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The Core Promoter Region of the Tumor Necrosis Factor α Gene Confers Phorbol Ester Responsiveness to Upstream Transcriptional Activators

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Activators of protein kinase C, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), are known to regulate the expression of many genes, including the tumor necrosis factor α (TNF) gene, by affecting the level or activity of upstream transcription factors. To investigate the mechanism whereby TPA activates the TNF promoter, a series of 5′-deletion mutants of the human TNF promoter linked to choramphenicol acetyltransferase was transfected into U937 human promonocytic cells. TPA produced a 7- to 11-fold activation of all TNF promoters tested, even those promoters truncated to contain only the core promoter with no upstream enhancer elements. The proximal TNF promoter containing only 28 nucleotides upstream and 10 nucleotides downstream of the RNA start site confers TPA activation to a variety of unrelated upstream enhancer elements and transcription factors, including Sp1, CTF/NF1, cyclic AMP-response element, GAL-E1a, and GAL-VP16. The level of activation by TPA depends on the TATA box structure, since the TPA response is greater in promoters containing the sequence TATAAA than in those containing TATTAA or TATTTA. These findings suggest that the core promoter region is a target for gene regulation by second-messenger pathways.

Tumor necrosis factor α (TNF) is a potent cytokine which is a vital mediator of the host defense against infection and tumor formation (4). Many of the processes of tissue inflammation and remodeling are stimulated by TNF, including the stimulation of collagenase (7), lipogenesis (14), bone resorption (22), prostaglandin E_2 (2), and superoxide anions (39). TNF is also the mediator of septic shock produced by bacterial endotoxin and of cachexia which results from chronic infections (4). Therefore, TNF exhibits both beneficial and toxic effects, a feature which requires tight control of its production. The TNF gene is regulated by a variety of agents, which may act at the transcriptional level. Since expression of the TNF gene needs to be exquisitely regulated, the identification of transcription factors that interact with the TNF promoter and the elucidation of how these factors alter the expression of the TNF gene are of key importance. Recently, it has been reported that the human TNF promoter is activated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA). However, the DNA sequences and transcription factors that mediate TPA activation of the TNF promoter have not been defined (12, 18). We recently reported that TNF activates its own promoter and that the DNA sequence mediating this effect resembles consensus binding sites for AP-1 and CREB, but these factors are not responsible for TNF activation (24).

Many extracellular signals are mediated by intracellular second messengers that activate specific protein kinases (3, 33, 40). Recent studies suggest that intracellular messengers may regulate gene expression by altering the phosphorylation state of transcription factors (5). Members of one family of DNA-binding proteins known as CREB are activated by cyclic AMP (cAMP) kinase (protein kinase A) (15, 29). The phosphorylation of CREB (13) by protein kinase A is required for CREB-inducible gene expression. Several other transcription factors (AP-1, AP-2, and NF-κB) are activated by phorbol esters such as TPA, which activate protein kinase C (1, 20, 23, 30). Recently, Boyle et al. (6) reported that the dephosphorylation of c-Jun (a component of AP-1 [10, 36]) enables it to bind to a TPA-responsive element. To further explore the molecular mechanisms of TPA-inducible gene expression, we investigated the effects of TPA on expression of the human TNF gene. We have found that in the case of the TNF gene, the core promoter near the TATA box region is sufficient to confer TPA regulation in the absence of upstream sequences. This finding implies that TPA regulation of the TNF gene occurs through an uncommon mechanism not involving the direct action of second messengers on upstream enhancer factors.

