

The RITE Assay: Identifying Effectors that Target the Transcription Machinery Using Phage Display Technology

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ABSTRACT

We describe an approach using phage display to identify effectors (activators and repressors) of transcription based on the particular component of the general transcription machinery that they target. We refer to this approach as the reverse identification of transcriptional effectors (RITE) assay. A library of phages containing cDNA-encoded peptides displayed on their surfaces is screened using as the target a specific region of one of the general transcription factors (e.g., the C terminus of hTAF_{II}135). The amino acid sequence encoded by the cDNA of an interacting phage is determined and analyzed in a database homology search to identify known or novel factors that may interact with the target protein. Candidate effectors from the homology search are synthesized from recombinant clones and tested for their abilities to bind to the target protein and to functionally modulate transcription in vivo when co-expressed with the transcriptional target protein. Because the RITE assay is a direct measure of the interactions between general transcription proteins and their effectors, it has an advantage over the well-known yeast two-hybrid system, which is not amenable to identifying transcription factor interactions.

INTRODUCTION

The greatest challenge to follow the human genome project will be to ascribe functions to the plethora of proteins defined by open reading frames (ORFs). In the field of transcriptional regulation, many recombinant proteins that affect gene expression (effectors) have been identified empirically by testing their abilities to activate or repress transcription. More recently, the components of the general transcription machinery have been elucidated and shown to interact with specific effectors (2,7). To accelerate the identification of transcriptional effectors, we have developed an approach called the reverse identification of transcriptional effectors (RITE). The RITE assay is unlike typical approaches in which the effectors are known and then tested for their abilities to bind specific components of the transcription machinery. In the RITE assay, the effectors are unknown and are identified by their abilities to bind a particular component of the transcription machinery. Using the RITE assay, we have asked what peptide sequences expressed from a phage display cDNA library (15) are capable of interacting with a specific component of the transcription machinery, in this case, hTAF_{II}135 (9–11,17).

It is important to point out that the RITE assay, which depends simply on direct protein-protein interactions, differs from the well-known yeast two-hybrid screen (3), which relies on transcriptional activity as a measure of protein-protein interactions. In the

yeast two-hybrid screen, the target protein (bait) fused to a DNA binding domain, which recognizes a site upstream of the reporter gene, stimulates transcription upon binding a cDNA-encoded protein (prey) fused to an activation domain. In many cases, the target protein, particularly one that has a function in transcription, is able to activate transcription of the reporter gene in the absence of a cDNA-encoded protein, making the yeast two-hybrid screen unusable (14). Indeed, in the absence of prey, regions of hTAF_{II}135, our target in the RITE assay, activate transcription when fused to a DNA binding domain in the yeast two-hybrid screen (16).

Here, we demonstrate the utility of the RITE assay to identify effectors that interact with a component of the transcription machinery. Moreover, we discovered through the RITE assay that effectors may contain similar motifs that target distinct surfaces of the transcription machinery.

MATERIALS AND METHODS

The RITE Assay

The procedure for the RITE assay is depicted in Figure 1. A human placenta cDNA phage display library (Clontech Laboratories, Palo Alto, CA, USA) was screened for effectors using a C-terminal portion of hTAF_{II}135 as the target. Phagemid cDNAs were expressed as N-terminal fusions to the phage coat protein pIII.

Expression Plasmids

To create the hTAF_{II}135 target, the C-terminal 578 amino acids of hTAF_{II}135 were fused to the N terminus of the biotinylated carboxylase carrier protein (BCCP). The BCCP portion

is biotinylated on a specific residue allowing the fusion protein to be coupled to streptavidin agarose beads, creating an immobilized target. The plasmid pXa-1 578R, encoding the BCCP-hTAF_{II}135 C-terminal fusion, was generated by subcloning a *Bam*HI/*Not*I

fragment from pET21d578R (10) into pXa-1 (Promega, Madison, WI, USA). Constructs pTM1RAR α and pTM1 Δ N91 RAR α were generated by PCR cloning using pSG5RAR α (12) as the DNA template. The plasmids pTM1C/EBP α , pTM1 Δ N96C/EBP α , and

