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The goal of this project was to understand the transcriptional regulation of the NF1 gene. Specifically to further characterize TLF-mediated regulation and identify new factors that participate in the control of NF1 regulation. In addition, an attempt was made to determine signaling pathways that affect NF1 levels.
We found that TLF(TRF2) activates a number of different genes that include the Neurofibromatosis I (NF1)gene. Overexpression of TLF increases the amount of NF1 mRNA in cells. *In vivo*, TLF binds and up regulates transcription from a fragment of the NF1 promoter. *In vitro*, purified TLF-TFIIA binds directly to the same NF1 promoter fragment that is required for TLF-responsiveness in cells. In contrast, TLF inhibits transcription driven by a fragment of the TATA-containing *c-fos* promoter by sequestering TFIIA. TBP affects the NF1 and *c-fos* promoters in a reciprocal manner to TLF, stimulating the *c-fos* promoter and inhibiting NF1 transcription. Thus, it appears that TLF is a functional regulator of transcription with targets distinct from those of TBP.

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INTRODUCTION

Neurofibromatosis Type 1 (NF1) is a common genetic disorder that affects approximately 1:3,500 people. The variety of mutations that result in the NF1 phenotype and the observation that cell lines from afflicted individuals display abnormally low levels of neurofibromin protein led to the hypothesis that the main cause of NF1 is haploinsufficiency. Therefore, the ability to upregulate NF1 could be of critical importance to the treatment of the disease, however, the factors regulating transcription of NF1 are unknown. This project was designed to understand this transcriptional regulation by further characterizing a new basal transcription factor, termed "TATA-binding protein (TBP)-like factor" (TLF) that upregulates the endogenous NF1 transcript greater than 4-fold. The goal of this project is to characterize TLF-mediated regulation and identify new factors that participate in the control of NF1 transcription.

The year one workplan called for the identification of specific regions of the NF1 promoter that are responsive to the NF1 promoter. Since this project terminated early due to the departure of the investigator neither task 2 or 3 of the workplan which were supposed to have been done in the second and third year of this award were initiated. However, considerable progress was made on the work associated with task 1 and this work is described below.

BODY

Here we show that human TLF and TBP can affect gene transcription in reciprocal and opposite fashions. We identify a number of human genes that respond to TLF over-expression in vivo, including the neurofibromatosis type I (NF1) gene. NF1 is a tumor suppressor and many of the symptoms of neurofibromatosis, which include café-au-lait spots, benign peripheral neurofibromas, and malignant plexiform neurofibromas, may result from haploinsufficiency at the NF1 locus (Cichowski and Jacks 2001). We show that TLF binds and increases transcription from a fragment of the NF1 promoter in vivo. In vitro purified TLF-TFIIA binds to the same region of the NF1 promoter that determines TLF responsiveness in vivo. TBP does not bind to the NF1 promoter in cultured cells, and transcription from an NF1 promoter fragment is inhibited by TBP over-expression. Conversely, TBP stimulates transcription from the c-fos promoter, while TLF inhibits it by sequestering TFIIA. Thus, TBP and TLF regulate the NF1 and c-fos promoters in a reciprocal manner.

RESULTS

Tissue and subcellular distribution of TLF

We identified hTLF by screening the human EST database with a sequence corresponding to the C-terminal half of TBP. As has been reported previously (Ohbayashi et al. 1999b; Rabenstein et al. 1999; Teichmann et al. 1999) Northern blot analysis revealed that this mRNA expresses ubiquitously at moderately low levels and at TLF is a Functional Regulator of Transcription Chong et al 5 high levels in testis (data not shown). To determine the protein distribution, we made a polyclonal anti-peptide antibody against the C-terminal region of TLF. This antibody recognized both bacterially expressed TLF and epitope-tagged TLF from mammalian cells. In protein extracts from mouse tissue, the antibody recognized a 21 kDa band that corresponded to the predicted

molecular weight of TLF. Addition of excess peptide blocked this interaction (data not shown).

When multiple tissues from mouse and human were probed on Western blots, a significant disparity between RNA and protein levels was apparent. TLF protein was expressed at highest levels in mouse liver and pancreas (Figure 1A) with strong reactivity in heart at a slightly higher molecular weight. Longer exposures revealed a band from brain tissue corresponding to 21 kDa. This distribution differs from that reported by Perletti et al. who observed very high levels of TLF in brain. The differences in these results may be attributed to differing solubilization conditions and normalization procedures.

The different patterns of TLF mRNA and protein distribution indicate a high degree of post-transcriptional regulation. Post-transcriptional regulation, particularly obvious in testis, is a feature of several basal transcription machinery proteins (Schmidt and Schibler 1995; Dikstein et al. 1996; Schmidt and Schibler 1997; Perletti et al. 1999). Comparison of our Western blot results with previously published data on the distribution of TBP indicated that the ratio of TBP to TLF varies from tissue to tissue. Western blot analysis TLF is a Functional Regulator of Transcription Chong et al 6 of different human cell lines confirmed that the amounts of TLF and TBP varied with respect to one another while the amount of TFIIB was relatively constant (Figure 1B).

Since our antibody does not recognize TLF in its native form, we used GFP fusion proteins to determine the localization of TLF and TBP in live, unpermeabilized, cells. A TLF-GFP fusion protein, GFP, and RFP were introduced into HEK-293 (Figure 1C, D) and COS-7 cells (Figure 1G, H, J). While GFP and RFP did not localize to any particular subcellular organelle (Figure 1C, D), the TLF-GFP fusion protein localized to the nucleus (Figure 1D). Strikingly, TLF-GFP associated strongly with nucleoli (Figures 1G, H, J) in both HEK-293 and COS-7 cells. In contrast, TBP-GFP localized to the nucleus but was virtually excluded from the nucleoli (Figure 1E, F, I).

As is true for native TBP (Jordan et al. 1996), TBP-GFP is extra-nucleolar. However, mild detergent treatment dramatically altered its localization. After the ten minute incubation with 0.2% Triton X-100 that has been reported to be required to visualize TBP in the nucleolus (Jordan et al. 1996), TBP-GFP filled the entire nucleus, including the nucleolus (data not shown). This treatment did not change the distribution of TLF-GFP. TLF-GFP persistently localized to the nucleolus even during cytokinesis. Nucleoli of TLF-GFP transfected cells rapidly recovered from photobleaching, indicating that their association with TLF-GFP is dynamic. The differential localization of TLF and TBP in the nucleus indicates that they may subserve different functions and that TLF may regulate transcription of rRNA.

