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Responsive site on the thrombospondin-1 promotor to down-regulation by phorbol 12-myristate 13-acetate in porcine aortic endothelial cells

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Abbreviations: TSP-1, thrombospondin-1; PMA, phorbol 12-myristate 13-acetate; PAE, porcine aortic endothelial; EMSA, electrophoretic mobility shift assay; PKC, protein kinase C; fpu, footprint units; PMSF, phenymethylsulfonyl fluoride

Abstract

Thrombospondin-1 (TSP-1), a multifunctional extracellular matrix protein, inhibits neovascularization and is implicated in the regression of tumor growth and metastasis. We found that the synthesis of TSP-1 in porcine aortic endothelial (PAE) cells was decreased in a dose-dependent manner by phorbol 12-myristate 13-acetate (PMA) treatment in porcine aortic endothelial (PAE) cells. In this study, a responsive site on the TSP-1 promotor affected by PMA treatment in PAE was characterized. The level of TSP-1 mRNA was also decreased by PMA after 1 h and persisted that way for at least 24 h. PMA treatment and c-Jun overexpression suppressed the transcription of TSP-1 promotor-luciferase reporter gene. A deletion between -767 and -657 on the TSP-1 promotor neutralized the PMA-induced down-regulation. In addition, oligo a (-767~-723) was responsive to PMA-induced repression, while oligo b (-734 ~-689) and c (-700~-656) was not. Electrophoretic mobility shift assays showed that this PMA responsive element specifically bound a nuclear protein and that the binding activity was diminished by PMA treatment in PAE cells but not in Hep 3B cells. In supershift assay, potential regulatory elements in this region, SP1 and GATA-1, were not responsive to the inhibition of TSP-1 expression by PMA. Our results suggest that the repression of TSP-1 synthesis by PMA is mediated by blocking a particular unknown nuclear protein binding to the responsive site (-767~-735), which is regulated by c-Jun.

Keywords: Thrombospondin-1; PMA; protein kinase C; c-Jun; AP-1

Introduction

Thrombospondin-1 (TSP-1) is a homotrimeric extracellular matrix glycoprotein of 450 kD (Lawler, 1986; Bornstein, 1992). It is synthesized and secreted by the wide range of cells including fibroblasts, smooth muscle cells, macrophages, endothelial cells, and transformed cells (Varani *et al*., 1989; Clezardin, 1993). Each monomer (180 kD) has functional domains capable of binding to many extracellular matrices and a variety of other proteins including several receptors, enzymes, and cytokines (Bornstein, 1995). Because of these multiple interactions, TSP-1 has been implicated in a number of biological processes such as development, inflammation, wound healing, tumor growth and metastasis (Mosher, 1990; Bornstein, 1992; Lahav, 1993).

The transcription factor AP-1 forms either homodimers or heterodimers with members of the Jun family (c-Jun, Jun B, and Jun D) or with proteins of the Jun and Fos (c-Fos, Fos B, Fra 1, and Fra 2) families (Angel and Karin, 1991). Among them, c-Jun is the major component of AP-1 complexes with c-Fos (Bohman *et al*., 1987; Angel *et al*., 1988). AP-1 is activated following stimulation by protein kinase C (PKC), and it regulates the expression of AP-1 responsive target genes.

We observed that PMA significantly induced the expression of TSP-1 in hepatocarcinoma Hep 3B cells and up-regulation of TSP-1 synthesis following PMA treatment was mediated by an increased AP-1 binding activity *via* PKC activation. The functional AP-1 binding site was also identified and was activated upon increased c-Jun expression. In addition, PMA was reported to reduce TSP-1 expression in human monocytes (Yesner *et al*., 1996) and c-jun lead to the repression of TSP-1 in rat fibroblasts (Mettouchi *et al*., 1994; Dejong *et al*., 1999). We also found that PMA inhibited TSP-1 synthesis in porcine aortic endothelial (PAE) cells but not on Hep 3B cells.

Based on cell type specific expression of TSP-1, the mechanism of TSP-1 gene expression in PAE cells was examined. A dose-dependent inhibition of TSP-1 expression by PMA was observed in PAE cells. The responsive element for the PMA-induced inhibition was contained in the region between -767 and -735 of the TSP-1 gene promoter. We found that this *cis* element played

an important role in the PMA-modulated down-regulation of the TSP-1 gene expression in PAE cells and that the c-Jun-induced repression of TSP-1 expression did not require direct binding of c-Jun to the TSP-1 promoter.