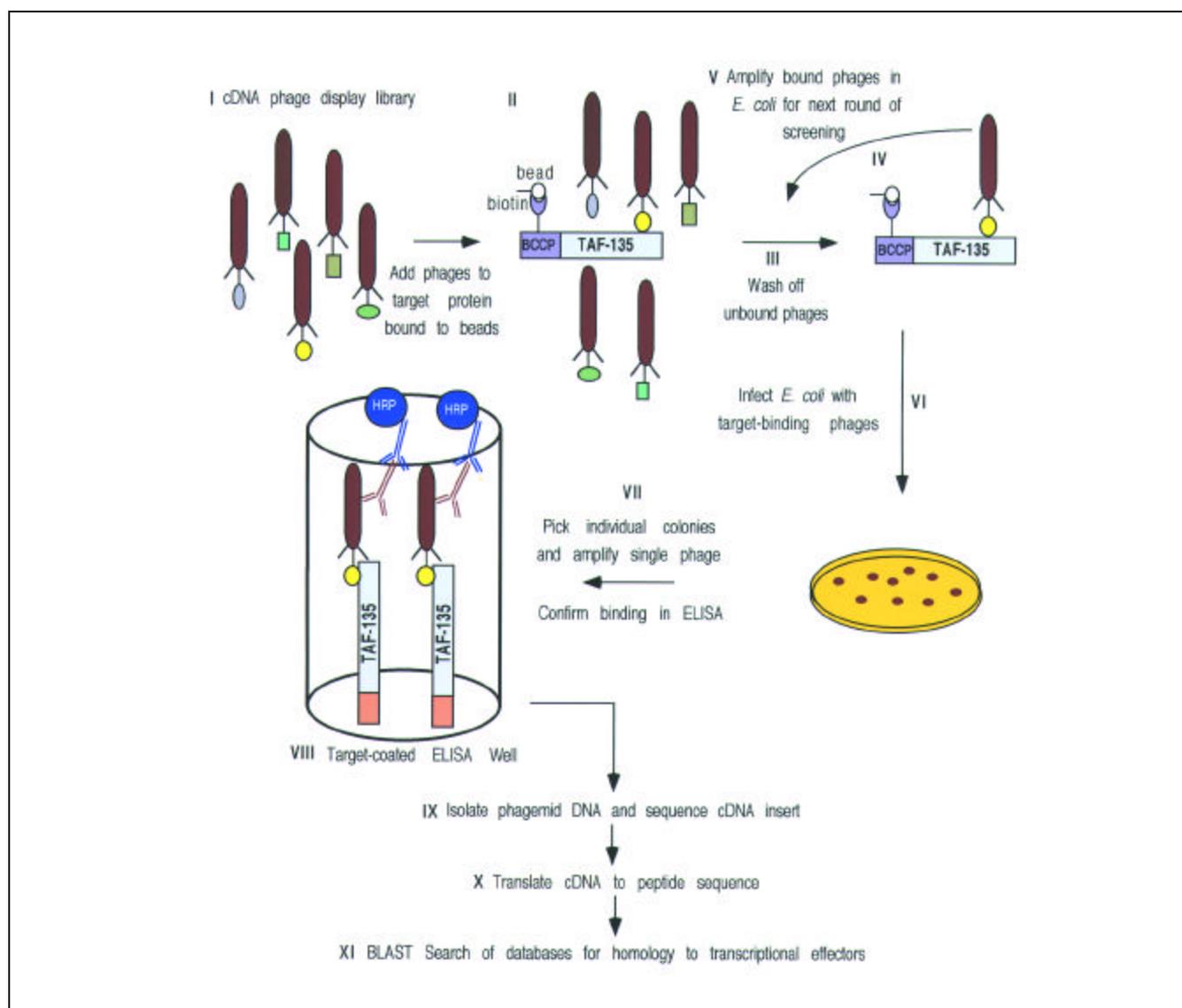


Figure 1. The RITE assay. The cDNA phage display library (10^{10} phage representing 10^6 independent clones and an insert size range of 60–660) (I) was added to the beads coupled with BCCP-hTAF_{II}135 fusion protein (about 1 μ g) (II) and allowed to bind at 37°C for 1 h in binding buffer (PBS containing 0.05% Tween[®]20 and 1% BSA). After washing the beads in PBS with 0.05% Tween 20 (III), the retained phage (IV) were amplified by infecting log phase XL-1 Blue cells at 37°C for 30 min (V). For the amplification, the infected XL-1 Blue cells were collected, grown at 37°C for 1 h in 2 \times yeast tryptone (YT)/glucose, following which helper phage were added and growth was continued for 1 h at 37°C in the presence of ampicillin. The infected cells were collected, resuspended in 2 \times YT containing ampicillin and kanamycin, and grown overnight at 37°C. Amplified phage were purified from the medium by polyethylene glycol precipitation and subjected to two additional rounds of screening with the BCCP-hTAF_{II}135 fusion protein (IV). Retained phage from the final screen were allowed to infect XL-1 Blue cells, which were then plated on 2 \times YT/ampicillin plates (VI). Fifty colonies containing phagemids were picked, and individual phage were amplified (VII) and added to ELISA wells coated with a glutathione S-transferase-hTAF_{II}135 C-terminal fusion (GST-hTAF_{II}135) and allowed to bind for 2 h (VIII). After washing the wells, phage that specifically recognized the C-terminal hTAF_{II}135 portion of the fusion were retained. Wells containing the positive-interacting phage were identified by the blue color in an ELISA assay using antibody against the phage coat protein pVIII followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (VIII). Phagemid DNAs from phage that were positive in the ELISA were isolated, and the cDNA inserts were sequenced (IX). When translated (X), the coding region of the largest cDNA insert (RITE-1 peptide) was analyzed by a BLAST search (XI) and shown to have homology to known transcriptional effectors.

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pTM1Δ70-200C/EBPα were created using either pSG5C/EBPα or pcDNAΔ70-200C/EBPα (13) as DNA templates. The plasmids pSG5PML-RARα (5) and pBSK⁺RXRα (8) have been described elsewhere.