Identification of TLF targets

To determine whether TLF regulated a unique subset of genes within the cell, we screened Affymetrix Hu35K gene chips representing roughly 35,000 different human ESTs. We probed the chips with biotinylated cRNA made from HEK-293 cells that transiently overexpressed both GFP and TLF, or GFP alone. The data from the gene chip screening were normalized to overall intensity (see supplementary data at **http://clapham.tch.harvard.edu/publications/supplementary.html)**. Since TLF can indirectly inhibit transcription from viral TATA-containing promoters by sequestering essential factors

(Moore et al. 1999; Teichmann et al. 1999), we focused on genes that were upregulated. TLF overexpression increased 125 (~0.35%) of the 35,000 ESTs represented on the chips more than 3-fold. This number is likely a significant underestimate of the number of genes TLF regulates because the criteria used to determine up-regulation were quite stringent. In addition, the concentration of TLF may not be the rate-limiting step in transcription from a promoter with multiple other transcription factor binding sites.

We verified the up-regulation of a number of targets including NF1 (3.7 fold) (Figure 2A), jumonji (5.2 fold), and ACF7 (14.4 fold) by RNase Protection Assay (data not shown). We chose the NF1 promoter as a model to test the activity of TLF because it had been previously characterized (Hajra et al. 1994; Feigenbaum et al. 1996), and its regulation has clinical significance (Cichowski and Jacks 2001).

Primer extension assays show that TLF stimulates transcription from the NF1 gene without altering the start sites (Figure 2B). Unlike alternate TBPs in Drosophila which upregulate their targets from a subset of transcriptional start sites (Hansen et al. 1997; Holmes and Tjian 2000), TLF displayed no obvious preference for start sites (its overexpression increased expression of all transcripts to a similar degree). Comparison with a sequencing ladder indicated multiple transcriptional start sites (see sequence in Figure 5A), which is not uncommon for promoters that lack a TATA-box (Ye et al.1993). Interestingly, the start sites that we observed in HEK cells differ from those reported in brain (Hajra et al. 1994) suggesting that tissue-specific factors lead to differential utilization of the NF1 promoter.

Having subcloned a 350 base pair fragment of the NF1 promoter into pCAT3-Basic, we sought to determine whether this reporter construct termed pNF1-CAT effectively mimicked the endogenous gene. Primer extension assays showed that this reporter gene construct employed the same transcriptional start sites as the endogenous NF1 promoter (Figure 2C). In addition TLF stimulated transcription from this promoter in a manner analogous to the endogenous, suggesting that pNF1-CAT is an appropriate model of promoter function.

We subsequently tested whether TLF up-regulated NF1 transcription by binding to the NF1 basal promoter by plasmid immunoprecipitation (PIP) assays (Figure 3A). This technique is a modified form of the Chromatin Immunoprecipitation technique (ChIP) which detects the binding of transcription factors to chromosomal targets (Kuras and Struhl 1999). HEK-293 cells were cotransfected with TLF-GFP, TBP-GFP or the parental eGFP and pNF1-CAT. A 1% formaldehyde treatment crosslinked protein to plasmid DNA and GFP containing complexes were isolated by immunoprecipitation. From these immunoprecipitates, we were able to amplify the NF1 sequence when cells were transfected with TLF-GFP, but not from samples containing either eGFP or TBP-GFP (Figure 3C). We confirmed expression of eGFP, TBP-GFP and TLF-GFP by Western blotting with the anti-GFP antibody (Figure 3B). pNF1-CAT was present in all inputs to the assay (Figure 3C). We conclude that TLF-GFP, but not TBP-GFP, interacts with the NF1 promoter. In contrast, the TATA-containing c-fos promoter co-immunoprecipitated with TBP-GFP, but not TLF-GFP (Figure 3D).

Reciprocal Regulation of NF1 and c-fos by TBP and TLF

Hypothesizing that TLF binding limits the rate of transcription from the NF1 promoter, we transfected pNF1-CAT into HEK-293 cells with GFP, TLF-GFP, or TBP-GFP. When compared to GFP alone, co-transfection with TLF-GFP increased the amount of CAT reporter activity from pNF1-CAT in a dose dependent manner. At the highest concentration of DNA, TLF-GFP caused a 4-fold increase in CAT activity as compared to GFP (n=6; Figure 4A).

To determine if the TLF mediated stimulation of the NF1 promoter is specific, we introduced TBP-GFP into cells with the pNF1-CAT construct. Surprisingly, TBP-GFP overexpression decreased activity from the NF1 promoter in a dose dependent manner, with the highest plasmid concentration leading to a more than 4-fold repression (n=6) (Figure 4B). Since even low levels of TBP produced an inhibitory effect, it is unlikely that the inhibition we see is a result of squelching. Non-fusion protein versions of TLF and TBP yielded identical results (data not shown). TBP-GFP mediated stimulation of the viral SV40 promoter confirmed that this TBP construct was functional (data not shown).

In order to identify which fragment in the NF1 promoter responded to TLF we performed deletion analysis. A fragment containing bases 1-103 of pNF1-CAT was necessary and sufficient for TLF-mediated stimulation (Figure 5B). This agrees well with our finding that TLF stimulates transcription from a group of start sites contained within this fragment (Figure 5A). TLF also stimulates transcription from some lesser sites downstream of this fragment. The fact that this downstream region was unresponsive to TLF overexpression in CAT assays has several possible explanations. Unlike TBP, TLF may stimulate transcription from start sites more than 30 bp from its binding site (Conaway and Conaway 1993). Alternatively, another transcription factor may bind in nucleotides 1-103 which is required for TLF-mediated NF1 transcription. TBP-mediated inhibition was specific in that the 103-222 nucleotide fragment was not stimulated by TLF and was not inhibited by TBP (data not shown).

To determine if TLF also binds the NF1 promoter in vitro, we performed electrophoretic mobility shift assays (EMSAs). Unfortunately, attempts to generate recombinant TLF in bacteria were fruitless. Consequently we used protein immunopurified from a HeLa cell line that stable expressed a FLAG-tagged TLF (Teichmann et al., 1999). In addition to TLF, the purified fraction contained stoichiometric quantities of the GTF, TFIIA. In mobility shift assays, the TLF protein specifically bound a radiolabelled probe corresponding to the 350 base pair promoter fragment from pNF1-CAT. Consistent with our deletion analysis, a 10-fold molar excess of unlabelled 1-103 fragment abolished this interaction. In contrast, a 10-fold excess of the 103-350 fragment did not affect retardation on the gel. To further delineate the TLF binding site we created four double-stranded oligonucleotides that covered the 1-103 region of the NF1 promoter. Incubation of an excess of oligonucleotide 3, but not 2 or 4, abolished the TLF-TFIIA mediated shift of the 1-103 probe (Figure 6A). Unlabelled oligonucleotide 1 also significantly reduced, but did not eliminate, this shift. The results of these binding experiments suggest the existence of multiple binding sites for TLF within the 1-103 fragment. The presence of multiple binding sites for TLF is consistent with the large number of start sites we observed.

TLF-TFIIA also effectively retarded the migration of a radiolabelled oligonucleotide 3 (Figure 6B).