Materials and Methods

Cell lines and culture

Porcine aortic endothelial (PAE) cells were provided by Kim H. P. (KNIH) and maintained in M199 medium (Gibco Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum. Human hepatocarcinoma Hep 3B cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 medium. Cells were maintained at 37°C under 5% CO_2. All experiments were performed in duplicate or triplicate hereafter.

Western blot analysis

PAE cells (2×10^5 cells/ml) were plated in 6-well culture plates. Two days later, the cells were washed twice with calcium/magnesium free-phosphate buffered saline (CMF-PBS, pH 7.2) and incubated at 37°C with serum free M199 in the presence or absence of PMA. After 24 h, the culture supernatants were harvested and concentrated in order to examine the level of TSP-1 secreted. Two milliliters of culture supernatants were concentrated using Centricon-50 (Amicon, USA) at 3,000 *g* for 20 min in the presence of protease inhibitors-PMSF (1 μg/ml), leupeptin (1 μg/ml) and aprotinin (2 μg/ml). The concentrates were mixed with an equal volume of 2× sample buffer (Laemmli, 1970) and electrophoresed through a 7.5% SDS-polyacrylamide gel, and the separated proteins were transferred onto a nitrocellulose membrane. After blocking with 5% skim milk, the membrane was incubated for 1.5 h with anti-TSP-1 antibody (1 : 1000, Calbiochem, San Diego, CA) and washed (four times for 5 min each) with TBS buffer containing 0.1% Tween 20 (TTBS). The membrane was then incubated with anti-rabbit peroxidase-conjugated antibody (1 : 4000, Sigma Co., St. Louis, MO) as the secondary antibody for 1 h and washed again with TTBS. TSP-1 was visualized using an ECL detection kit (Amersham, Buckinghamshire, U. K.). For Western blot analysis of c-Fos and c-Jun, nuclear extracts (30 μg) were prepared (Schreiber *et al.*, 1989) and analyzed after 12.5% SDS-PAGE. These blots were incubated with anti-c-Fos or anti-c-Jun antibody (1 : 1000, Oncogene Research Products, Cambridge, MA) for 1.5 h.

Northern blot analysis

Cells (2×10^5 cells/ml) were seeded into 60 mm dishes. After two days, the cells were washed twice with CMF-PBS and incubated at 37°C with serum free M199. Cells were treated with PMA for the indicated times and total cellular RNA was isolated using RNA STAT-60 (TEL-TEST, INC., Friendswood, TX). The RNA samples (15 μg) were then electrophoresed through a 1% agarose-formaldehyde gel and transferred to a nylon membrane. The blots were hybridized with a digoxigenin (DIG)-labeled probe and detected by an immunochemical method using a DIG chemiluminescence detection kit (Boehringer Mannheim, Germany). The probe encoding the sequence of TSP-1 was prepared by polymerase chain reaction (PCR) using human TSP-1 cDNA and DIG-11-dUTP (Boehringer Mannheim). A cDNA probe for human β-actin was also prepared and used as a control.

Reporter plasmid

A 2974 bp region of the human TSP-1 gene (-2220~+754) was prepared from Hep3B genomic DNA by PCR using a primer set, 5'-CAACTGAAGTATCATGATAAG AG and 5'-ATCCTGTAGCAGGAAGCACAAG, and r*Tth* DNA polymerase XL (PE Applied Biosystems, Foster City, CA). The amplification was conducted under the following conditions: denaturation, 94°C for 30 sec; annealing, 50°C for 30 sec; extension, 72°C for 3 min. The PCR product was then ligated into the *Kpn*I and *Xho*I sites of a promoterless pGL3 luciferase expression vector (Promega Co., Madison, WI).