MATERIALS AND METHODS

Plasmid constructions. TNF promoters of different lengths were cloned into a pUC19-based chloramphenicol acetyltransferase (CAT) expression vector already described (24). The −1044/+93 construct was created by cutting the native gene (32) with *Pst*I at −1044 and *Aat*II at +93 and cloning into the *Pst*I and *Acc*I sites of the pUC19 polylinker. The −505, −161, −85, and −32 deletions were created by cutting the −1044/+93 CAT vector with *Pst*I and *Stu*I, *Tha*I, *Sty*I, and *Bst*NI, respectively, blunting with T4 polymerase, and religating. The −39/+93 construct was obtained by Bal 31 digestion beginning at the *Sty*I site of the −85/+93 CAT vector, ligation of phosphorylated *Hin*dIII linkers, and then cutting at the *Xba*I site within the polylinker between the TNF promoter and the CAT gene at the 3′ end. The promoter fragments were isolated by polyacrylamide gel electrophoresis and then cloned into the CAT expression vector. The −161/+20 construct was obtained by isolating a

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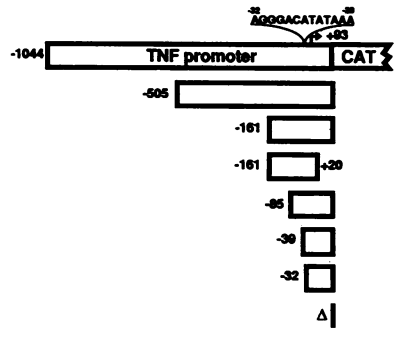
*Pvu*II-to-*Xba*I fragment from the −161/+93 CAT vector. The isolated fragment was cut at the −20 site with *Dde*I, blunt ended with T4 polymerase, and ligated with phosphorylated *Xba*I linkers. After digestion with *Hin*dIII and *Xba*I, the fragment was isolated and then cloned into the CAT vector.

To test several unrelated transcription elements in the TPA induction assay, double-stranded oligonucleotides containing either the consensus CTF/NF1 site (9) derived from the adenovirus replication origin (5′-TTT TGG CTT GAA GCC AAT ATG AG-3′; two copies), Sp1 sites (11) derived from the simian virus 40 (SV40) promoter (5′-GGG GCG GGG CGG GGC GGG GC-3′; two copies), the Sp1 site (24a) from the TNF promoter (5′-TTC TTC CCC GCC CTC CT-3′; two copies), the cAMP-responsive element (CRE) site (29) from the somatostatin promoter (5′-GGG GGC GCC TCC TTG GCT GAC GTC AGA GAG AGA G-3′; three copies), or the *GAL* DNA binding site (5′-GCG GAG TAC TGT CCT CCG A-3′; one copy) were cloned upstream of a synthetic minimal TNF promoter (−28/+10; see below), which was linked to a thymidine kinase (+14 [*Pst*I]/+45 [*Bgl*II]) leader and the bacterial CAT gene.

To test mutations within the TATAAA sequence, double-stranded promoters were chemically synthesized. The five synthetic promoters used had upstream *Xba*I and downstream *Pst*I linkers and had sequences derived from the native −28/+10 TNF promoter (5′-CTA GAC ATA TAA AGG CAG TTG TTG GCA CAC CCA GCC AGC AGA TCC CTG CA-3′), a TNF promoter with one mutation (5′-CTA GAC ATA TTA AGG CAG TTG TTG GCA CAC CCA GCC AGC AGA TCC CTG CA-3′) or two mutations (5′-CTA GAC ATA TTT AGG CAG TTG TTG GCA CAC CCA GCC AGC AGA TCC CTG CA-3′) in the TATA box, a native SV40 early promoter (5′-CTA GAC ATA TTT ATG CAG AGG CCG AGG CCG CCT CGG CCT CTG AGC CTG CA-3′), and an SV40 promoter that contains the TNF TATA box (5′-CTA GAC ATA TAA ATG CAG AGG CCG AGG CCG CCT CGG CCT CTG AGC CTG CA-3′). The underlined nucleotides represent the mutations within the TATA box.

