Identification of a Tumor Necrosis Factor-responsive Element in the Tumor Necrosis Factor α Gene*

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The regulation by tumor necrosis factor α (TNF) of its own promoter has been investigated by transient transfection and nuclear protein binding assays. In human K562 erythroleukemia cells TNF produced an 8–10-fold activation of the human TNF promoter linked to the chloramphenicol acetyltransferase gene. The TNF-responsive element was localized to the −125 to −82 region by examining the TNF activation in 5′-deletion or site-directed mutants of the TNF promoter and by demonstrating that the −125 to −82 fragment confers TNF responsiveness to the thymidine kinase promoter. This region contains a palindromic 5′-TGAGCTCA 3′, that resembles the consensus binding sequences for the transcription factors, activator protein-1 (AP-1), cyclic AMP-responsive element binding protein (CREB), and activation transcription factor (ATF). An internal deletion in the palindrome abolished the TNF responsiveness, whereas known AP-1 and CREB/ATF elements were unresponsive to TNF. In band shift analyses a nuclear factor from U937 cells specifically bound to the −125 to −82 TNF-responsive fragment in or near the palindromic sequence. Oligonucleotides containing AP-1 or CREB/ATF sites did not effectively compete for the binding, indicating that the U937 cell factor is different from these factors. Anti-c-fos antiserum did not affect binding of the U937 cell factor, whereas anti-c-jun antiserum did block its binding, indicating that either c-jun or a protein antigenically related to c-jun is a component of the factor. These results suggest that the TNF-responsive element is not activated by AP-1 or CREB in U937 cells and that a novel DNA binding factor is important for constitutive and inducible TNF gene expression.

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* The abbreviations used are: TNF, tumor necrosis factor α; CAT, chloramphenicol acetyltransferase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; AP-1, activator protein-1; CRE, cyclic AMP-responsive element; CREB, cyclic AMP-responsive element binding protein; ATF, activation transcription factor; PBS, phosphate-buffered saline; TNF-RE, TNF-responsive element; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; NFκB, nuclear factor κB.
In the present study, we demonstrate that TNF activates its promoter in K652 and U937 cells and that the core of an eight-nucleotide palindromic sequence, 5'-TGAGCTCA-3', located at -100 to -101 is essential for this activation. This palindrome resembles the consensus element TNF-RE located at the factors AP-1 and CREB. However, both our functional and DNA binding studies suggest that the TNF activation of the TNF promoter is not mediated by these factors but may utilize a novel DNA binding protein.

**Experimental Procedures**

**Materials**—The human erythrocytoma K652 and human monocyte U937 cell lines, RPMI-1640, Dulbecco's phosphate-buffered saline (PBS), glutamine, and penicillin-streptomycin were obtained from the cell culture facility at the University of California, San Francisco. Biobrene was purchased from Applied Biosystems. Recombinant TNF was a generous gift from Dr. Jeffrey Andresen (Amgen Biologics). Riboprobe system and rabbit reticulocyte lysate were purchased from Promega.

**Plasmid Constructions**—A PsI to Aha11 fragment (-1044 to +93) was excised from the human TNF gene (pLT) (28), which was generously provided by Dr. David Goeddel (Genentech) and cloned into a PsI site in a plasmid vector in a pUC19-based vector containing the rabbit 5'-globin polyadenylation signals cloned upstream in the HindIII site and the chloramphenicol acetyltransferase (CAT) gene and an SV40 intron and polyadenylation signals cloned downstream in the BamHI site (29). This vector had also been deleted in the plasmid sequences from the polymerase Asp-718 site to the Aha11 site. The 5'-deletions were constructed using unique restriction sites, BstXI for the -345 deletion, ApaI for the -125 deletion, then ligated with phosphorylated HindIII linkers. The DNA fragments from pUC19 vectors into which the test binding sites had been cloned into a blunted SalII site. The sequences of the test binding sites are shown in Fig. 3. Radiolabeling was done by 5'-end labeling using T4 polynucleotide kinase and [γ-32P]ATP. DNA binding reactions were performed in a 20-μl volume containing a 10,000-cpm probe in a final concentration of 12 mM Hepes, pH 7.6, 48 mM potassium chloride, 0.8 mM EDTA, 10% glycerol, and 5 μg of poly(dI-dC). The binding reaction was initiated by the addition of 1 μl of nuclear extract, and the samples were then incubated for 15 min at room temperature. The samples were placed on ice and then loaded on a 6% polyacrylamide gel and electrophoresed at 200 V with running buffer consisting of 25 mM Trizma base, 25 mM sodium borate, and 1 mM EDTA.

**In Vitro Transcription and Translation**—A plasmid containing the rat c-fos cDNA was linearized using EcoRI, and capped mRNA was synthesized (Promega Riboprobe System) with T7 RNA polymerase. The reactions were incubated for 1-2 h at 37 °C. RNA was treated with RNase-free DNase, extracted with phenol/chloroform, dissolved in H2O, and stored at -70 °C.

**RESULTS AND DISCUSSION**

TNF activated a -1044 TNF promoter by 10-fold. The activation by TNF was similar in promoters deleted to -345 and -125 but was abolished by deleting the TNF promoter to -100 (Fig. 1). These results suggest that the TNF promoter contains a TNF-responsive element (TNF-RE), located in the -125 to -101 region. Nucleotides -108 to -101 of the promoter consist of the eight-nucleotide palindromic sequence, 5'-TGAGCTCA-3'. A four-nucleotide deletion (AGCT) within this sequence is critical for both of these activities.