Transient transfection and reporter assay

Cells (3×10^5) were plated in a 6-well culture plate. The next day, the cells were transiently transfected with 2 μg plasmid DNA using FuGENE 6 (Boehringer Mannheim). The pTSP-LUC, pCR3.1 coding for β-galactosidase, and the c-Jun expression vector pSG5 were used in these transfection assays. Eight hours later, the cells were washed and treated with the respective agents. After 12 h, the cells were harvested, extracted, and subjected to luciferase assay (Boehringer Mannheim) and β-galactosidase assay (Sambrook *et al.*, 1989). Luciferase activity was normalized to the β-galactosidase activity used as control for the efficiency of transfection. Relative luciferase activity was expressed as mean ± SD in triplicate.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from about 3×10^6 cells as described by Schreiber *et al.* (1989) with some modification. Briefly, cells were harvested and washed once with cold CMF-PBS, and nuclear proteins were extracted in the presence of protease inhibitors including 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μg/ml aprotinin. The three double-stranded oligonucleotides used in this assay were distributed over the region of -767 to -656 of the TSP-1 promoter (oligo a, -767 to -723; oligo b, -734

to -689; oligo c, -700 to -656). The probes were end-labeled with [α-^{32}P]dATP using the Klenow fragment. Binding reactions were performed with 5 μg of nuclear protein as described previously (Vincenti *et al.*, 1992), and the samples were analyzed on a 5% polyacrylamide gel in 1$\times$ TBE buffer. The gels were vacuum-dried and then analyzed by autoradiography.

Results

Effects of PMA on TSP-1 synthesis in PAE cells

In order to confirm the PMA effect on endothelial cells, the level of TSP-1 synthesis in PAE cells was investigated and found that TSP-1 synthesis was inhibited in a dose-dependent manner upon treatment with PMA (Figure 1A). TSP-1 mRNA levels by Northern blot analysis also showed decrease in the PAE cells and suggest possible suppression of the TSP-1 gene expression. When the PAE cells were treated with 100 nM PMA, the level of TSP-1 mRNA started to decrease after 30 min and steadily decreased after 6 h. This inhibitory effect persisted at least 24 h (Figure 1B).

Induction of c-Jun and transcriptional regulation of the TSP-1 gene promoter.

Possilibity of PMA effect on the level of c-Jun and c-Fos, the major components of AP-1 as observed in other cell types were examined. Although the level of c-Fos expression did not change, the level of c-Jun increased dramatically upon PMA treatment (Figure 2). After the addition of PMA, the c-Jun level started to increase within 1 h, and the increased level was sustained for 12 h.

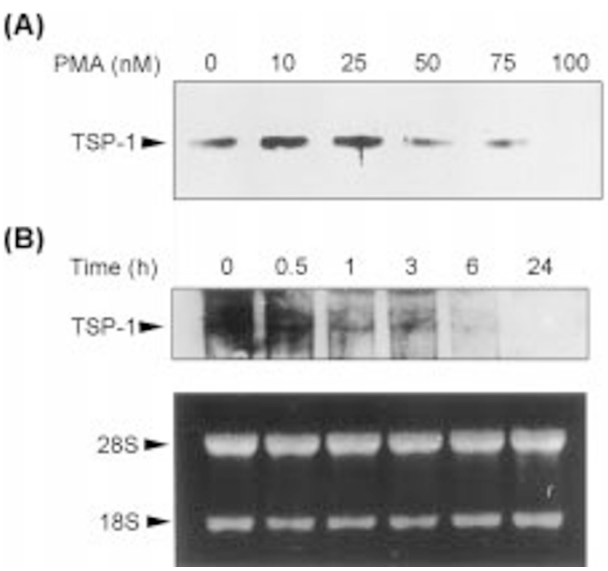

Figure 1. Regulation of TSP-1 synthesis by PMA in PAE cells. (**A**) Cells were incubated with the indicated concentrations of PMA for 24 h and the culture supernatants were analyzed by Western blot. (**B**) Cells were treated with 100 nM PMA for a 24 h period, and total cellular RNA was extracted. Fifteen mg total RNA was used for Northern blot analysis. Ethidium bromide staining of the same gel was used to monitor nucleic acid loading in each lane.