Purification of Fusion Proteins and Binding Assays

Expression of the BCCP-hTAF_{II}135 C-terminal fusion was induced in the strain JM109 in the presence of 2 μM biotin. The cells were lysed, and the cleared supernatant was added to TetraLink™ Tetrameric Avidin Resin (Promega) and incubated at 4°C for 4 h. The resin was then washed in TBS-E and kept at 4°C until used in the RITE assay. Purification of GST-hTAF_{II}135 and the binding to radiolabeled proteins were as described (10) with the excep-

tion that the binding and washing buffers utilized in the assay are as described in Figure 1. Labeled proteins were synthesized from DNA templates by in vitro transcription/translation (Coupled TNT System; Promega).

Transfections

COS cells were transfected by calcium phosphate precipitation, and the total amount of plasmid DNA used in each transfection was normalized by the addition of pAT6 empty vector. Cell extracts were assayed after 48 h for reporter activity. In each assay, the luciferase activity was normalized based on protein concentration (Bio-Rad Laboratories, Hercules, CA, USA). The cytomegalovirus (CMV) expression vectors, pAT6 and pAT6-hTAF_{II}135 (372-1083) (11), have been described.

RESULTS AND DISCUSSION

In the RITE assay depicted in Figure 1, the C terminus of hTAF_{II}135 was used as the target protein for binding to phage displaying peptide sequences from a human placenta cDNA library. The placenta library was comprised of 10⁶ independent phage. Fifty phage obtained from repeated rounds of screening against the biotinylated-hTAF_{II}135 fusion protein were examined for their interaction with the GST-hTAF_{II}135 fusion protein. The utilization of two different fusion tags in the screening ensures specific selection of phage that target the hTAF_{II}135 moiety (Figure 1). The cDNA inserts of seven interacting phage were sequenced. Since the human peptides are expressed as fusions to the phage coat protein pIII and the cDNAs encoding the peptides reside within

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RITE-1 peptide 1 HASAGVSSPLSSHIEHSPHIQSCPGEPRGTLTLLSGSPDDLPEIDLPSVCCPSGT 55