Cell culture and transfection. U937 cells were cultured and transfected by electroporation as previously described (24). For each transfection, 15 μg of the CAT reporter plasmid was cotransfected with 5 μg of a plasmid that expresses β-galactosidase as a control for transfection efficiency. Triplicate CAT assays and β-galactosidase assays were performed as previously described (24). For primer extension analysis, total RNA from transfected U937 cells was isolated by guanidine thiocyanate extraction and CsCl gradient fractionation according to Maniatis et al. (27). Total RNA (20 μg) was mixed with 7×10^5 cpm of a DNA primer (5′-CTG TAG CCA TTG CAG CTA-3′) which is complementary to the CAT mRNA sequence and hybridized in a 10-μl volume of 2 mM Tris (pH 8.0)–0.2 mM EDTA–0.25 M KCl–10 U of RNasin (Promega Biotec, Inc.) at 65°C for 5 min with cooling to 42°C over a 30-min period. Then 25 μl of a reverse transcriptase mixture containing 0.5 mM each dGTP, dATP, dTTP, and dCTP, 2 mM Tris (pH 8.7), 10 mM $MgCl_2$, 5 mM dithiothreitol, 20 U of RNasin, and 10 U of avian myeloblastos virus reverse transcriptase (Boehringer Mannheim) was added, and the mixture was incubated at 42°C for 40 min. The reaction was stopped by the addition of 1 μl of 0.5 mM EDTA and 1 μl (20 μg) of RNase with incubation at 37°C for 20 min. Then 100 μl of 2.5 M ammonium acetate was added, after which the sample was phenol and chloroform extracted, ethanol precipitated, and then loaded onto an 8% acrylamide sequencing gel. For size markers, both the Rous sarcoma virus-CAT and TNF-CAT reporters were sequenced by dideoxy-chain termination using the primer extension primer.



Control	TPA	Fold
98	753	7.6
78	676	8.7
69	664	9.6
17	120	7.2
23	256	11.0
1.7	13.9	8.0
1.6	13.1	8.5
1.0	0.7	0.7

FIG. 1. Localization of TPA responsiveness of the human TNF promoter to the core promoter region. U937 cells were transfected with 15 μg of TNF promoters of different lengths linked to the CAT gene, treated without or with 100 nM TPA for 18 h, and assayed for CAT activity. Five micrograms of a plasmid that expresses β-galactosidase was cotransfected as a control for transfection efficiency. Each value represents the mean of triplicate samples from a representative experiment. The standard error of the mean was 7% or less for each value.

RESULTS

To localize TPA-responsive elements, we prepared 5′-deletion mutants of the TNF promoter and linked these to the CAT reporter gene. After transient transfection into U937 cells (24), TPA produced a 7- to 11-fold activation of each 5′-deleted TNF promoter tested, including a −32/+93 construct which extended only 5 nucleotides upstream of the TATA box (Fig. 1). A −161/+20 construct had a lower basal activity compared with the −161/+93 construct, but the sequences downstream of +20 were not essential for TPA inducibility. Only the promoterless CAT construct was not induced by TPA. These results demonstrate that the activation of the TNF promoter by TPA does not require sequences upstream of −32 or downstream of +20, suggesting that TPA is acting through a factor(s) associated with the core promoter.

If TPA acts directly at the core transcriptional complex, then the core TNF promoter should confer TPA inducibility to factors that bind to elements distinct from the core promoter. To address this hypothesis, several enhancers (CTF/NF1, Sp1, and CRE) which have not been reported to be activated by TPA (9, 11, 29) were cloned upstream of a synthetic minimal TNF promoter (−28/+10). The −28/+10 construct was activated threefold by TPA. The lower TPA activation of the −28/+10 construct compared with the −32/+93 and −161/+20 constructs may be due to differences in basal activity, since the −28/+10 construct was almost devoid of basal activity. The activation by TPA was potentiated by each of the upstream enhancer elements. TPA activated CTF/NF1-TNF-CAT by 6-fold, Sp1-TNF-CAT by 6- or 8-fold, and CRE-TNF-CAT by 19-fold (Fig. 2). A TNF-CAT reporter plasmid containing a DNA binding site for *GAL4* (26) (GAL-RE) gave no potentiation of the TPA response when cotransfected with a vector expressing only the *GAL* DNA binding domain (amino acids 1 to 147), whereas an 11-fold activation was observed with a vector

