in the process of transcription (16–19). This is an obvious starting point in the search for targets of N-CoR mediated repression of transcription, since these factors are central and essential components of the transcription initiation complex. $TAF_{II}32$ recruits TFIIB (16–18) and interacts very efficiently with $TAF_{II}70$ (20,21) that efficiently binds $TAF_{II}250$ in the TFIID complex during transcriptional activation (21). $TAF_{II}250$ is histone acetyltransferase (22) and obviously any mechanism that affects a pathway of histone acetylation is an ideal target/pathway for corepressor action that leads to net deacteylation (16–26).

The present study utilised mammalian two hybrid and direct *in vitro* binding assays to characterise the specific regions in N-CoR and its variants, RIP13a and RIP13Δ1, that interact with key targets in the process of transcriptional initiation. These experiments demonstrated that the N-terminal repressor region and the C-terminal receptor interaction domains of N-CoR made direct contact with three key components of the transcription initiation complex, TFIIB, $TAF_{II}32$ and $TAF_{II}70$. These studies suggested that N-CoR represses transcription via a mechanism that involves direct non-competitive interaction with the TAFs and TFIIB, and regulates the crucial interaction between $TAF_{II}32$ and TFIIB. Interestingly, these are key components of the initiation process and a major rate limiting step in the initiation process. We suggest that the corepressor interactions with the basal transcriptional factors freezes them into a non-functional state or conformation that is not permissive to basal transcription.

MATERIALS AND METHODS

Primer sequences

GMUQ296 5′-GCGAATTCACCATGGTNAAA/GA/TCNA-AG/AAAG/ACA-3′
GMUQ297 5′-GCGAATTCACCNCA/TA/GTCNG/CA/TN-AA/GNGTT/CTCG/ATAT/CTG-3′
GMUQ332 5′-GCGGTCGACTAACCATGGCTGAGGAG-AAGAAGCTG-3′

GMUQ333 5′-GCGGTCGACTTCACGGAGCAGGCTG-AGGGG-3′
GMUQ340 5′-GCGGAATTCACCATGTCAAGTTCAGG-TTATCCT-3′
GMUQ341 5′-GCGGAATTCCCACTCCCTGTTTGGA-CT-3′
GMUQ390 5′-GCGGAATTCACCATGTGTGCTTCCTG-CTCT-3′

Plasmids

The expression plasmids pGAL0 (27), pNLVP16 (28) and pG5E1bCAT (29). pGAL0 contains the GAL4-DBD and pNLVP16 contains the acidic activation domain of VP16. All PCR amplifications were performed with *Pfu* DNA (Stratagene) or *Pwo* DNA polymerase (Boehringer Mannheim) according to the manufacturer's instructions. End-filling reactions were performed with Klenow DNA polymerase according to the manufacturer's instructions. All pBluescript and pBS clones and GAL and VP16 chimeras were sequenced by double stranded sequencing to verify identity and confirm the reading frame.

Full length RIP13a was amplified from pCDM8-RIP13a (4) using the two primers GMUQ390 and GMUQ297. The product was cleaved with *Eco*RI and ligated to pGAL0/*Eco*RI and pSG5/*Eco*RI to create GAL4-Rip13a and pSG5-Rip13a, respectively. To construct pSG5-RIP13Δ1, GAL4-RIP13Δ1 was cleaved with *Eco*RI and the resulting fragment was ligated to pSG5/*Eco*RI. To construct pSG5-N-CoR/RIP13a-RID, the RID was excised from GAL4-ID-I+II (14) with *Eco*RI and the resulting fragment was ligated to pSG5/*Eco*RI. N-CoR amino acids 1–1017 was amplified with *Pwo* DNA polymerase from GAL4-NCoR using the two primers GMUQ340 and GMUQ341. The product was cleaved with *Eco*RI and the resulting fragment was ligated to pGAL0/*Eco*RI and pSG5/*Eco*RI to form pGAL4-N-CoR amino acid 1–1017 and pSG5-N-CoR amino acids 1–1017, respectively. RIP13Δ1 amino acids 1–627 was excised from pSG5-RIP13Δ1 with *Eco*RI/*Nco*I and the resulting 1.8 kb fragment was end-filled and ligated to end-filled pNLVP16/*Nde*I to create VP16-RIP13Δ1 amino acids 1–627. VP16-RIP13Δ1 amino acids 1–627 was cleaved with *Sal*I/*Xba*I and the resulting fragment was ligated to pGAL0/*Sal*I/*Xba*I.