RITE-1 peptide          9 PLSSHIEHSPHIQSCPGEPRGTLTLLSGSPDDLPEIDLPSVCC 51
P+S + SP                E + S PSPP LP I P C
RAR alpha              49 PVSGYSTPSPATIETQSSSSEEIVSPSPSPPLPRIYKPCFVC 91

RITE-1 peptide          9 PLSSHIEHSPHIQSCP--GEPREGTLTLLSGSPDDLPE 43
P+SSH++ PH S G PR      P+PP PE
C/EBP alpha          14 PMSSHLQSPPHAPSSAAFGFPRGAGPPKPPAPPAPE 50

RITE-1 peptide          9 PLSSHIEHSPHIQSC 23
P SH +HSP++QSC
XDLL1                76 PYMSHSQHSPYLQSC 90

RITE-1 peptide          12 SHIEHSPHIQSCPGEPRGTLTLLSGSPDDLPE 42
+H H P Q CP P L PS P P
DSXM                 439 AHFTHLPLTLQICPPTPEPLALSRSPSSPSGP 469

RITE-1 peptide 1 HASAGVSSPLSSHIEHSPHIQSCPGEPRGTLTLLSGSPDDLPEIDLPSVCCP 52
H + V L+H+ + +S G P + P PP LP LP+S P
ELL2              339 HLTNRVPPTLNGHLNPTSE-KSAAGLPLPPAAAAIPTPPPLPSTYLPISHPP 389
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Figure 2. The novel RITE-1 peptide has restricted homology to known transcriptional effectors. A BLAST homology search showing that the RITE-1 peptide, which interacts with the C terminus of hTAF_{II}135, has regions of limited homology to five different transcriptional effectors (see text for details). Identical amino acids are in bold, and those similar in character are denoted by a plus sign.

phage virions, there is a genotypic linkage between the displayed sequence and the cDNA encoding the sequence (15). The longest cDNA insert from a phage, which bound the hTAF_{II}135 target, was shown to encode a novel 55 amino acid sequence, referred to as the RITE-1 peptide (Figure 2, top line).

A basic alignment search tool (BLAST) homology search (1) revealed that the RITE-1 peptide has regions in which the identity and positions of particular residues appear to be conserved in five different transcriptional effectors: retinoic acid receptor alpha (RAR α), CCAAT/enhancer binding protein alpha (C/EBP α), *Xenopus* homeobox protein

distal-less 1 (XDLL1), *Drosophila* doublesex protein (DSXM), and RNA polymerase II elongation factor (ELL2) (Figure 2). RAR α and C/EBP α are two well-characterized transcriptional activators. Interestingly, homology to the RITE peptide-1 is within the N-terminal activation domains of both RAR α (12) and C/EBP α (13), suggesting that these regions likely contact the transcription initiation complex through hTAF_{II}135.

To test for a possible interaction between RAR α and hTAF_{II}135, we used the GST-hTAF_{II}135 fusion protein (Figure 1) and radiolabeled RAR α proteins. As shown in Figure 3A, RAR α exhibited strong binding to the GST-