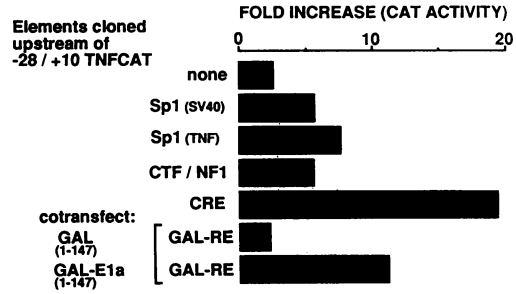


FIG. 2. Evidence that the core TNF promoter confers TPA responsiveness to unrelated DNA transcription elements. The indicated elements were each tested for the ability to amplify the TPA regulation when cloned upstream of the −28/+10 TNF promoter. U937 cells were transfected by electroporation using 15 μg of reporter CAT plasmids and 5 μg of a β-galactosidase plasmid. For experiments using the GAL-RE reporter plasmids, cells were cotransfected with 5 μg of a plasmid that expresses a protein with only the *GAL* DNA binding domain or a GAL-E1a fusion protein (26). Triplicate cell cultures were treated without or with 100 nM TPA for 18 h. The standard error of the mean was less than 9% for each value.

expressing a GAL-E1a fusion protein which contains the E1a (26) transcriptional activation domain. These results demonstrate that the core TNF promoter is capable of conferring TPA activation to a variety of unrelated upstream enhancers and activators. The magnitude of TPA responsiveness is not dependent on the level of basal activation by the introduced enhancer. For example, transfection of up to 25 μg of GAL-E1a expression vector resulted in only minimal increases in basal CAT activity over that of the untransfected cells, whereas TPA treatment increased the levels of CAT activity by 27-fold (Fig. 3).

Mutations of the TATA box were examined since the TATA box is a fundamental element in the core promoter responsible for the assembly and initiation of transcription (8, 31). CRE sites, which conferred the greatest response to TPA, were placed upstream of five different synthetic promoters of identical length. The five promoters included a minimal native TNF promoter (−28/+10) containing the sequence TATAAA, a TNF promoter with one mutation

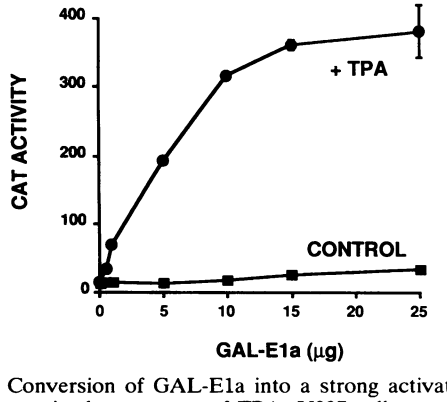


FIG. 3. Conversion of GAL-E1a into a strong activator of the TNF promoter in the presence of TPA. U937 cells were cotransfected with 15 μg of GAL-RE–TNF–CAT reporter plasmid and increasing amounts of the GAL-E1a expression vector. Triplicate cell cultures were treated without or with 100 nM TPA for 18 h. Bars represent the standard error of the mean.

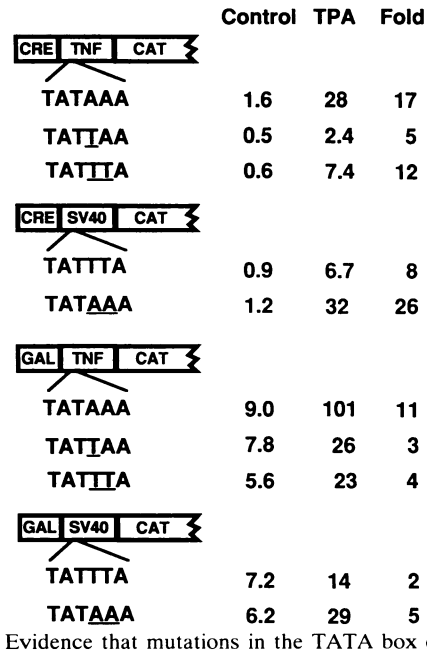


FIG. 4. Evidence that mutations in the TATA box of the TNF and SV40 promoters alter the magnitude of TPA activation. Three somatostatin CRE sites or one GAL-RE was cloned upstream of synthetic promoters. The underlined nucleotides represent the mutations within the TATA box. U937 cells were cotransfected with 15 μg of CRE-CAT or GAL-RE reporter plasmid and 5 μg of GAL-E1a expression vector. Triplicate cell cultures were treated without or with 100 nM TPA for 18 h. Standard error of the mean was less than 8% for each value.