hTAF$_{II}$-32 was cut from pBS-KS$^+$-hTAF32 (an unpublished clone from the Tjian laboratory) with *Nde*I/*Eco*RI, end-filled and ligated to end-filled pNLVP16/*Xho*I. hTAF$_{II}$-70 was amplified with *Pfu* DNA polymerase from phTAF$_{II}$-70 (an unpublished clone from the Tjian laboratory) using the primers GMUQ332 and GMUQ333 and was ligated to pBluescript KS/*Eco*RV. Antisense pBluescript-TAF$_{II}$-70 was cleaved with *Sal*I and the resulting fragment was inserted into pGAL0/*Sal*I and pNLVP16/*Sal*I to create pGAL4-TAF$_{II}$-70 and VP16-TAF$_{II}$-70, respectively. TFIIB was excised from pGEX-KT-TFIIB with *Eco*RI end-filled and ligated to end-filled pGAL0/*Sal*I or end-filled Vp16/*Sal*I create pGAL4-TFIIB or VP16-TFIIB, respectively. GAL4-TBP and VP16-TBP were constructed by cleaving pTβ-hTBP (an unpublished clone from the Tjian laboratory) with *Nde*I and ligating the resulting fragment into pGAL0/*Nde*I and pNLVP16/*Nde*I, respectively. All other plasmids and primers have been described previously (5,11–15).

Mammalian two hybrid assay

Each well of a six well plate of JEG-3 cells (60–70% confluence) was co-transfected with 3 µg pG5E1bCAT reporter, 1 µg GAL chimeras and 1 µg VP16 chimeras in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 5% charcoal-stripped foetal calf serum (FCS) by the DOTAP (Boehringer Mannheim) mediated procedure as described previously (30,31). After 24 h, the medium was replaced and cells were harvested for the assay of CAT activity 36–48 h after transfection. Each transfection was performed at least three times to overcome the variability inherent in transfections.

CAT assays

Cells were harvested and CAT activity measured as described previously (32). Aliquots of cell extracts were incubated at 37°C, with 0.1–0.4 µCi of ^{14}C-chloramphenicol (ICN) in the presence of 5 mM acetyl CoA in 0.25 M Tris–HCl, pH 7.8. After a 1–4 h incubation period, 1 ml ethyl acetate was used to extract the chloramphenicol and its acetylated forms. Extracted materials were analysed on Silica gel thin layer chromatography plates as described previously (32). Quantitation of all CAT assays was performed with an AMBIS β-scanner.

In vitro binding assays

GST and GST-fusion proteins were expressed in *Escherichia coli* (BL21) and purified using glutathione agarose affinity chromatography as described previously (31). The GST-fusion proteins were analysed on 10% SDS–PAGE for integrity and to normalise the amount of each protein. The Promega TNT-coupled transcription-translation system was used to produce ^{35}S-methionine labelled N-CoR, TFIIB, TAF$_{II}$32 and TAF$_{II}$70 proteins that were visualised by SDS–PAGE. *In vitro* binding assays were performed with glutathione agarose beads (Sigma) coated with ~500 ng of GST-fusion protein and 2–30 µl of ^{35}S-methionine-labelled protein in 200 µl of binding buffer containing 100 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 5 µg ethidium bromide and 100 µg BSA. The reaction was allowed to proceed for 1–2 h at 4°C with rocking. The affinity beads were then collected by centrifugation and washed five times with 1 ml of binding buffer without ethidium bromide and BSA. The beads were resuspended in 20 µl SDS–PAGE sample buffer and boiled for 5 min. The eluted proteins were fractionated by SDS–PAGE, the gel was treated with Amersham Amplify fluor, dried at 70°C and autoradiographed (11).

RESULTS

In vivo and *in vitro* interaction assays demonstrate that the repression and receptor interaction domains of N-CoR and the RIP13 variants interact specifically with TAF$_{II}$32

In an initial investigation to address whether the corepressors interact with the basal transcriptional machinery that is required for initiation, we examined the ability of the corepressors to interact with TFIIB, TAF$_{II}$32 and TAF$_{II}$70 *in vivo* and *in vitro*. We tested these components of the basal transcriptional machinery since N-CoR mediates the repression of GAL4VP16 mediated transactivation by the orphan nuclear receptors, and it has been demonstrated that the VP16 acidic activation domain interacts directly with (i) TFIIB and TBP and (ii) TAF$_{II}$32 and TAF$_{II}$70.

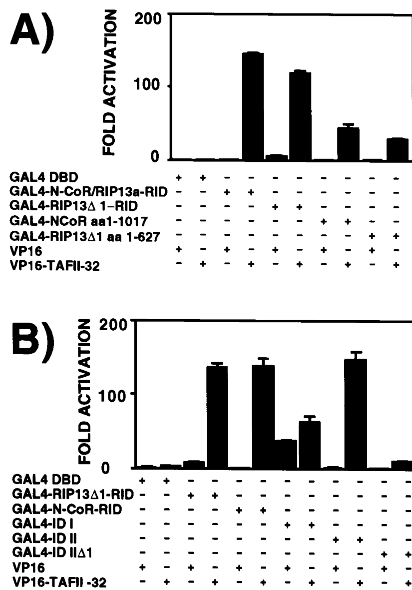

Figure 2. $TAF_{II}32$ interacts with the N-terminal and C-terminal regions of N-CoR and its variants: analysis of the interaction of the N-CoR and its variants with $TAF_{II}32$ by the mammalian two hybrid assay. JEG-3 cells were co-transfected with the indicated plasmids (+) in the presence of pG5E1bCAT reporter plasmid and assayed for CAT activity. Results shown are mean ± standard deviation and were derived from three independent transfections. VP16 or the indicated VP16-$TAF_{II}32$ (+) were co-transfected with GAL4 chimeras of (**A**) the receptor interaction domains (RIDs) and N-terminal regions of N-CoR and the RIP13 variants and (**B**) the indicated GAL4 chimeras of the independent interaction domains I and II were co-transfected with VP16 or VP16-$TAF_{II}32$. Fold activation is expressed relative to CAT activity obtained after co-transfection of GAL4 DBD and the VP16 vector alone arbitrarily set to 1.0 (A and B).