These findings suggest that the 24 base pair sequence of oligonucleotide 3 is sufficient for TLF-TFIIA binding. In addition, TLF interacts with the same portion of the NF1 promoter in vivo and in vitro. TLF and TBP expression affect the TATA-containing c-fos promoter, in a reciprocal manner. As expected (Colgan and Manley 1992; Chatterjee and Struhl 1995; Moore et al. 1999), overexpression of TBP-GFP more than doubled CAT activity from the basal cfos promoter. Transfection with TLF-GFP, in contrast, decreased the rate of transcription from the c-fos promoter in a dose-dependent fashion (Figure 7), consistent with previous reports (Moore et al., 1999; Teichmann et al., 1999)

The reports cited above also showed that in vitro TLF-mediated inhibition could be overcome by addition of excess TFIIA. To determine if competition for TFIIA also inhibited TBP-regulated promoters in vivo, we mutated a single amino acid of the proposed TFIIA binding site in TLF (TLFN37Y; Figure 8A). We chose to mutate this region based on its homology with the TFIIA binding site in TBP (Bryant et al., 1996). TLFN37Y-GFP and TLF-GFP expression levels were similar (Figure 8C) and both localized to the nucleolus (Figure 8B). To determine if TLFN37Y was deficient in binding to TFIIA, we performed co-immunoprecipitation experiments using an anti-GFP antibody. As expected, TFIIA co-immunoprecipitated with TLF-GFP but not TLFN37Y-GFP. Interestingly, TLF-GFP stably associated with p55, the uncleaved precursor of TFIIA□ and □ as well as the cleaved alpha subunit (Figure 8D, 8E). TLFN37Y was much less effective in inhibiting the c-fos promoter than TLF (Figure 8F) and it failed to stimulate the NF1 promoter (data not shown). Additionally, overexpression of TFIIA alleviated TLF-mediated inhibition of c-fos transcription (Figure 8F). The simplest interpretation of these results is that TLF sequesters TFIIA to inhibit TBP-mediated transcription.

Mutating the TFIIA binding site in TBP dramatically decreased transcription from the cfos promoter and decreased cell viability. Unlike TFIIA.s abrogation of TLF-mediated repression, overexpression of TFIIA did not relieve TBP-mediated repression of pNF1- CAT (data not shown). This suggests that TBP competes with TLF for a factor other than TFIIA. This competition for an as yet unidentified general transcription factor (GTF) may be specific for TLF-responsive promoters since TBP over-expression did not diminish the activity of the pNF1-CAT 103-222 construct.

KEY RESEARCH ACCOMPLISHMENT

Our results suggest that TLF is not simply a .false face., but a functional activator of transcription. However, TLF can inhibit transcription from promoters driven by the TBP, and TBP can inhibit transcription from TLF-driven promoters.

REPORTABLE OUTCOME

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PERSONNEL:

Drew Chong, Ph.D.

CONCLUSIONS

Our results show that human TLF is a functional transcription factor with a distinct role in gene regulation. TLF increases transcription from the endogenous NF1 promoter, using the same start sites employed in the production of the endogenous transcript. TLF also binds and increases the rate of transcription from an NF1 reporter construct. Finally purified TLF-TFIIA binds the same region of the NF1 promoter in vitro as TLF-GFP does in cells.

TBP, in contrast, does not bind the NF1 promoter and inhibits its transcription. TBP and TLF also affect the TATA-containing c-fos promoter in a reciprocal manner: TBP increased, while TLF decreased transcription from this promoter. These findings are summarized in a model in which TLF and TBP compete for members of the basal transcription machinery (Figure 9). The model incorporates the .false face. hypothesis for TLF in which TLF acts to buffer proteins that bind TBP, and establishes TLF as a functional activator of transcription.

TLF and TBP are differentially expressed

TBP and TLF protein levels are uncorrelated and variable in different cell types. Unlike TBP, TLF protein is highest in liver or pancreas. Both TLF and TBP are expressed at low levels in brain. The ratio of TLF to TBP differed in HeLa, HEK-293, and SK-N-SH human cell lines. These distinct expression patterns suggest that TLF and TBP may regulate distinct suites of genes.
In support of their distinct roles, TLF-GFP strikingly localized to the nucleolus while TBP was largely excluded from the nucleolus. The nucleolar localization of TLF raises the question of whether TLF participates in Pol I-mediated transcription. Although TBP is associated with SL1 and rDNA transcription, the majority of TBP is extra-nucleolar (Hernandez, 1993; Roeder, 1996) and we propose that detergent treatment enhances the appearance of TBP in the nucleolus.

The TLF-deficient transgenic mouse (Martianov et al. 2001; Zhang et al. 2001) displayed only a defect in spermiogenesis, in contrast to severe defects observed in other TLF-null animals. This is surprising since TLF is highly expressed in many other tissues and TLF overexpression does not specifically up-regulate testis-specific transcripts. RNA interference and antisense approaches in zebrafish, Xenopus, and C. elegans indicate that TLF is crucial to gastrulation. One resolution to this conundrum is that multiple loci may encode murine TLF; the hTLF locus on human chromosome 6q22.1-q22.3 (Ohbayashi et al. 1999a) is 95% identical to a locus on chromosome 3.

Overexpressed TLF and TBP compete for GTFs.

We have demonstrated that TLF mediated repression of the c-fos promoter depends on both the ability of TLF to interact with TFIIA, and TFIIA availability. By binding TFIIA, TLF decreases the amount of TFIIA available for TBP binding and thus indirectly regulates the c-fos promoter, as

was suggested in the false face hypothesis. Mutation of the TFIIA binding site on TBP led to a significant decrease in all reporter constructs tested, leading us to hypothesize that disrupting TBP-TFIIA interactions reduces overall transcription and cell viability. In the case of the NF1 promoter however, expression of additional TFIIA does not increase activity above that provided by TBP expression alone. One likely explanation for this observation is that TBP competes with TLF for a non-TFIIA GTF.

SO WHAT

Two conclusions can be made from this study. First, while TLF has the potential to buffer TFIIA, TLF's main function is to substitute for TBP on a group of promoters and activate transcription of genes, NF1 being one example. Second, the basal transcription machinery is not simply permissive but rather provides specificity for gene regulation.