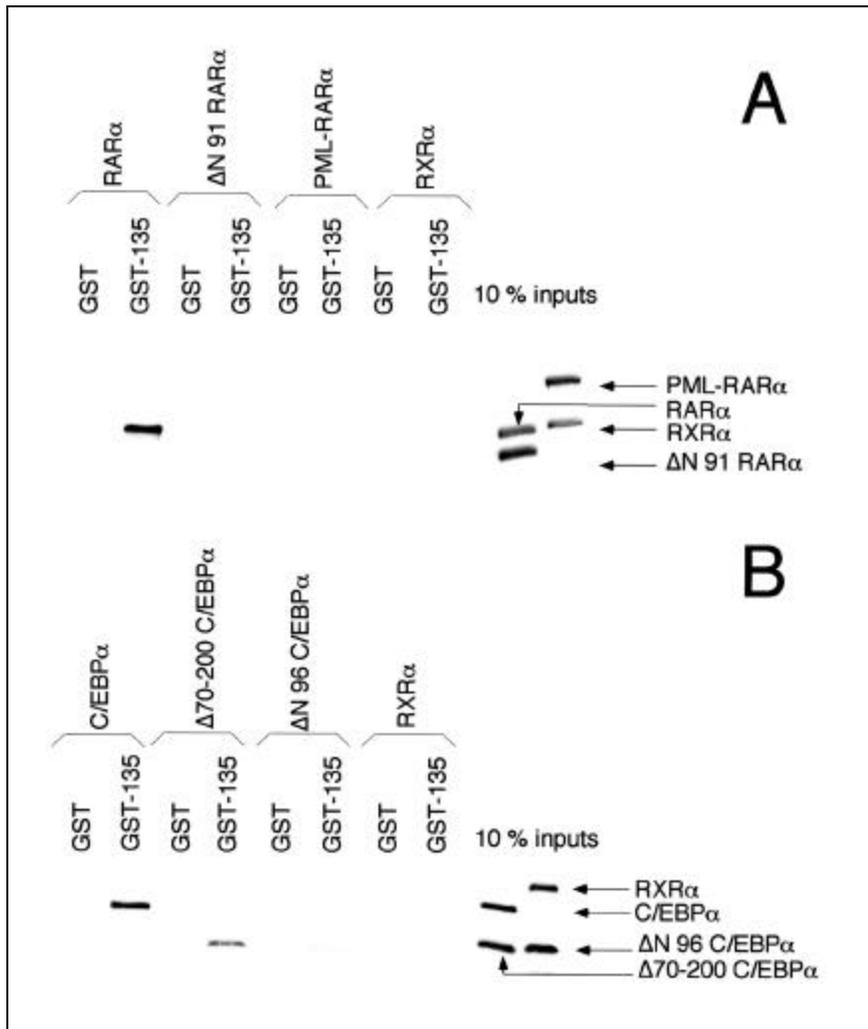


Figure 3. RAR α and C/EBP α interact with the C terminus of hTAF_{II}135. (A) A GST-hTAF_{II}135 C-terminal fusion peptide binds to labeled RAR α , but not to Δ N 91 RAR α , which lacks the region of homology to the RITE-1 peptide, nor to PML-RAR α or RXR α . (B) A GST-hTAF_{II}135 C-terminal fusion binds to labeled C/EBP α and Δ 70-200C/EBP α but not to Δ N96C/EBP α , which lacks the region of homology to the RITE-1 peptide, nor to RXR α . Ten percent of the input counts for each labeled protein are indicated.

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hTAF_{II}135 C-terminal fusion protein. Binding specificity was demonstrated by the inability of a different receptor, the retinoid X receptor alpha (RXR α), to bind the hTAF_{II}135 fusion protein. We then tested a mutant RAR α protein (Δ N 91 RAR α), in which 91 amino acids were deleted from the N terminus,

the region containing homology to the RITE-1 peptide. Indeed, Δ N 91 RAR α proved to be incapable of binding hTAF_{II}135. In promyelocytic leukemia (PML), a chromosomal translocation disrupts the domain of RAR α , which appears to interact with hTAF_{II}135, creating the fusion protein PML-RAR α

(5). As shown in Figure 3A, PML-RAR α also failed to bind hTAF_{II}135. These findings were confirmed by co-immunoprecipitation experiments. A hemagglutinin (HA)-tagged full-length hTAF_{II}135 protein was mixed with either radiolabeled RAR α , Δ N 91 RAR α , PML-RAR α or RXR α . Only RAR α was co-immunoprecipitated with hTAF_{II}135 using the antibody specific for the HA epitope (not shown).

We then investigated whether C/EBP α and hTAF_{II}135 were able to associate. As shown in Figure 3B, C/EBP α does bind to GST-hTAF_{II}135. However, when the N-terminal 96 amino acid coding region of C/EBP α , containing the region of homology to the RITE-1 peptide, was deleted (Δ N 96 C/EBP α), binding to hTAF_{II}135 was dramatically reduced. By contrast, Δ 70-200 C/EBP α , which retains the region of homology to the RITE-1 peptide, was still able to bind hTAF_{II}135. Again, binding specificity was demonstrated by the inability of RXR α to bind to hTAF_{II}135.