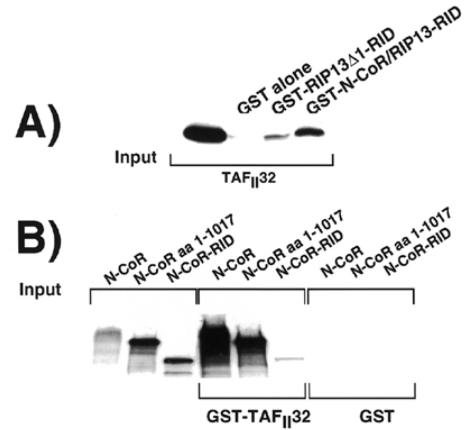

Figure 3. N-CoR directly interacts with $TAF_{II}32$ *in vitro*. Interaction of $TAF_{II}32$ and corepressors *in vitro*. (**A**) $TAF_{II}32$ was radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone, GST-RIP13Δ1-RID and GST-N-CoR/RIP13a-RID. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. (**B**) N-CoR, N-CoR amino acids 1–1017 and the N-CoR/RIP13a-RID were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-$TAF_{II}32$. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input.

Furthermore, $TAF_{II}32$ is recruited into the TFIID complex by $TAF_{II}70$ (16–19,25,26).

Recruitment of $TAF_{II}32$ is critical to the initiation of transcription. Hence, we examined the ability of the N-CoR/RIP13a and RIP13Δ1-RIDs (Fig. 1) to interact with full length $TAF_{II}32$ linked to VP16 in the mammalian two hybrid assay. We observed that both RIDs very efficiently (>100-fold) interacted with $TAF_{II}32$ (Fig. 2A). The N-terminal regions of RIP13Δ1 (lacking the RID) and N-CoR, which has an additional ~1000 N-terminal amino acids (relative to the RIP13 splice variants) that encode two repression domains (between amino acids 1 and 1017) that are not in RIP13a or RIP13Δ1 (Fig. 1), both interacted with $TAF_{II}32$, although 2–3-fold less efficiently than the RIDs (Fig. 2A). This suggested that the corepressor C-terminal RIDs and the N-terminal repression domains were part of the interaction surface of the corepressor with the basal transcription machinery.

As discussed earlier, the N-CoR/RIP13a and RIP13Δ1 RIDs are each composed of two interaction domains (I and II) that can independently interact with some nuclear receptors, albeit weakly. ID-I corresponded to the region between amino acids 2218 and 2451 of N-CoR (1), and to amino acids 1164–1397 in RIP13a (ID-I in N-CoR, RIP13a and RIP13Δ1 are identical). ID-II corresponded to the region between amino acids 1848 and 2163 of N-CoR and to amino acids 794 and 1109 in RIP13a (ID-II in N-CoR and RIP13a are identical). ID-IIΔ1 from RIP13Δ1 has an internal deletion of 120 amino acids, that lacks amino acids 805–925 from the RIP13a ID-II. We therefore examined the ability of ID-I and ID-II and ID-IIΔ1 from N-CoR, RIP13a and RIP13Δ1 to independently interact with $TAF_{II}32$. As demonstrated above, both RIDs interacted very efficiently with $TAF_{II}32$. ID-I poorly interacted with $TAF_{II}32$ (<2-fold); however, ID-II very strongly interacted with $TAF_{II}32$ (~130-fold). In contrast, ID-IIΔ1 weakly interacted with $TAF_{II}32$ (~10-fold) (Fig. 2B). In summary, this indicated that ID-II in the N-CoR/RIP13a-RID, was required for $TAF_{II}32$ binding. In contrast, ID-IIΔ1 could not independently interact with $TAF_{II}32$, suggesting that the amino acids deleted were critical to $TAF_{II}32$ binding, but interestingly were redundant in the context of the entire RIP13Δ1 RID.

The demonstration of interaction between $TAF_{II}32$ and the corepressors in the *in vivo* mammalian two hybrid assay strongly suggests these proteins may interact by a direct mechanism. However, this does not eliminate the possibility of an indirect mechanism in which additional factor(s) mediate the interaction. We tested this hypothesis using a biochemical approach, the *in vitro* GST pulldown assay.

