Title

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Journal

Journal of Experimental Medicine, 170(1)

ISSN

0022-1007

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et al.

Publication Date

1989-07-01

DOI

10.1084/jem.170.1.321

Peer reviewed

Brief Definitive Report

GENETIC ANALYSIS OF THE HUMAN TUMOR NECROSIS FACTOR α/CACHECTIN PROMOTER REGION IN A MACROPHAGE CELL LINE

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TNF-α/cachectin is an important immunoregulatory cytokine with a broad range of biological properties (1–3). These include cytotoxicity for tumor cells (4), augmentation of humoral and cellular immunity (5, 6), and activation of macrophages (7) and neutrophils (8). TNF has been implicated as a proximal mediator of Gram-negative endotoxemia (9) and circumstantially in the toxicity of IL-2-based immunotherapy regimens (10).

Although predominantly a product of mononuclear phagocytes, TNF/cachectin is also produced by T cells, B cells, and NK cells (11–13). A number of cytokines and bacterial products can induce TNF-α/cachectin production; phorbol esters (PMA) are strong agonist signals (14).

Little is known about regulation of TNF/cachectin gene expression at the level of transcription. Murine TNF transcription can be induced by inhibition of protein synthesis implicating a transcriptional repressor (15). However, no promoter region studies are available. In this report, we describe genetic studies using recombinant constructs containing the human TNF-α/cachectin promoter linked to the luciferase (Luc) reporter gene in a transient transfection system (16). TNF/cachectin promoter constructions were inducible by PMA in the U937 macrophage cell line.

Materials and Methods

Reagents. RPMI 1640, FCS, and antibiotics were obtained from Flow Laboratories, Inc., McLean, VA. PMA, ATP, and firefly luciferase were purchased from Sigma Chemical Co., St. Louis, MO. D-luciferin was purchased from Analytical Luminescence Laboratorires, San Diego, CA. The promoterless luciferase plamids, pXP 1 and 2, used in this work were the generous gift of Dr. S. Nordeen, University of Colorado (17). The TNF-α/cachectin genomic clone (2) was the generous gift of Dr. Michael Shepard of the Genentech Corporation, So. San Franscisco, CA.

Cell Lines. The U937 human macrophage cell line was obtained from the American Type

This work was supported by National Institutes of Health grants CA-09010, CA-12582, and CA-29605. J. Economou is the recipient of a National Cancer Institute Clinical Investigator award (1 KO8 01360-01). Address correspondence to James S. Economou, Division of Surgical Oncology, Factor 9-267, Center for the Health Sciences, University of California, Los Angeles, CA 90024.

Culture Collection, Rockville, MD, and passaged in RPMI 1640, 10% FCS, and antibiotics. Cells were split the day before transfection.

Transfection. Cells were transfected using the technique of electroporation essentially as described by Cann et al. (18). Cells were resuspended in RPMI 1640 + 20% FCS at 5×10^6 in 0.25 ml, to which 25 μg plasmid DNA was added. Electroporation was performed with a single pulse from a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) with a capacitance extender unit (250 V, 960 μFd). Cells were incubated on ice for at least 10 min before and after electroporation and then resuspended in 4 ml RPMI 1640 + 10% FCS in 6-well plates (Costar, Cambridge, MA). The decay constant for these electroporations was generally between 45 and 55 ms. Cell viability immediately after electroporation averaged 50–60%.

Luciferase Assay. Transfected cells were retrieved with a plastic policeman after 24 h of culture, washed, and resuspended in 100 μl lysis buffer (17). Cells were lysed by three freeze-thaw cycles and the cellular debris was sedimented in a microfuge. 10–25 μl of supernatant was assayed for luciferase according to de Wet et al. (16) using a luminometer (DuPont Co., Wilmington, DE). The luminometer records data as relative light units (peak), which is directly proportional to luciferase concentration over a 7-log range. This instrument is sensitive to the level of 10^{-7} to 10^{-8} U of firefly luciferase. Protein concentration of extracts was measured using the Coomassie Blue G250 binding assay (Bio-Rad Laboratories) with BSA as a standard, and generally averaged 15–20 $\mu g/10^6$ cells.