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FIGURE LEGENDS

Figure 1. TLF and TBP have different tissue and subnuclear localizations. (A) Western blot showing the presence of TLF in Heart (He), Kidney (Ki), Liver (Li), Lung (Lu), Pancreas (Pa) and Testis (Te) from adult mouse. Longer exposures showed TLF was also present in Brain (Br). (B) Western blots showing TLF:TBP ratios in different cell lines. Equal amounts of protein from HEK-293, HeLa, and SK-N-SH cells were loaded. Parallel blots were stained either for TFIIB (left panel), TLF (upper right) or TBP (lower right). HEK-293 cells have a lower TLF:TBP ratio than either SK-N-SH or HeLa cells. The amount of protein in each lane was normalized by BCA protein assay and verified by Ponceau S staining. (C) eGFP does not localize to the nucleus. The upper cell is completely filled with eGFP including the nucleus (stained with Hoechst dye). The lower cell was untransfected allowing visualization of the nucleus. (D) TLF-GFP localizes to the nucleus. HEK-293 cells were co-transfected with RFP and TLF-GFP. RFP fills the cell, while TLF-GFP is restricted to the nucleus. (E through H) TBP-GFP and TLF-GFP have different sub-nuclear localizations. Arrowheads mark the position of the nucleoli. (E and F) Transmission DIC and fluorescence images of COS-7 cells transfected with TBP-GFP fusion protein. (G and H) Transmission DIC and fluorescence images of COS-7 cells transfected with TLF-GFP fusion protein. Arrowheads mark the position of the nucleoli. (I and J) High-resolution images show that TBP-GFP is excluded while TLF-GFP is concentrated in the nucleoli. Scale bars = 10 □m.

Figure 2. TLF upregulates the NF1 transcript using the endogenous start sites. (A) TLF is a Functional Regulator of Transcription Chong et al 23
RNase protection assay comparing levels of NF1 transcript in HEK cells expressing GFP and those expressing TLF. The probe generated again NF1 recognizes a 306 bp fragment of the 3. UTR. A □-actin probe served as a control to ensure equivalent precipitation of the samples. (B) Primer extension analysis shows that TLF upregulates NF1 transcription from the endogenous start sites. RNA from cells transfected with TLF-GFP or GFP alone was used for primer extension analysis with an antisense primer corresponding to bases 328-346 of the NF1 promoter. The start sites of the NF1 transcript (arrows) were identical in samples transfected with TLF-GFP and those transfected with GFP indicating that TLF is regulating NF1 in a physiologically relevant manner. (C) Primer extension analysis showing that pNF1-CAT uses the same start sites as the endogenous NF1 transcript. As indicated by the numbers at the bottom, when cells were transfected with pNF1-CAT, 15 □g of RNA was used per reaction, as compared to 100 □g for cells transfected with GFP alone.

Figure 3. Plasmid immunoprecipitation shows that TLF binds directly to the pNF1-CAT reporter construct. (A) Diagrammatic representation of the PIP procedure. pNF1-CAT and GFP, TLF-GFP or TBP-GFP are cotransfected into HEK cells. After 40 hours, a 1% formaldehyde treatment crosslinked the proteins to DNA. Subsequent immunoprecipitation with an anti-GFP antibody and Proteinase K digestion allows identification of DNA regions that associate with the fusion protein. (B) Expression of GFP containing TBP and TLF proteins was verified by Western blot analysis using an anti-GFP antibody. (C) NF1 immunoprecipitates with TLF-GFP, but not TBP-GFP or

GFP. Cells transfected with the NF1 promoter plasmid and GFP, TLF-GFP, or TBP-GFP

(as indicated) were subjected to PIP and the 350 bp band detected by agarose gel electrophoresis (lanes marked PIP products). The presence of the NF1 plasmid in the cultures was verified by performing PCR on an aliquot of the lysates removed just prior to mixture with agarose beads, and purified by Proteinase K digestion and phenol:chloroform extraction (lanes marked PIP inputs). (D) The c-fos promoter associated with TBP-GFP, but not TLF-GFP. Cells transfected with the c-fos promoter plasmid and TLF-GFP, or TBP-GFP (as indicated) were subjected to PIP and the 2100 bp band was detected by agarose gel electrophoresis (lanes marked PIP products). The presence of the c-fos plasmid in the cultures was verified by performing PCR on an aliquot of the lysates.

Figure 4. TLF, not TBP, increased transcription from the NF1 promoter. (A) CAT assays showing that TLF stimulates transcription from an NF1 promoter fragment in a dose dependent manner. Top: Thin layer chromatography (TLC) plate showing an increase in pNF1-CAT activity, measured by the acetylation of the FAST-CAT substrate (Molecular Probes), in response to TLF overexpression. Bottom: Quantification of the effects of TLF on pNF1-CAT activity. The effects of TLF on pNF1-CAT were quantified by fluorometry and were compared to cells co-transfected with pNF1-CAT and eGFP. The error bars represent the standard error of the mean (SEM; n=6). The numbers on the bottom indicate μg of transfected TLF-GFP. Total DNA transfected in each condition was equalized to 6 μg by adding eGFP-C3. (B) TBP decreased pNF1-CAT activity in a dose-dependent manner. Top: thin layer chromatography (TLC) plate showing a decrease in pNF1-CAT activity, measured by the acetylation of the FAST-

CAT deoxy green substrate (Molecular Probes), in response to TLF overexpression. Bottom: Quantification of the effects of TBP on pNF1-CAT activity. The effects of TBP on pNF1-CAT were quantified by fluorometry and were compared to cells co-transfected with pNF1-CAT and eGFP. The error bars represent the standard error of the mean (SEM; n=6). The numbers on the bottom indicate the μg of transfected TBP-GFP. Total DNA transfected in each condition was equalized to 6 μg by addition of eGFP-C3.

Figure 5 Deletion analysis shows that nucleotides 1-103 of pCAT-NF1 are necessary and sufficient for responsiveness to TLF. (A) The sequence of the NF1 promoter fragment from pNF1-CAT. This sequence differs slightly from the published sequence of the NF1 promoter (Hajra et al., 1994), but is identical to genomic sequences in the public database that correspond to the NF1 locus. The responsive region is shaded grey (1-103). Putative transcriptional start sites determined by primer extension analyses are underlined. Oligonucleotides 1 and 3, which contain potential TLF binding sites are indicated. (B) The effects of TLF on each reporter construct were quantified by fluorometry and compared to pNF1-CAT transfected with eGFP (± SEM; n=6).

Figure 6 TLF binds directly to the NF1 promoter in vitro. (A) Electrophoretic mobility shift assays show that TLF binds directly to the 1-103 fragment. The first lane shows the height of the radiolabelled probe in the absence of proteins. The second lane shows that