Glutathione agarose immobilised GST-RIP13Δ1-RID and the GST-N-CoR/RIP13a-RID were tested for direct interaction with *in vitro* ^{35}S-radiolabelled full length native $TAF_{II}32$. GST-RIP13Δ1- and N-CoR/RIP13a-RIDs both showed a direct interaction with full length $TAF_{II}32$ (Fig. 3A). We then examined the ability of *in vitro* ^{35}S-radiolabelled full length/native N-CoR, the repression region (amino acids 1–1017) and the receptor interaction domains to interact with the immobilised GST-$TAF_{II}32$ fusion protein. Full length N-CoR very efficiently interacted with $TAF_{II}32$ *in vitro*. The amino acids between 1 and 1017 encoding the repression domain also interacted efficiently with $TAF_{II}32$. The receptor interaction domain also interacted with $TAF_{II}32$ *in vitro*, although less efficiently than the native protein and the repression domain (Fig. 3B).

In conclusion, these *in vivo* and *in vitro* studies demonstrate that the repression region and receptor interaction domain of the

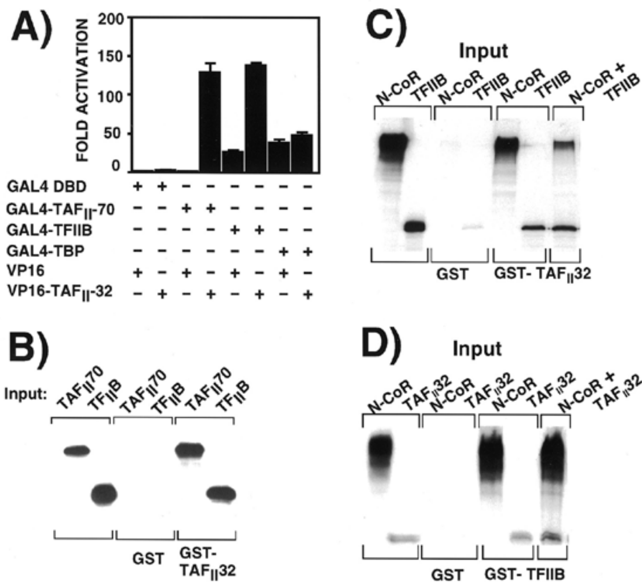

Figure 4. N-CoR directly interacts with TFIIB and $TAF_{II}32$. Analysis of the interaction of the N-CoR family of co-repressors with factors known to be involved in transcriptional activation by the mammalian two hybrid assay. JEG-3 cells were co-transfected with the indicated plasmids (+) in the presence of pG5E1bCAT reporter plasmid and assayed for CAT activity. Results shown are mean ± standard deviation and were derived from three independent transfections. (**A**) Interaction of factors known to be involved in transcriptional activation with $TAF_{II}32$. GAL4-DBD or GAL4-$TAF_{II}70$-TFIIB or -TBP were co-transfected with VP16 or the VP16-TAF_{II}-32 chimera. (**B**) $TAF_{II}70$ and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-$TAF_{II}32$. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes in all gels contain ~10% input. (**C**) N-CoR and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-$TAF_{II}32$. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes in all gels contain ~10% input. (**D**) N-CoR and $TAF_{II}32$ were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-TFIIB. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input.

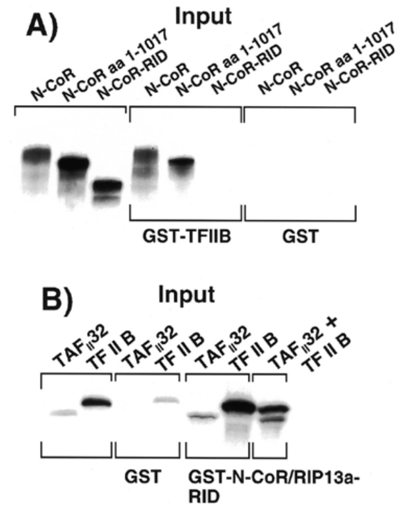

Figure 5. TFIIB interacts with the N-terminal and C-terminal regions of N-CoR: interaction of TFIIB and corepressors *in vitro*. (**A**) N-CoR, N-CoR amino acids 1–1017 and the N-CoR/RIP13a-RID were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-TFIIB. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input. (**B**) $TAF_{II}32$ and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-N-CoR/RIP13a-RID. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input.

corepressors are involved in the interaction with $TAF_{II}32$. There is a quantitative (but not qualitative) discrepancy between the *in vivo* and *in vitro* assays with respect to the strength of the interactions between $TAF_{II}32$, and the repression and RID regions of N-CoR. Whether this is reflects the true situation, or assay anomalies, has not been resolved.

N-CoR, $TAF_{II}32$ and TFIIB can simultaneously interact with each other: the repression and receptor interaction domains of N-CoR interact specifically with TFIIB

$TAF_{II}32$ interacts with $TAF_{II}70$ and TFIIB during the formation of the transcriptional initiation complex (18,23), which we verified by the *in vivo* mammalian two hybrid assay and *in vitro* GST-pulldown assay (Fig. 4A and B). Since TFIIB interactions with nuclear receptors have been implicated in transcriptional regulation (33–38) and N-CoR and its variants RIP13a/RIP13Δ1 interact with $TAF_{II}32$, we investigated the ability of $TAF_{II}32$ and TFIIB to form a complex with N-CoR.