It was important to ascertain whether the hTAF_{II}135-RAR α interaction affected *in vivo* promoter activity. Transcription from a reporter construct, pRAR β 2Luc (4) containing a RAR α binding site in its promoter was stimulated when cotransfected with a RAR α expression vector in the presence of retinoic acid (Figure 4A). Expression of hTAF_{II}135 alone was also able to activate the RAR β promoter to the same extent, suggesting that the stimulating effect of exogenous hTAF_{II}135 maybe mediated through endogenous RAR α . However, co-expression of RAR α and hTAF_{II}135 resulted in significant stimulation of the RAR β promoter. Consistent with this finding is a recent study indicating that transfected hTAF_{II}135 is able to potentiate RAR α activation, although a direct interaction between RAR α and hTAF_{II}135, as shown here, was not reported (11).

We then tested if the addition of hTAF_{II}135 affects transcription mediated by C/EBP α . As seen in Figure 4B, when an obese promoter construct (6) containing a C/EBP α binding site was cotransfected with a C/EBP α construct into COS cells, there was an almost 50-fold increase in promoter activity. When C/EBP α was cotransfected with

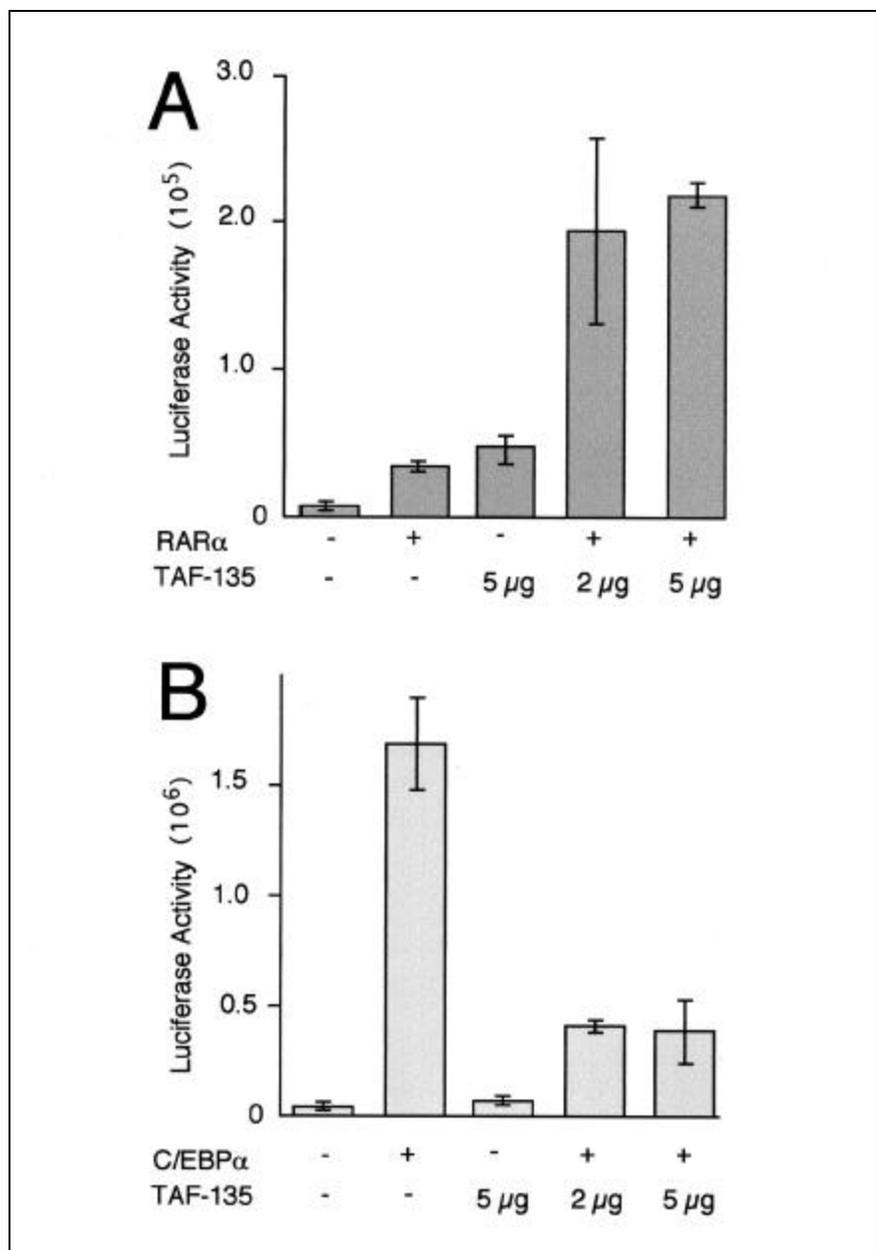


Figure 4. Expression of hTAF_{II}135 affects transcription mediated by RAR α and C/EBP α . (A) RAR α expression plasmid (2 μ g), RAR β promoter luciferase reporter plasmid (5 μ g), and 2 or 5 μ g of the hTAF_{II}135 expression vector [hTAF_{II}135 (372-1083)] were cotransfected into COS cells in the presence of retinoic acid (10 μ M). (B) C/EBP α expression vector (2 μ g), a luciferase reporter plasmid driven by the obese gene promoter (pObLuc-159) containing a C/EBP α binding site and the hTAF_{II}135 expression vector were cotransfected into COS cells. Luciferase activities were measured after 48 h. Results represent multiple experiments performed in duplicate.

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increasing concentrations of hTAF_{II}135, the promoter activity was reduced dramatically (Figure 4B). This result was corroborated by showing that activation mediated by Gal4-C/EBP α was also inhibited by hTAF_{II}135 overexpression, while activation mediated by Gal4-VP16 was completely unaffected by hTAF_{II}135 overexpression (not shown). The specific involvement of hTAF_{II}135 in this inhibition was further demonstrated by showing that overexpression of another human TAF (hTAF_{II}55) did not affect C/EBP α activation (not shown).