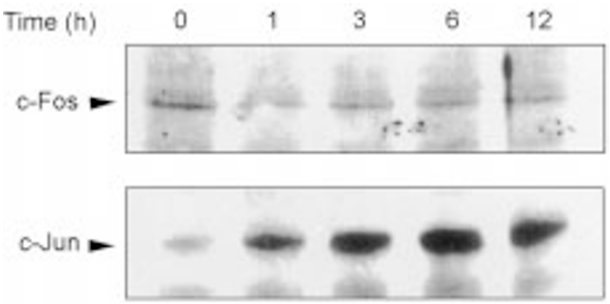

Figure 2. Induction of c-Jun in PMA-treated PAE cells. PAE cells were treated with 100 nM PMA for the time periods indicated. The levels of c-Fos and c-Jun expression in the nuclear extract was then analyzed by Western blot. c-Fos and c-Jun positive bands were revealed using an enhanced chemiluminescence detection system.

To determine whether the inhibition of TSP-1 expression occurred through the regulation of a *cis*-acting element in the TSP-1 promoter, we constructed a reporter plasmid which consisted of the TSP-1 promoter region (-2220 to +754) linked to a promoterless luciferase gene. PAE cells were transiently transfected with this TSP-Luc reporter plasmid and the effect of PMA on the reporter expression was examined. TSP-1 promoter-mediated luciferase activity was declined to 0.32 fold of control activity following treatment with 100 nM PMA for 12 h in the transfected PAE cells (Figure 3). Since PMA dramatically induced c-Jun expression in PAE cells (Figure 2), an affect of the c-Jun expression on TSP-1 promoter

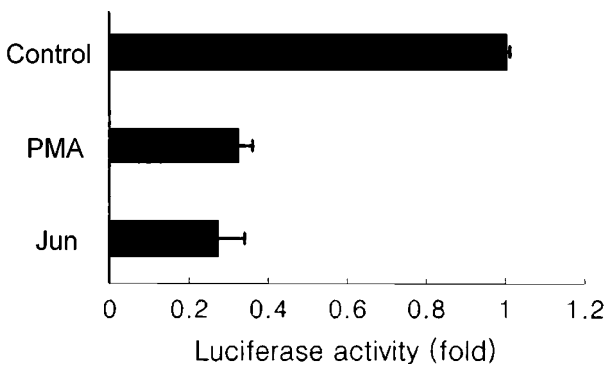

Figure 3. Transcriptional inhibition of a TSP-Luc reporter plasmid by PMA and c-Jun in PAE cells. Cells were transiently transfected with TSP-Luc reporter plasmid alone or in combination with a c-Jun expression plasmid. After transfection, cells were left untreated (control and c-Jun transfectant) or treated with 100 nM PMA for 12 h. Luciferase activity is expressed relative to the luciferase activity obtained from the untreated control cells. The luciferase activity was normalized to the cotransfected β-gal activity. These data represent the mean $\pm$ SD of three independent experiments.

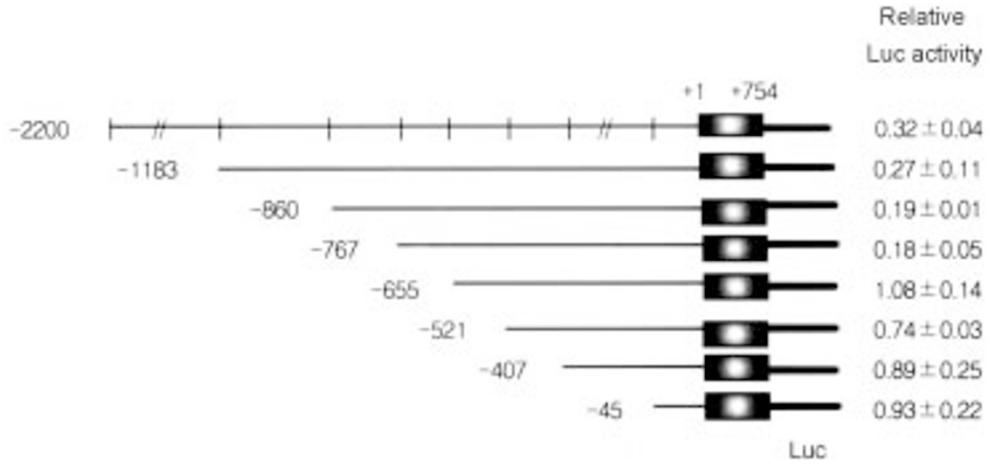

Figure 4. Structure and activity of various deletion fragments of the TSP-1 promoter. PAE cells were transfected with each deletion construct. After 8 h, the cells were treated with 100 nM PMA, and luciferase activity was assayed after 12 h. The luciferase activity of each plasmid is expressed relative to the luciferase activity obtained from the untreated control cells. The luciferase specific activity of each construct was normalized to the cotransfected β-gal activity. The data represent the mean ± SD of three independent experiments.