(TATTAA), a TNF promoter with two mutations (TATTTA), a native SV40 early promoter containing the sequence TATTTA, and a mutant SV40 promoter containing the TNF TATA box (TATAAA). TPA increased the activity of the native CRE-TNF-CAT construct by 17-fold, compared with 8-fold for the native CRE-SV40-CAT construct (Fig. 4). The activation of the TNF promoter by TPA was reduced by 70 or 30%, respectively, by one or two mutations in the TNF TATA box. When the TATA box in the SV40 promoter was mutated to the TNF TATA box, the TPA response (26-fold) was completely restored, compared with the native TNF promoter (17-fold). Whereas a variety of transcription proteins in the CREB/ATF/AP-1 family bind to the CRE (16), this element has been reported to be unresponsive to TPA (19, 27, 37), suggesting that the CRE itself does not mediate the TPA inducibility. The effects of TPA on constructs that contain a GAL-RE upstream of the five synthetic promoters were also examined by cotransfection with GAL-E1a. TPA stimulated the native TNF promoter by 11-fold, compared with 2-fold for the native SV40 promoter in cells cotransfected with GAL-E1a (Fig. 4). Mutations of the TATAAA sequence in the TNF promoter reduced the TPA activation to three- or fourfold. Introduction of the TATAAA sequence into the SV40 core promoter increased the induction by TPA from twofold to fivefold.

The effects of mutating the TATAAA sequence were verified by using primer extension analysis of the CAT RNA (Fig. 5). For these studies, the strong activator GAL-VP16 (35) was chosen because it produced higher CAT mRNA levels which were easier to detect. The primer extension products observed for the TNF −28/+10 promoter mapped to positions 22 to 31 nucleotides downstream of the

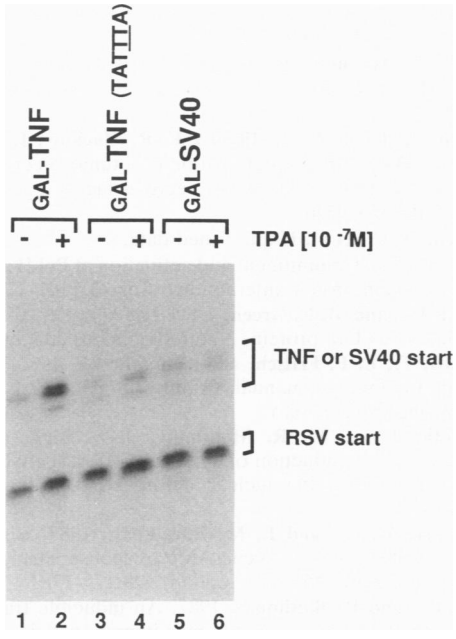


FIG. 5. Evidence that TPA activation of CAT mRNA varies with TATA box and other core promoter sequences. One GAL-RE was cloned upstream of three different synthetic promoters containing either the native −28/+10 TNF sequence (lanes 1 and 2), the TNF sequence with the mutant TATTTA sequence (lanes 3 and 4), or the native SV40 sequence (lanes 5 and 6). U937 cells (15×10^5) were cotransfected with 15 μg of GAL-RE–CAT and 5 μg of Rous sarcoma virus (RSV)-CAT reporter plasmids plus 5 μg of an expression vector producing GAL-VP16 (35) by using a human actin gene promoter. Transfected U937 cells (30×10^6) were treated without (−) or with (+) 10^{-7} M TPA for 18 h. The RNA was analyzed by primer extension, and the products were quantified by laser densitometry.