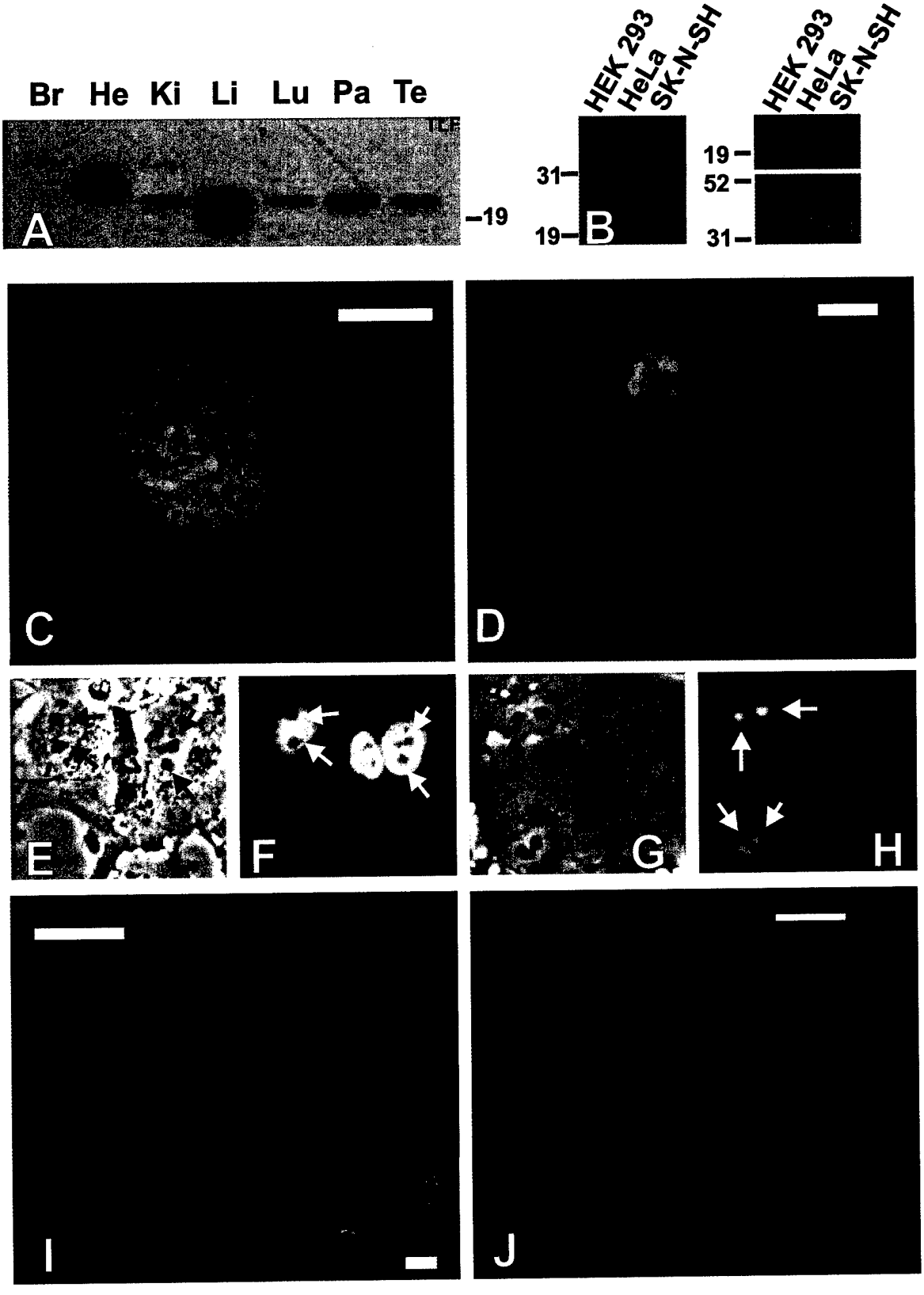
the addition of approximately 10 ng of TLF-TFIIA caused retardation of some of the probe. This retardation was reduced by a ten-fold excess of unlabelled oligonucleotide 1 (lane 3) and was eliminated by a ten-fold excess of unlabelled oligonucleotide 3 (lane 5), suggesting that these oligonucleotides contained TLF binding sites. Oligonucleotides 2

and 4 did not affect the shift (lane 4). (B) Electrophoretic mobility shift assays show that TLF directly bound oligonucleotide 3. The first lane is the radiolabelled probe in the absence of proteins. In the second lane, addition of ~10 ng of TLF-TFIIA retarded probe migration.

Figure 7 TLF and TBP have reciprocal effects on a fragment of the c-fos promoter. (A) Quantification of the effects of TBP on fos-CAT activity. The effects of TBP on fos-CAT were quantified by fluorometry and were compared to cells co-transfected with fos-CAT and eGFP (SEM; n=6). The numbers on the bottom indicate μg of transfected TBP-GFP. Total DNA transfected in each condition was equalized to 6 μg by addition of eGFP-C3. (B) Quantification of the effects of TLF on fos-CAT activity. The effects of TLF on fos-CAT were quantified by fluorometry and were compared to cells co-transfected with fos-CAT and eGFP (SEM; n=6).

Figure 8 TLF inhibits c-fos reporter gene activity by sequestering TFIIA. (A) Asparagine 37 of TLF-GFP was substituted by tyrosine in the construct TLFN37Y-GFP to disrupt TLF.s TFIIA binding site. (B) TLF-GFP and TLFN37Y-GFP localization in COS-7 cells was similar. Scale bars = 10 □m. (C) Western blot analysis shows that TLF-GFP and TLFN37Y-GFP are expressed at similar levels. BCA protein assays were used toensure equal amounts of total protein were loaded in each lane. (D) TLF-GFP and TLFN37Y-GFP coimmunnoprecipitated with an anti-GFP antibody (Molecular Probes) with similar efficiencies from transfected HEK cells. (E) Western blot with an anti-TFIIA□ antibody to GFP immunoprecipitated materials products from TLF-GFP or TLFN37Y-GFP-transfected cells. TFIIA did not co-immunoprecipitate TLFN37Y-GFP, indicating that the mutation disrupted TFIIA-TLF interactions. Interestingly the uncleaved p55 product (TFIIA □ and □) also efficiently immunoprecipitated with TLF. (F) Repression of the c-fos reporter by TLF-GFP, TLFN37Y-GFP, GFP + TFIIA, and TLF-GFP+TFIIA. Addition of excess TFIIA or mutation of the TLF TFIIA binding site alleviated TLF-mediated inhibition of c-fos reporter gene activity (± SEM.; n=6, 5, 3 and 5, respectively).