activity was also tested. As shown in Figure 3, the cells contransfected with the TSP-Luc reporter plasmid in combination with a c-Jun expression vector, showed reduced luciferase activity by 0.3 fold in comparison with that of control activity, which is similar to what we observed in the PMA treated cells. The results suggested that the decrease in TSP-1 synthesis induced by PMA was due to transcriptional regulation and that the TSP-1 gene must contain some regulatory element which is affected by c-Jun expression.

Identification of regulatory elements for the inhibition of TSP-1 expression

In order to determine which sequence on the promoter was involved in the repression of TSP-1 expression, PAE cells were transiently transfected with the TSP-Luc plasmid (-2220 to +754) or deletion constructs thereof. Deletion of the TSP-1 promoter region up to -768 did not modify the transcriptional repression by PMA (Figure 4). However, further deletion, up to -656, abolished the response to PMA. The same results were obtained from the cells cotransfected with the c-Jun expression vector (data not shown).

Since the binding of nuclear protein(s) to a *cis* element may cause the PMA-dependent repression of the TSP-1 gene expression and because the region between -767 and -656 contained no known AP-1 responsive element, any protein binding to this region was examined by EMSA. Three overlapping fragments were used as probes: oligo a; -767 to -723, oligo b; -734 to -689, and oligo c; -700 to -656. A specific shift of band was observed only with oligo a. When the cells were treated with PMA, this band shift disappeared in a time depen-

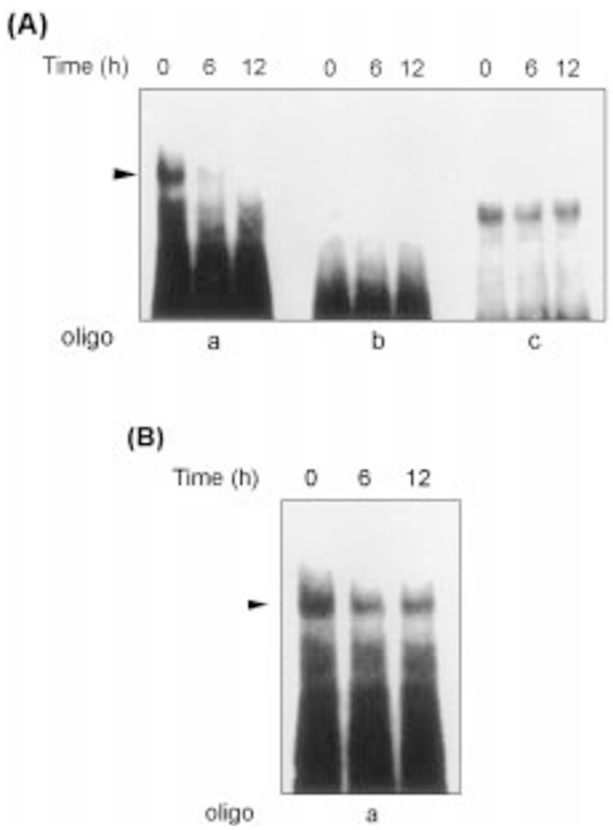

Figure 5. EMSA of nuclear extract binding to the TSP-1 promoter region -767 to -656. PAE (**A**) and Hep3B (**B**) cells were treated with 100 nM PMA for the indicated time periods and nuclear extracts were prepared. Ten μg of nuclear protein was combined with [α-^{32}P]dATP-labeled oligo a, b, and c and analyzed by EMSA as described in "Materials and Methods." The arrow head points to the specifically shifted bands. Oligo a, -767 to -723; oligo b, -734 to -689; oligo c, -700 to -656.

dent manner (Figure 5A), which decrease at 6 h and completely faded away at 12 h. On the other hand, no significant change was observed in a DNA-protein complex in Hep 3B cell extracts (Figure 5B). A computer analysis revealed the potential presence of Sp1 and GATA-1 binding sites in this region (-767 to -735). To find out whether these nuclear proteins could indeed bind to this region, we performed EMSA using Sp1, GATA-1, and AP-1 antibodies. But the protein band, whose level was diminished upon PMA treatment, was not affected by any of these antibodies (data not shown). This result suggests that Sp1, GATA-1, and AP-1 transcription factors were not responsible for the PMA-dependent inhibition of TSP-1 expression in PAE cells.