Plasmid Construction. The techniques described were performed essentially as described by Maniatis et al. (19). The TNF promoter region, a 705-bp Aha II/Eco R1 fragment, was excised from the human TNF-α gene (which had been cloned into pUC13pML and supplied by Genentech, So. San Francisco, CA) and blunt-end ligated into the multiple cloning site of pUC18 at the Bam HI site. This fragment was then excised with Hind III and Kpn I and ligated downstream from the luciferase gene contained in pXP1 and pXP2. The plasmids pXP1 and pXP2 contain opposite orientations of a multiple cloning site, thus allowing insertion of the TNF promoter in forward and reverse orientations. Unidirectional deletions were then made in the forward orientation promoter using exonuclease III, as described by Heinkoff (20). Briefly, the plasmid containing the TNF promoter in the forward orientation just downstream of the luciferase gene, pTNF (−615) Luc, was digested with Hind III and the overhangs were filled in with thionucleotides to protect the ends from digestion with exonuclease III. The plasmid was then digested with Sal I to expose a 5′ overhang just 5′ of the TNF promoter, which would be susceptible to digestion with Exo III and allow digestion into the promoter region. The Exo III digest was performed at 37°C and samples were taken at 30-s intervals for 10 min. All samples were S1 nuclease treated to blunt end and then religated. To determine the extent of deletion into the 5′ end of the promoter, each mutant was sequenced as described by Maxam and Gilbert (21). Briefly, each deleted promoter was labeled at the 5′ end at the Bam HI site and digested again with Sst I or Dde I, depending on the extent of the deletion, to leave a single end-labeled probe.

Results and Discussion

The putative TNF-α/cachectin promoter region was isolated from the 5′ flanking region of the genomic clone with Eco R1/Aha II, which yielded a 705-bp fragment, 615 bp of which are upstream from the transcription start site (TSS position 0). The Aha II site is upstream from the initiation codon. This flanking region was ligated into the luciferase (Luc) reporter gene in the forward (pTNF [−615]Luc) and reverse (pTNF [+615]Luc) orientations. A series of 5′ truncations of pTNF (−615)Luc was generated by unidirectional exonuclease III digestion, which produced promoter region constructs ranging in length from −615 to −36 bp upstream from the TSS.

These constructs were transfected into the U937 cell line using electroporation. Transfected cells were cultured for 24 h in the presence or absence of 10 ng/ml PMA, washed, lysed, and assayed for Luc using a luminometer. The Luc reporter gene

TABLE I
Induction of Full-length TNF-α Promoter Construct by Phorbol Ester in U937 Cells

Plasmid	Induction signal	Luciferase concentration in experiments: 1	2	3	4	5	6
CMV-Luc		20,800	17,600	33,800	683,000	151,000	1,030,000
pXP		440	40	30	370	100	730
pXP	PMA	40	30	0	0	60	250
pTNF(-615)Luc		770	30	470	4,190	400	1,400
pTNF(-615)Luc	PMA	5,900	910	4,830	19,900	1,280	16,600
Fold induction		(7.6)	(33)	(10.2)	(4.7)	(3.2)	(11.8)

U937 cells were transfected with indicated plasmid and cultured in the presence or absence of PMA. Cells were harvested and assayed for luciferase as described in Materials and Methods. Luciferase concentration is expressed as relative light units (per 10^6 transfected cells). The index of PMA induction of construct pTNF(-615)Luc is expressed in parentheses. Replicate luciferase assays are reproducible to within 7-10%.

under control of the CMV viral promoter served as a positive transfection control. There is some variability in the transfection efficiency of these cells from experiment to experiment with better responses achieved in freshly split log phase cells and with the appropriate electroporation decay constant. As previously noted by others (17), the promoterless Luc plasmid (pXP) has very weak cryptic promoter activity. The TNF-α/cachectin promoter in the reverse orientation (pTNF[+615]Luc) is inactive. In the forward orientation, the full-length promoter construct had either weak or absent basal (uninduced) activity in U937 cells. PMA caused a mean 12-fold induction (range, 3.2-33) of the full-length TNF promoter construct (see Table I). The series of 5′ promoter truncations were studied (Fig. 1). Marked induction of promoter activity was observed when transfected cells were activated with PMA, especially with some of the shorter constructs. PMA-inducible promoter activity is still retained in constructs with only 95 bp, but not 36 bp, upstream from TSS. The U937 cell line generally does not constitutively produce TNF mRNA or protein, but PMA will induce TNF mRNA with peak expression at 4 h and release 0.5 U TNF/10^6 cells (data not shown; reference 2). These results demonstrate PMA-inducible promoter/enhancer activity in the 5′ flanking region of the human TNF-α/cachectin gene.