Figure 9. Model for TLF function. TLF and TBP bind to the NF1 and c-fos promoters, respectively. In the model, TLF competes with TBP for TFIIA, thus inhibiting TBP-responsive promoters. TBP competes with TLF for an undetermined GTF to inhibit TLF-responsive promoters.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HeLa
SK-N-SH
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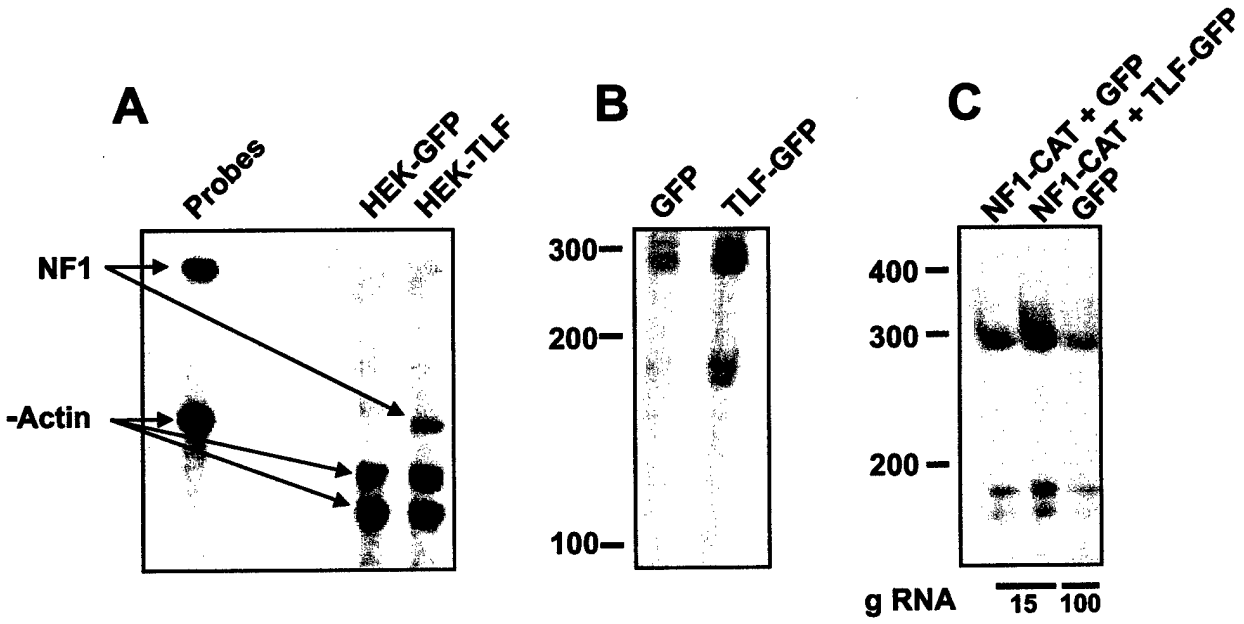
31
C
D
E
F
G
H
I
J

Figure 1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HEK-TLF
NF1
-Actin
B
GFP
TLF-GFP
300
200
100
C
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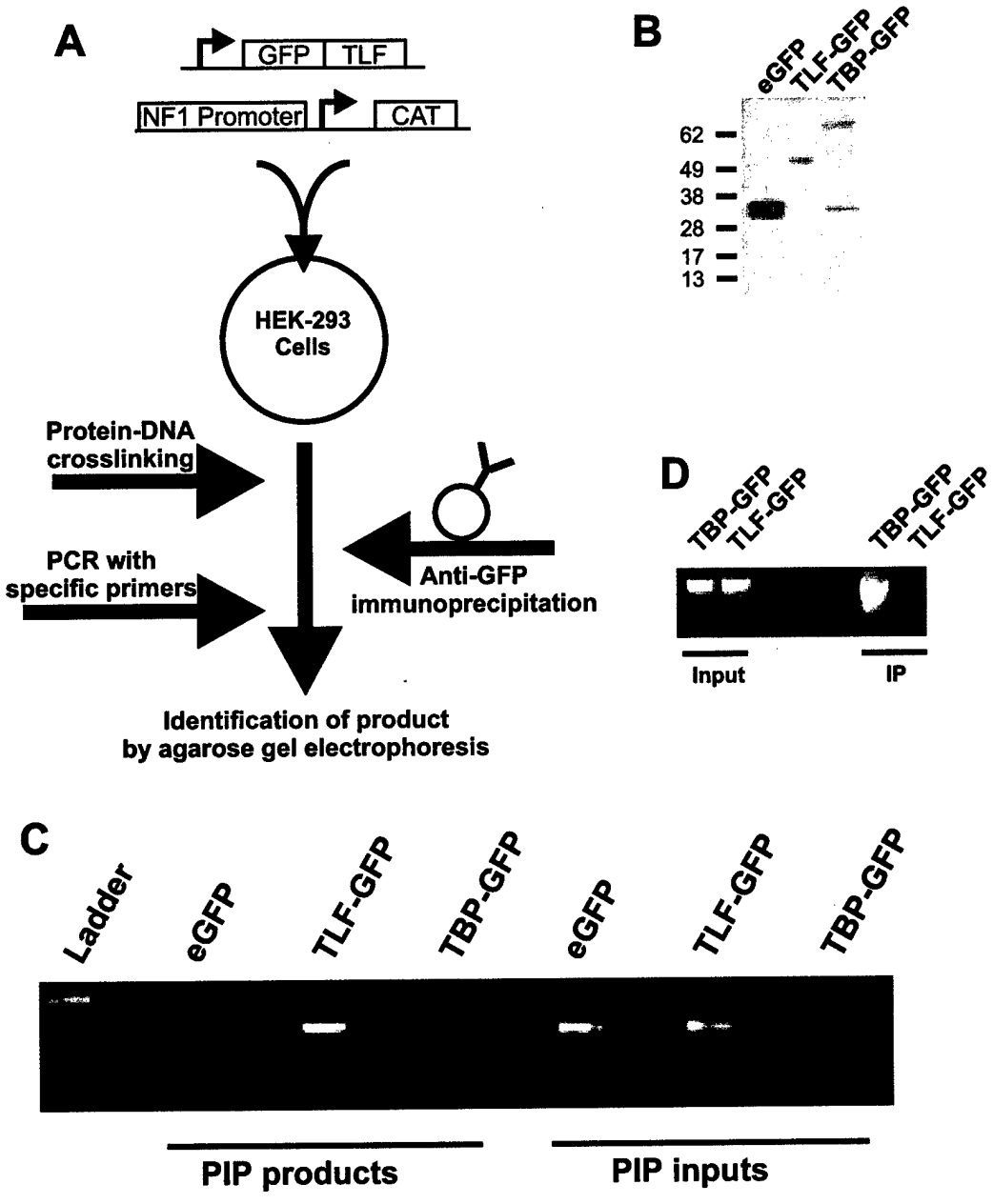
NF1-CAT + TLF-GFP
GFP
400
300
200
g RNA
15
100