We examined the ability of glutathione agarose immobilised GST-$TAF_{II}32$ and GST-TFIIB for direct interaction with *in vitro* ^{35}S-radiolabelled full length native N-CoR and TFIIB or $TAF_{II}32$, respectively (Fig. 3C and D). Full length N-CoR and TFIIB independently and directly interacted with GST-$TAF_{II}32$ (Figs 3B, 4B and C). However, we also observed that GST-$TAF_{II}32$ could simultaneously pulldown N-CoR and TFIIB. To discriminate whether this observed interaction between $TAF_{II}32$, N-CoR and TFIIB involved tethered complexes or direct interaction between TFIIB and N-CoR, we performed further GST-pulldowns using GST-TFIIB (Fig. 4D). We observed that full length N-CoR and $TAF_{II}32$ independently and directly interacted with GST-TFIIB (Fig. 4D). We also observed that GST-TFIIB could simultaneously pulldown N-CoR and $TAF_{II}32$. These studies suggested that N-CoR could directly interact with TFIIB and $TAF_{II}32$.

We then examined the ability of *in vitro* ^{35}S-radiolabelled full length/native N-CoR, the repression region (amino acids 1–1017) and the receptor interaction domains to interact with the immobilised GST-TFIIB fusion protein. Full length N-CoR very efficiently interacted with TFIIB *in vitro*. The amino acids between 1 and 1017 encoding the repression domain also interacted very efficiently with TFIIB (Fig. 5A). The receptor interaction domain also specifically interacted with TFIIB *in vitro*, although significantly less efficiently than the native protein and the repression domain (observed in longer exposures and Fig. 5B). To verify that the corepressor receptor interaction domains could interact with TFIIB, we examined the ability of glutathione agarose immobilised GST-N-CoR/RIP13a-RID for direct interaction with *in vitro* ^{35}S-radiolabelled full length native TFIIB and $TAF_{II}32$. GST-N-CoR/RIP13a-RIDs showed a direct interaction with full length $TAF_{II}32$ and TFIIB (Fig. 5B). Further analysis demonstrated that the N-CoR-RID could simultaneously pulldown $TAF_{II}32$ and TFIIB.

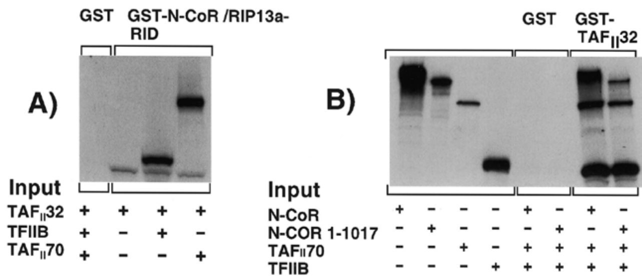

Figure 6. N-CoR directly interacts with TAF$_{II}$32, TAF$_{II}$70 and TFIIB. (**A**) Interaction of TFIIB, TAFs 32 and 70 with N-CoR *in vitro*. TAF$_{II}$32, TAF$_{II}$70 and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-N-CoR/RIP13a-RID. (**B**) N-CoR, N-CoR amino acids 1–1017, TAF$_{II}$70 and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for simultaneous interaction with GST-alone, and GST-TAF$_{II}$32. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input.

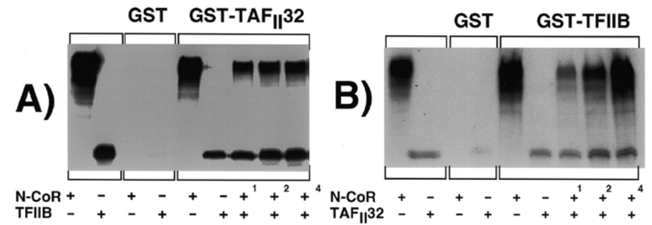

Figure 7. N-CoR, TAF$_{II}$32 and TFIIB interact in a non-competitive manner. (**A**) N-CoR and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-TAF$_{II}$32. We examined the effect of increasing amounts of N-CoR (1, 2 and 4 µl) on the efficiency of the interaction between GST-TAFII32 and TFIIB. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input. (**B**) N-CoR and TAF$_{II}$32 were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-TFIIB. We examined the affect of increasing amounts of N-CoR (1, 2 and 4 µl) on the efficiency of the interaction between GST-TFIIB and TAF$_{II}$32. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~10% input.

These studies confirmed the interaction between N-CoR and TFIIB and suggested that the corepressor, TFIIB and TAF$_{II}$32 were directly involved in the repression of transcription.

N-CoR can simultaneously interact with TFIIB, TAF$_{II}$32 and TAF$_{II}$70

Since N-CoR had been independently shown to interact with TAF$_{II}$32 and TFIIB, and that TAF$_{II}$32 could directly interact with TFIIB and TAF$_{II}$70, we investigated whether N-CoR could simultaneously interact with TAF$_{II}$32 and TAF$_{II}$70 in the GST-pulldown assay. Hence, we examined the ability of glutathione agarose immobilised GST-N-CoR/RIP13a-RID to interact with a mixture of *in vitro* ^{35}S-radiolabelled full length native TAF$_{II}$32 and TFIIB, and TAF$_{II}$32 and TAF$_{II}$70 (Fig. 6A). We observed that GST-N-CoR/RIP13a-RID could simultaneously pulldown TAF-$_{II}$70 and TAF$_{II}$32.