TATAAA sequence (Fig. 5, lane 1). The location and number of start sites were not altered either by treatment with TPA (lane 2) or by mutation of two bases within the TATA box sequence. The pattern observed with the SV40 −28/+10 sequence was a little more heterogeneous, mapping to positions 22 to 34 nucleotides downstream of the TATTTA sequence. The levels of CAT mRNA produced from the TNF or SV40 promoter constructs were quantified by laser scanning densitometry and normalized to the amounts of CAT mRNA produced from the cotransfected RSV-CAT plasmid. TPA stimulated the TNF −28/+10 promoter 3.9-fold (lanes 1 and 2), whereas it stimulated the TNF promoter with the mutant TATTTA sequence only 1.9-fold (lanes 3 and 4) and actually caused a 40% decrease in the CAT mRNA produced by the SV40 promoter construct (lanes 5 and 6). These data confirm that the TATAAA sequence is optimal for TPA activation but that other nucleotides in the core promoter also affect TPA regulation.

DISCUSSION

Previous studies suggest that second messengers control gene expression exclusively by activating upstream factors such as CREB (29), AP-1 (1, 23), and NF-κB (30). However, the data reported here show that the core TNF promoter confers TPA activation to a variety of unrelated upstream factors, including CTF/NF1, Sp1, CREB/ATF, GAL-E1a, and GAL-VP16. Whereas it is possible that phorbol esters activate all of these factors by altering their phosphorylation state, this seems unlikely since it was recently shown that Sp1 is not a substrate for protein kinase C (21). Our results are more consistent with a model in which TPA activates a protein(s) associated or interacting with the core promoter which then facilitates the action of upstream transcription factors. Apparently, once the core TNF promoter is activated by TPA, upstream transcription factors, such as Sp1, become more potent activators of the promoter. The possibility that the TPA stimulation is due to elements in the plasmid sequences outside of the TNF promoter can be ruled out, since we repeatedly observed that a −32/+45 thymidine kinase promoter in the identical CAT plasmid was not activated by TPA (data not shown). Although AP-1 binding has been observed in pUC plasmids (42), the region which binds AP-1 has been deleted in the plasmids used in this study.

Previous studies have shown that TATA box structure can affect the actions of upstream transcription factors. These include the induction of the *HIS3* gene in *Saccharomyces cerevisiae* (17), cell-specific expression of the myoglobin promoter (41), and E1a activation of the c-*fos* promoter (38). The finding that the TNF TATA box is the more effective in either the TNF or SV40 promoter context indicates that optimal TPA regulation requires a specific TATA box structure. It is possible that TPA produces a conformational change in the core promoter complex for which the TATAAA sequence is more permissive or complementary than are the other less responsive TATA box sequences. One possibility is that the different TATA box sequences could bind factor TFIID with different affinities, which could alter the kinetics of transcription in a way which affects the TPA inducibility. The observation that GAL-E1a did not activate the SV40 promoter containing the TATAAA sequence as well as the native TNF promoter suggests that additional nucleotides besides the TATAAA sequence within the core promoter and other factors may also be important for TPA activation.

Our results provide an additional mechanism for phorbol ester regulation. To our knowledge, this is the first instance of activation of the core promoter by any signal transduction pathway, and it therefore introduces a new target for control of gene expression that may be a general phenomenon. The factor in the TNF core promoter that is activated by TPA could be a general component such as TFIIA, -B, -D, or -E or RNA polymerase (8, 31). However, in preliminary experiments we found that TPA does not activate the Sp1 TNF-CAT construct in HeLa cells (data not shown), suggesting that the TPA-activated transcription factor may be cell specific, although it is also possible the TPA induction pathway in U937 cells is specific. Other possible targets for TPA are putative coactivator factors that may facilitate interactions of upstream and downstream factors with core promoter elements (25, 34). The identification of the factor that is regulated by TPA will require the purification of coactivators and possibly other components in the core transcriptional complex that have thus far eluded purification and characterization.

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