Discussion

Increasing number of the data strongly support a role of TSP-1 as a negative regulator of solid tumor progression and angiogenesis. Understanding regulation of TSP-1 expression thus may provide insights into a new approaches to cancer therapy. Although several factors known to affect TSP-1 expression, the regulatory mechanism of the TSP-1 gene expression is not well understood. Among the factors effecting TSP-1 expression, PMA exhibited a typical cell type inhibitory agent in a specific effectiveness.

In human hepatocarcinoma Hep 3B cells, we found that PMA markedly stimulated TSP-1 synthesis. In addition, the regulatory mechanism of the *tsp-1* gene expression was found to involve the activation of PKC and the transcription factor AP-1 which were the critical events in the PMA-mediated *tsp-1* gene activation. *Tsp-1* gene promotor has a functional AP-1 binding site, and it is activated *via* the increased expression of c-Jun in Hep 3B cells.

Interestingly, PAE cells treated with PMA showed inhibition of TSP-1 synthesis in a dose-dependent manner *via* the PKC pathway. The fact that c-Jun overexpression in cells transfected with an expression vector resulted in inhibition of TSP-1 expression, suggested that this repression may be linked to transcriptional control by c-Jun. But experiments with serial deletions of the TSP-1 promoter demonstrated that TSP-1 repression by c-Jun did not utilize any of the known AP-1 sites present in the TSP-1 promoter. Instead, we identified a 33 bp region, -767 to -735 that was responsive to the negative regulation of TSP-1 transcription. A computer analysis revealed the presence of several potential regulatory elements, including Sp1 and GATA-1 in this region. However, supershift assays with antibodies against Sp1 or GATA-1 demonstrated that these transcription factors were not responsible for the PMA induced down-regulation of TSP-1 (data not shown). Supershift experiments with anti-AP-1 antibody reconfirmed that AP-1 did not bind to this 33-bp region.

The interaction between DNA binding proteins and *cis*-acting elements plays an important role in controlling gene expression. The same DNA sequence may function as either a positive or negative control element depending on the DNA binding factors present in different cell types or stages of differentiation. Although the PMA-responsive regulatory factor of TSP-1 exists in both PAE and Hep 3B cells, PMA treatment suppressed its binding activity only in PAE cells (Figure 5). Our results indicate that c-Jun does not participate directly in the repression of TSP-1 transcription by binding to its promoter. Recently, PMA-responsive down-regulatory factors, thyroid transcription factor 1 (TTF-1) and hepatocyte nuclear factor 3 (HNF3), have been reported (Kumar *et al.*, 1997). But, the consensus motif was different from any nucleotide sequence in the 33-bp region of TSP-1.

A c-Jun homodimer can bind to other proteins or transcriptional coactivators. AP-1 requires transcriptional coactivator CBP, cAMP response element-binding protein (CREB) binding protein for its transcriptional activation (Arias *et al.*, 1994; Bannister *et al.*, 1995). It is possible that the unknown factor bound to the 33-bp region may require another protein for stability. C-Jun and the unknown factor may compete for the same transcriptional coactivator. It has been demonstrated that both AP-1 and retinoic acid receptor (RAR) require CREB for their transcriptional activation, and because of competition for CREB, they were regulated in opposite ways (Willy *et al.*, 1995; Soprano *et al.*, 1996). To elucidate this unknown factor, the PMA-dependent down-regulation of TSP-1 gene expression is currently under further investigation.

In summary, we demonstrated that TSP-1 expression is effectively inhibited by PMA in PAE cells. In this PMA induced inhibition of TSP-1 expression, c-Jun does not participate directly in the repression of TSP-1 transcription by binding to its promoter, but this effect may be mediated by another regulatory factor which could bind to the -767 to -735 region of the promoter.

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