Since TAF$_{II}$32 had been independently shown to interact with N-CoR, TFIIB and TAF$_{II}$70, we investigated whether these proteins could simultaneously interact with TAF$_{II}$32 in the GST-pulldown assay. The previous experiments had demonstrated that TAF$_{II}$32 could independently interact with all these proteins that were part of the basal transcription machinery. Hence, we examined the ability of glutathione agarose immobilised GST-TAF$_{II}$32 to interact with a mixture of *in vitro* ^{35}S-radiolabelled full length native N-CoR (or the repression domain, amino acids 1–1017), TFIIB and TAF$_{II}$70 (Fig. 6B). We observed that GST-TAF$_{II}$32 could simultaneously pulldown N-CoR (or its repression domain, amino acids 1–1017), TAF$_{II}$70 and TFIIB.

These studies added weight to the suggestion that N-CoR directly interacted with TFIIB and two TAFs that are critical components of the transcriptional initiation process. Interestingly, the TAFs normally function as essential coactivators, but may have been targeted by the corepressor to be frozen in a non-functional state during transcriptional repression.

N-CoR, TAF$_{II}$32 and TFIIB interact in a non-competitive manner

We then embarked on experiments designed to analyse whether competitive binding between these interacting proteins was an issue with respect to the formation of a ternary complex. We examined the ability of increasing amounts of N-CoR (1, 2 and 4 µl) to affect the efficiency of interaction between GST-TAF$_{II}$32 and radiolabelled TFIIB (Fig. 7A), and GST-TFIIB and radiolabelled TAF$_{II}$32 (Fig. 7B). We did not observe a reduction in the efficacy of binding among these interacting proteins when the concentration of N-CoR was substantially elevated.

Based on these observations and the previous experiments, we suggest a structure/model involving multiple contacts among these components that secures them in a non-productive complex and/or blocks the functional assembly of an initiation complex.

The RVR-corepressor complex also interacts with the general transcriptional machinery, and the physical association of TFIIB with N-CoR occurs in the presence of Sin3B and HDAc-1

We subsequently investigated whether the corepressor, N-CoR, could still interact with TFIIB and the TAFs when it was bound or anchored to a nuclear receptor. Since we had demonstrated that N-CoR could interact with TFIIB, TAF$_{II}$32 and TAF$_{II}$70, we investigated whether N-CoR could maintain these contacts to the generalized transcription machinery when bound to the orphan nuclear receptor, RVR, in the GST-pulldown assay. Hence, we examined the ability of glutathione agarose immobilised GST-RVR to interact with a mixture of *in vitro* ^{35}S-radiolabelled full length native N-CoR, TFIIB, TAF$_{II}$32 and TAF$_{II}$70 (Fig. 8A). We observed that N-CoR bound to GST-RVR could simultaneously pulldown the TAFs and TFIIB.

Repression of transcription by the nuclear receptors and the Max associated Mad/Mxi1 family involves the formation of multiprotein complexes that are comprised of N-CoR/SMRT, Sin3A/B and the histone deacteylases. We decided to examine whether the corepressor, N-CoR, anchored to the orphan nuclear receptor, RVR/Rev-erbβ, could interact with (i) Sin 3B, that functions to recruit proteins that condense chromatin structure and (ii) HDAc-1, a histone deacteylase that is involved in nucleosome condensation, in the presence of TFIIB. Hence, we investigated the ability of glutathione agarose immobilised RVR to interact with N-CoR, TFIIB, Sin3B and HDAc-1. We observed

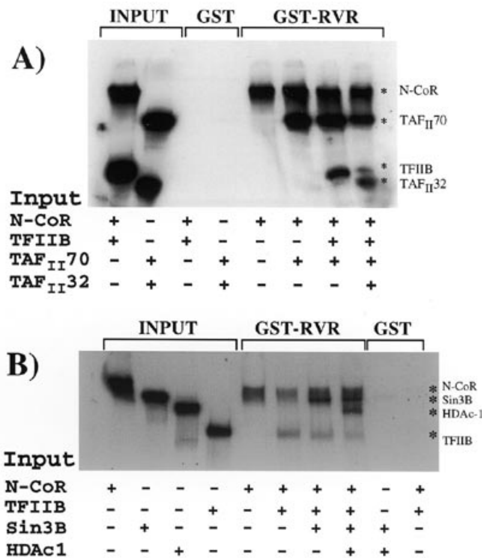

Figure 8. RVR forms a complex with N-CoR, the general transcriptional machinery and the Sin3B-HDAc-1 complex. N-CoR, $TAF_{II}70$, $TAF_{II}32$, Sin3B and HDAc-1 and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-RVR (Rev-erbβ) *in vitro*. (**A**) N-CoR, TFIIB and the TAFs were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-RVR. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~5–10% input. (**B**) N-CoR, Sin3B, HDAc-1 and TFIIB were radiolabelled with ^{35}S-methionine by *in vitro* transcription/translation and tested for interaction with GST-alone and GST-RVR. Inputs of the radiolabelled ^{35}S-methionine protein are also shown. The input lanes contain ~5–10% input.