Figure 2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PCR with
specific primers
Anti-GFP
immunoprecipitation
Identification of product
by agarose gel electrophoresis
B
eGFP
TLF-GFP
TBP-GFP
62
49
38
28
17
13
C
Ladder
eGFP
TLF-GFP
TBP-GFP
eGFP
TLF-GFP
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PIP products
PIP inputs
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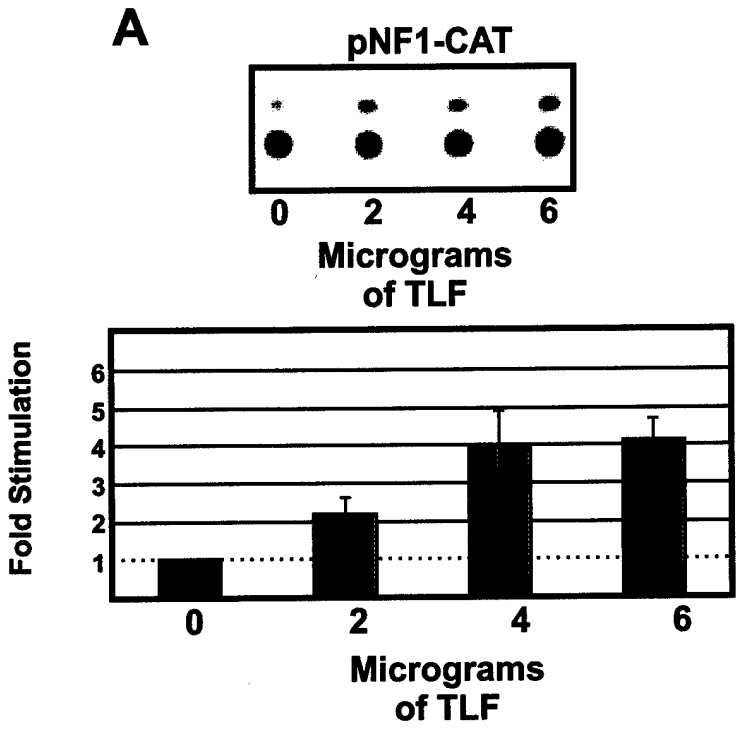
TBP-GFP
TLF-GFP
TBP-GFP
TLF-GFP
Input
IP

Figure 3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4
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of TLF
Fold Stimulation
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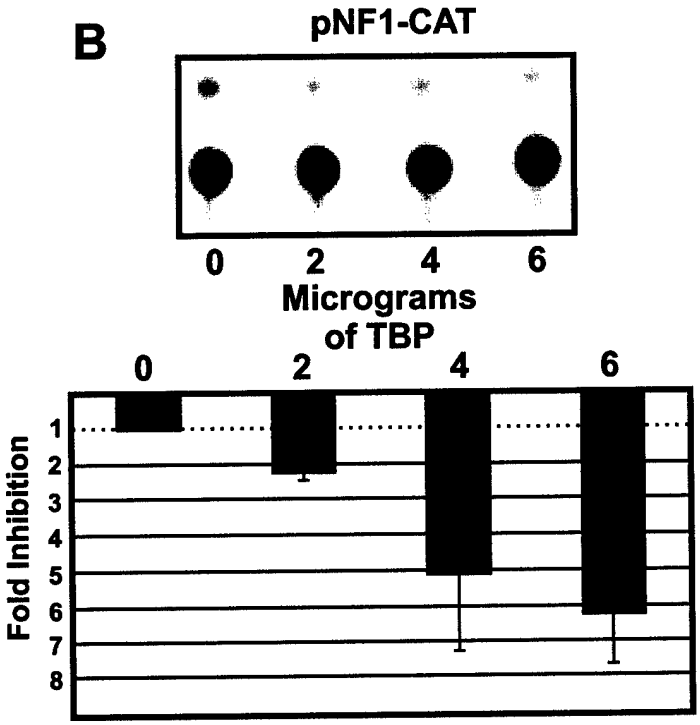
5
4
3
2
1
0
2
4
6
Micrograms
of TLF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2
4
6
Micrograms
of TBP
0
2
4
6
Fold Inhibition
1
2
3
4
5
6
7
8

Figure 4

Oligo 1

1 gctacgaagaacctgaaaatcggaggtcgtgtaccttatttttctgaga

Oligo 3

51 gcttaagctgagagcacagcctccccaggagattagcggcagagatccgc

101 gcgctgggagaaaggctagccccagggcgccctaacttccaactccggga

151 gcaatccaaacccggaggccggcgggggaggggacagctgtagggggcgg

201 tggggatggagtggatgctcccgggtcagctctggcactcgccagctga

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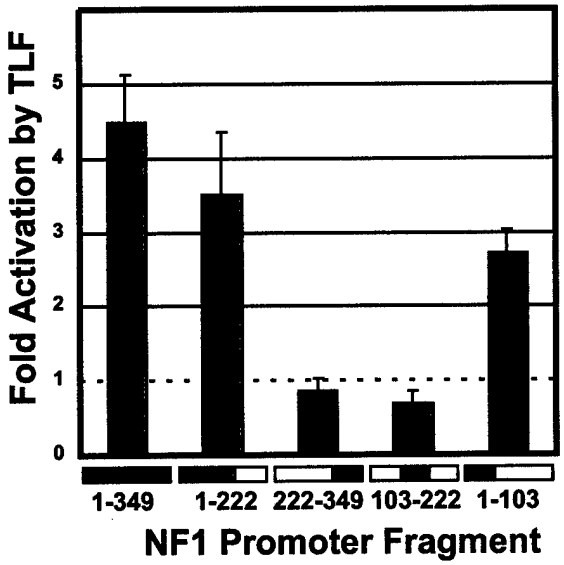
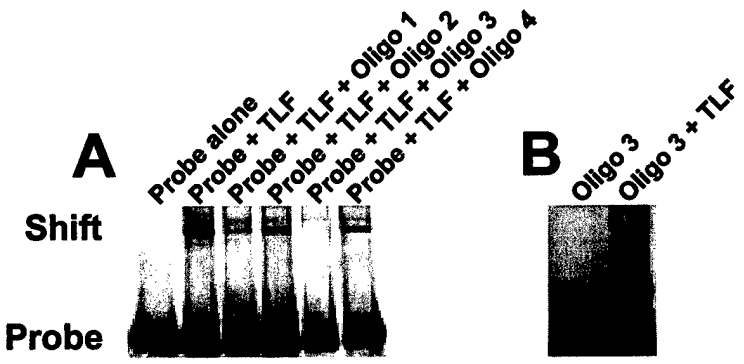


Figure 5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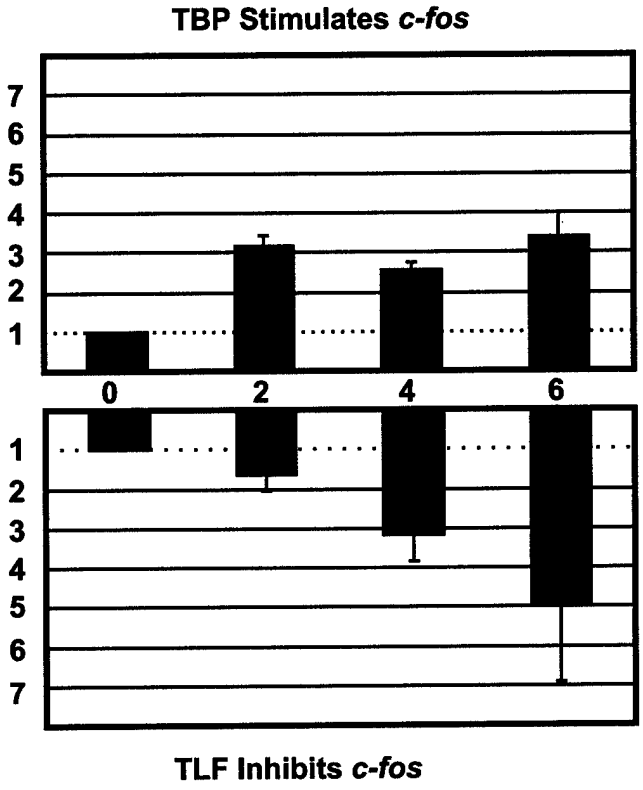
Probe alone
Probe + TLF
Probe + TLF + Oligo 1
Probe + TLF + Oligo 2
Probe + TLF + Oligo 3
Probe + TLF + Oligo 4
Shift
Probe
B
Oligo 3
Oligo 3 + TLF