Both RAR α and C/EBP α are activators, and yet it is not surprising that overexpression of hTAF_{II}135 caused an increase in transcription by RAR α and a decrease in transcription by C/EBP α . Indeed, depending on the activator, such differences in the effect mediated by the overexpression of hTAF_{II}135 have been observed (9,11,16) and may reflect distinct ways in which exogenous hTAF_{II}135 either forms or precludes contact of the activator with transcription factor IID (TFIID).

In summary, the RITE assay is a novel approach to identify transcriptional effectors based on their abilities to target specific components of the general transcription machinery. All or part of a general transcription factor is presented to millions of human cDNA-encoded peptides expressed by a phage display library. In this study, we have used the RITE assay to screen for peptides that interact with the C terminus of hTAF_{II}135 and have identified a novel peptide (RITE-1). Remarkably, 50% of the 10 different proteins exhibiting homology to the RITE-1 peptide were known transcriptional effectors, while the remaining proteins were known to have other cellular functions. Indeed, we showed that hTAF_{II}135 could bind to and affect the transcriptional activities of two of these effectors. Importantly, binding to hTAF_{II}135 was destroyed by deleting the regions of these effectors, which show homology to the RITE-peptide. We anticipate that the other known transcriptional effectors will also associate with hTAF_{II}135. The fact that RITE-1 represents a novel amino acid sequence suggests that it is part of a new effector that is capable of interacting with hTAF_{II}135.

By utilizing the sequence information assembled by the human genome project, the RITE assay should make it possible to assign effector functions to new genes. Of practical significance, analysis of different tissue-specific phage display libraries by the RITE assay should lead to the discovery of novel effectors that are important in development and differentiation. Also, the RITE assay may define motifs belonging to certain groups of effectors, which interact with a specific surface of the transcription machinery, thereby lending insights into the regulation of gene expression. Most significantly, the RITE assay represents an invaluable approach in discovering activators and repressors that, when either mutated or abnormally expressed, may be involved in oncogenesis.

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