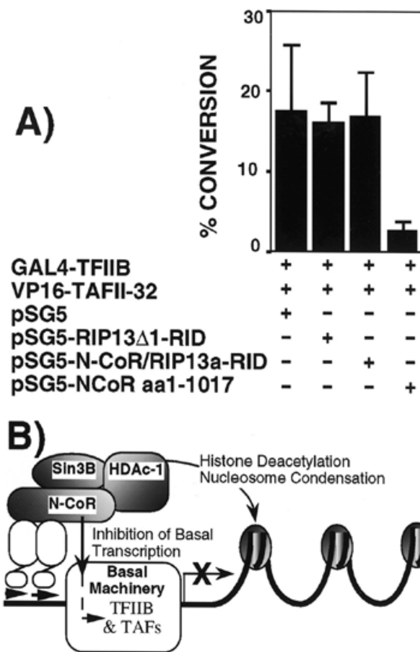

Figure 9. The N-CoR repression domain inhibits the functional interaction between TFIIB and $TAF_{II}32$ *in vivo*. (**A**) JEG-3 cells were co-transfected with GAL4-TFIIB (1 µg) and VP16-TAFII-32 (1 µg) and with the indicated pSG5 plasmids (1 µg) (+) in the presence of pG5E1bCAT reporter plasmid and assayed for CAT activity. Percent conversion of ^{14}C-chloramphenicol to its acetylated forms was calculated. Results shown are mean ± standard deviation and were derived from three independent transfections. (**B**) A cartoon that highlights the dual role of N-CoR in the repression of gene expression. N-CoR is involved in mediating nucleosome condensation and inhibiting the basal transcriptional machinery.

that Sin3B and HDAc-1 could still bind to N-CoR, in the presence of TFIIB (Fig. 8B). We have presented a weak exposure so as to discriminate the binding of N-CoR, Sin3B and HDAc-1; stronger exposures very clearly show TFIIB binding in the presence of Sin3B and HDAc-1.

These studies suggested that repression of transcription by the orphan nuclear receptor RVR/Rev-erbβ and the the corepressor N-CoR probably involves intimate contacts with the generalised transcriptional machinery and the proteins (Sin3 and HDAc-1) involved in nucleosomal condensation.

The N-CoR repression domain inhibits the functional interaction between TFIIB and $TAF_{II}32$ *in vivo*

To further understand the consequence of these interactions between the corepressors and the general transcription factors *in vivo*, we investigated the effect of the expression of the corepressor receptor interaction domain and repressor region on the ability of $TAF_{II}32$ to interact with TFIIB in the *in vivo* mammalian two hybrid assays. As observed in Figure 9A, pSG5-N-CoR 1–1017, which encodes the two N-CoR repression domains, inhibits the TFIIB-$TAF_{II}32$ interaction, whereas the RIDs do not.

This suggested that N-CoR binding to $TAF_{II}32$ and TFIIB may repress/regulate transcription by controlling the functional/productive interaction between these key interactions. This interaction is central to the formation of the transcriptional initiation complex. $TAF_{II}32$ normally interacts with activators/coactivators to mediate the signal to the basal transcription machinery. The targeting of $TAF_{II}32$ indicates that repressors/corepressor(s) may function by recruiting the TAFs and TFIIB in a non-productive manner that blocks the subsequent formation of an active transcription complex.

DISCUSSION

Sequence specific binding proteins regulate transcription by protein–protein interactions with the basal transcriptional machinery, which includes TFIID a complex containing TBP and the TBP-associated factors (TAFs) that function as co-activators. Site specific regulatory protein interactions with the TFIID multiprotein complex have been proposed to function in the productive recruitment of TFIIB and/or a conformational change in the TFIID/TFIIB promoter–protein complex that facilitates the binding of other components of the transcription complex (19,21).

Nuclear receptor mediated repression of transcription is mediated by the cofactor(s) N-CoR, SMRT and RIP13, that function as corepressors (1–4). Here we present *in vivo* and *in vitro* studies demonstrating that N-CoR directly interacts with $TAF_{II}32$, $TAF_{II}70$ and TFIIB. Our results suggest that a model for N-CoR mediated repression must explain interactions between these components of the basal transcriptional machinery and the corepressor. Although we have demonstrated that N-CoR can directly interact with these central components of the basal transcription apparatus and that the corepressor can simultaneously pulldown TFIIB, $TAF_{II}32$ and $TAF_{II}70$, the presence of a ternary complex has not been

identified directly. However, we have analysed the distinct interactions of each pair of potential partners. Furthermore, our investigation did not identify any indication for competitive binding between these components of the inhibitory complex. Hence we propose a structure/model involving multiple contacts among these components that locks them in a non-productive network and/or blocks the functional assembly of an initiation complex. This suggestion is supported by our observation that N-CoR inhibits the functional interaction or the transmission of the signal between TFIIB and $TAF_{II}32$ in the mammalian two hybrid assay, even in the presence of VP16.