Figure 6


TBP Stimulates c-fos
7
6
5
4
3
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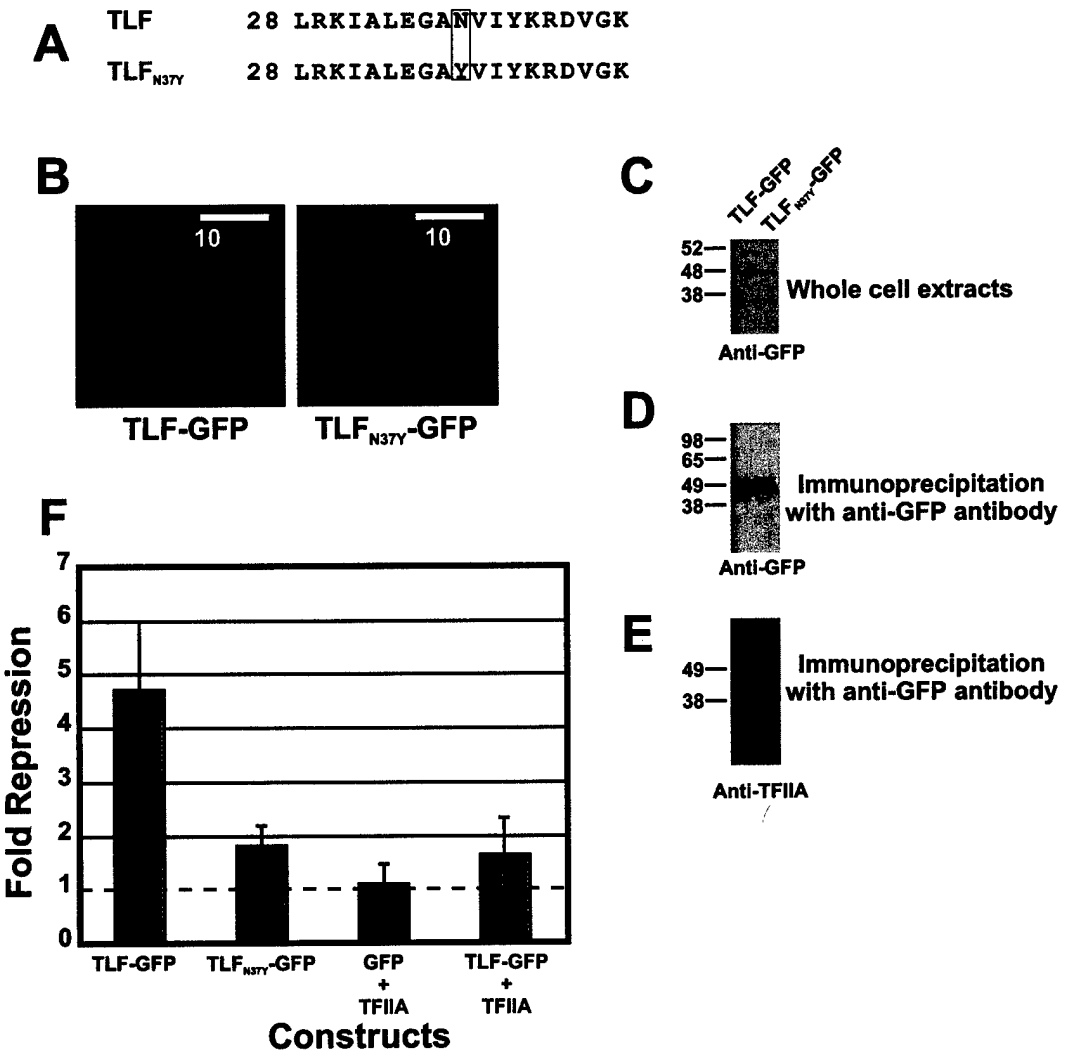
1
0
2
4
6
1
2
3
4
5
6
7
TLF Inhibits c-fos

Figure 7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TLF$_{N37Y}$ 28 LRKIALEGAYVIYKRDVGK
B
10
10
TLF-GFP
TLF$_{N37Y}$-GFP
C
TLF-GFP
TLF$_{N37Y}$-GFP
52
48
38
Whole cell extracts
Anti-GFP
D
98
65
49
38
Immunoprecipitation with anti-GFP antibody
Anti-GFP
E
49
38
Immunoprecipitation with anti-GFP antibody
Anti-TFIIA
F
Fold Repression
0
1
2
3
4
5
6
7
TLF-GFP
TLF$_{N37Y}$-GFP
GFP + TFIIA
TLF-GFP + TFIIA
Constructs

Figure 8

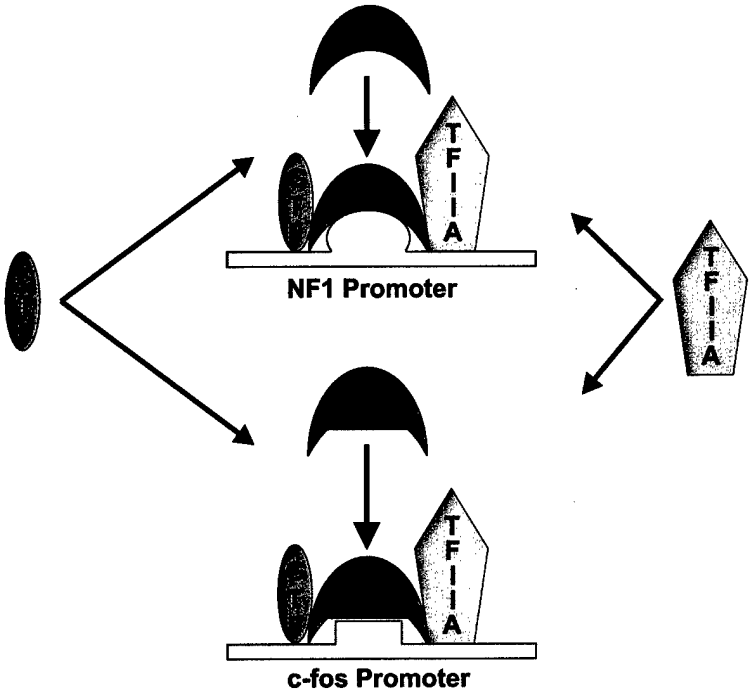

Figure 9