Analysis of the TNF promoter region (Fig. 2) reveals a cAMP-responsive element

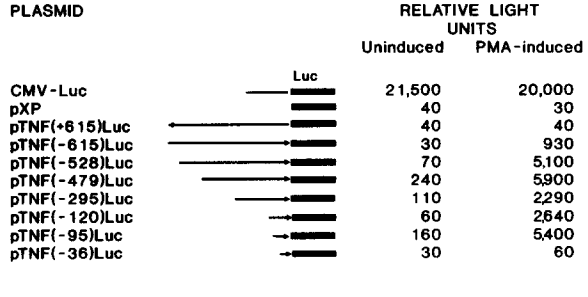

FIGURE 1. Activity of TNF/cachectin promoter region constructs in uninduced and PMA-induced U937 cells. Cells were transfected with 25 μg of indicated plasmid and cultured for 24 h in the presence or absence of 10 ng/ml PMA. Cells were then washed, lysed, and assayed for luciferase. Results are expressed as relative light units per 10^6 transfected cells. The data presented are reproducible in replicate assays to within 7-10%. This pattern of construct induction is representative of five experiments with U937 cells.

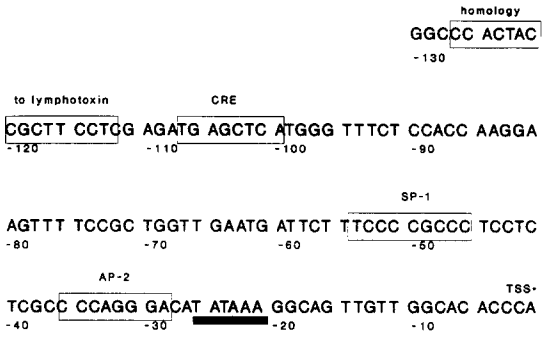

FIGURE 2. Sequence of human TNF-α promoter region. The 130-bp region upstream from transcription start site (TSS) is shown with putative *cis*-acting sequence motifs identified by boxes. The TATAA box is underlined.

(CRE) at positions −107 to −99 bp upstream from the TSS. The CRE is reportedly not responsive to phorbol esters (22), consistent with our findings of PMA inducibility in construct pTNF (−95)Luc, in which this enhancer element is eliminated. An AP-2 consensus sequence is present at position −36 to −28 (23). AP-2 is responsive to phorbol esters and cAMP, apparently through different signal-transduction pathways.

An AP-1 sequence is present at position −66 to −60 and is also responsive to phorbol esters. AP-1 and/or AP-2 may be responsible for inducibility. When the TNF-α and TNF-β (lymphotoxin) promoters are compared, there is a 15-bp sequence of exact homology (112–127 bp upstream from the TSS in the TNF-α gene) without recognizable similarity to known promoter/enhancer motifs (24). This sequence would also not appear to play a role in PMA induction of the TNF-α promoter. DNase I footprinting analysis and site-directed mutagenesis will be required to correlate these genetic data with the interaction of *trans*-acting factors with this promoter region.

Summary

The 615-bp 5′ flanking region of the human TNF-α/cachectin gene was isolated and ligated to the luciferase reporter gene. In addition, a series of truncated promoter constructs was generated by exonuclease III digestion. The promoter activity of these constructs was studied in a transient transfection system using the TNF-α-producing U937 cell line. Full-length and truncated TNF promoter constructions extending from −615 to −95 bp relative to the transcription start site (TSS) could be induced by phorbol esters. A construct truncated to within 36 bp of the TSS (and within 11 bp of the TATAA box) was inactive. Therefore, the phorbol ester responsive is localized in the TNF/cachectin promoter to a relatively short region proximal to the TATAA box.

The valuable advice of A. Cann, S. Nimer, and S. Golub is acknowledged as is the technical assistance of S. Kallaos.

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