Analogously, Tjian and colleagues (16) demonstrated VP16 mediated transactivation involves direct non-competitive interactions between VP16, TFIIB and $TAF_{II}32/40$ resulting in a conformational change in the TFIID/TFIIB complex that is permissive for complex assembly and subsequent initiation.

Interestingly, our study suggested that the N-terminal region of N-CoR encoding the repression domains very efficiently interacted with TFIIB; this is in agreement with the observations from Privalsky and colleagues who have shown that TFIIB interacted in a functional and efficient manner with the silencing domains in SMRT (M.Privalsky, personal communication; 39).

Many studies have demonstrated that $TAF_{II}32/40$ and $TAF_{II}60/70$ directly interact with each other, bind TBP and are key mediators in the transmittance of the signals from the activators and the initiation complex (16–19). Furthermore, $TAF_{II}32/40$ functions in TFIIB recruitment (16–19) and $TAF_{II}60/70$ interacts with $TAF_{II}230/250$ (20,23), a histone acetyltransferase that is involved in the transcriptional access to chromatin. The process of transcriptional initiation and activation has been viewed in two alternate models; one suggests that activators induce the step-wise formation of a multiprotein complex, whereas the alternate idea is that the complex is preassembled and that the activator recruits the complex to the DNA and produces functional conformational changes (19). Analysis has been complicated by data that suggests the components are involved in preinitiation/basal transcription and activation. These data for example have shown that TFIIB mutants are competent in basal transcription, but do not function in activation; furthermore, antibodies to $TAF_{II}32/40$ inhibit activation but not basal transcription (16,17 and references therein). Our studies in this context suggest that N-CoR forms a complex with $TAF_{II}32$, $TAF_{II}70$ and TFIIB that is non-functional and may prevent the incorporation of these components into the initiation complex and/or produce conformational changes that are detrimental to transcription. The interaction of N-CoR with $TAF_{II}70$ is very interesting; $TAF_{II}70$ strongly binds $TAF_{II}250$, a histone acetyltransferase. These observations, in light of the corepressor interactions with Sin3 and the histone deacetylases, suggest that the corepressor multiprotein complex may actively induce deacetylation and passively repress acetylation to assure minimum leakage. This hypothesis is supported by our observation that the orphan nuclear receptor/corepressor complex (RVR-N-CoR) also interacts with TFIIB and the TAFs, and that the physical association of TFIIB with N-CoR can occur in the context of interactions with Sin3B and HDAc-1 (Fig. 9B). Our data are consistent with the observations from Privalsky and colleagues who have shown that TFIIB also interacts with Sin3A and propose that SMRT/Sin3A mediated repression involves interactions with TFIIB (M.Privalsky, personal communication; 39).

The strong interactions of the corepressor with TFIIB correlate with the many reports of nuclear receptor interactions with TFIIB, although these reports have been very contradictory spanning the entire spectrum of positive and negative effects (24,33–35). Specifically, it has been reported that in the absence of ligand, the thyroid hormone receptor (TR) LBD interacts with TFIIB, and that this interaction may freeze TFIIB in a non-functional conformation that is relieved by hormone binding which facilitates the assembly of functional initiation complex. Concomitant with these events is the reduction of non-productive interactions between TR and the initiation factors (24,33–35). Another report has suggested that TFIIB interacts with the AB region of TR, and that the interaction has decreased affinity in the presence of T3, although the association is still stable (36). Many other reports have demonstrated that TFIIB mediates vitamin D and retinoid receptor dependent gene expression, via mechanisms that involve direct binding (37,38).

TFIIB appears to be a key target in the activation and repression of gene expression. Furthermore, the cofactor SRC-1 (40), a coactivator for the steroid receptor superfamily, encodes histone acetyltransferase activity, interacts with p300 and PCAF and also makes strong contacts with TFIIB (41). SRC-1 is a member of the a gene family that includes SRC-1, TIF-2/GRIP-1/SRC-2 and RAC-3/ACTR/AIBI/SRC-3 (which is amplified in breast cancer; 40 and references therein). Our studies and the other reports suggest interaction with TFIIB is a crucial rate limiting step in the positive and negative regulation of transcription, and may be a key target in the control of cell division and differentiation. Furthermore, TFIIB and the TAFs are obviously key targets in the repression of transcription during oncoprotein (PLZF-RARα, LAZ3/BCL6 and Mad/Mxi-1) mediated repression (6–10).

We conclude that N-CoR mediated repression of transcription by the nuclear receptors involves direct interactions with central components of the preinitiation complex that locks them into a non-functional state or conformation that passively leads to reduced acetylation. This process occurs in a background of active deacetylation induced by the corepressor mediated Sin3 and histone deacetylase interactions. We speculate that the resolution of the corepressor structures will provide valuable insights into the mechanics of the process and provide clues as to which other members of the initiation complex will be likely targets of corepressor action.

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