The palindromic sequence is similar to the consensus motifs

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B. L. West, unpublished data.

V. Baichwal, unpublished data.
for three previously characterized families of DNA binding proteins, c-jun, CREB, and ATF. AP-1 is a heterodimeric complex comprised of c-jun- and c-fos-related factors (35-37). AP-1 regulates gene transcription by binding to the DNA sequence 5' TGACTCA 3', which is known as the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE) since AP-1 is activated by phorbol esters (35). The CREB and the ATF families both bind to the sequence, 5' TGACGTCA 3', and are activated by cyclic AMP (38) and E1a from adenovirus (39), respectively. Of these three classes, only AP-1 has been reported to be activated by TNF (22). Therefore, a possible role for AP-1 in the TNF induction of the TNF promoter was examined.

Three copies of the human metallothionein AP-1 element (TRE) were cloned upstream of the -32 to +45 thymidine kinase promoter, which does not contain any upstream enhancer elements and is inactive and not inducible by TNF in K652 and U937 cells. If AP-1 mediates the effects of TNF, then authentic AP-1 binding sites should confer TNF responsiveness to a heterologous promoter. For comparison we also transferred the -125 to -82 fragment of the TNF promoter and the somatostatin CREB binding element upstream of the thymidine kinase promoter. As shown in Fig. 2, three copies of the -125 to -82 TNF fragment conferred a substantial increase in basal activity, as well as a 7- or 11-fold stimulation by TNF. The -125 to -82 TNF fragment containing an internal deletion of four nucleotides (AGCT) within the palindrome was unable to confer either basal or TNF stimulation. The addition of three metallothionein TRE sites upstream of the thymidine kinase promoter markedly increased basal activity. However, this construction responded to TNF with a minor decrease in CAT activity in both K652 and U937 cells. This result may indicate that TNF regulation of AP-1 is more complex than previously appreciated (22), possibly depending on either tissue specificity and/or promoter context. CRE binding sites did not enhance basal activity or confer TNF responsiveness to the thymidine kinase promoter. These results demonstrate that only the native -125 to -82 region containing the intact palindromic sequence 5' TGAGCTCA 3' is capable of conferring TNF responsiveness to the heterologous thymidine kinase promoter even though the TRE and CRE sequences are almost identical to the TNF promoter palindromic sequence. These observations also suggest that AP-1 and CREB do not mediate the effects of TNF in these cells.

We next used a band shift assay to detect proteins in U937 or K652 cells that bind to the -125 to -82 region of the TNF promoter. Several shifted bands were observed, one of which was specific since this complex (Fig. 3A, lane 2, solid arrow) was inhibited by the addition of excess unlabeled -125 to -82 TNF fragment (lane 3). Treatment of U937 cells with TNF for 5 or 18 h did not alter the pattern observed (data not shown). These results indicate that the factor that binds to the TNF promoter fragment is expressed constitutively and is not inducible by TNF. The mutated -125 to -82 TNF fragment (lane 4) did not compete for binding, indicating that the deleted four nucleotides are critical for binding of the factor to the promoter. Oligonucleotides containing the TRE (lane 5) or CRE (lane 6) competed for binding minimally, indicating that the binding site for the U937 cell factor differs from AP-1 or CREB. Similar results were obtained when the band shift assays were performed with nuclear extracts prepared from K652 cells (data not shown). DNase I footprinting of the TNF promoter using affinity-purified c-junA protein revealed only a weak footprint over the palindromic region (data not shown), confirming that the proximal promoter does not contain a high affinity AP-1 binding site.

Although the functional and competition binding studies rule out that the factor from U937 cells is AP-1, a possible relationship of the factor to AP-1 was further examined by comparing their electrophoretic mobilities and anti-c-jun and anti-c-fos antiserum to determine if c-jun or c-fos are components of the factor that binds to the TNF palindrome. AP-1 was generated by binding purified bacterially expressed c-jun to an in vitro-synthesized c-fos. Fig. 3B shows that c-jun-c-fos (lane 2, open arrow) binds very weakly to the -125 to -82 TNF fragment and the band occurs at a lower position in the gel compared with the factor derived from U937 cells (lane 1, solid arrow). The difference in migration indicates that the U937 factor cannot be identical to the c-jun-c-fos heterodimer. Binding of c-jun-c-fos (Fig. 3C, lane 4) to an oligonucleotide containing the TRE site was much stronger than to the TNF promoter (Fig. 3B, lane 2). As shown in Fig. 3C, anti-c-fos antiserum did not block binding or supershift the factor bound to the -125 to -82 TNF fragment (lane 2), indicating that c-fos is not a component of the complex. Anti-c-jun antiserum did inhibit binding of the U937 cell factor (lane 3). Therefore, the U937 cell factor either contains c-junA or an antigenically related protein such as a CREB/ATF protein (39, 40). In a control experiment, anti-c-jun antiserum abolished binding of the c-jun-c-fos complex to the TRE (lane 6), whereas anti-c-fos antiserum produced a supershift of the complex (lane 5), demonstrating that the antibodies are capable of recognizing c-jun and c-fos.

In this study, we demonstrated that the -125 to -82 TNF promoter region containing the 5' TGAGCTCA 3' sequence functions as a constitutive enhancer and a TNF-responsive element in K652 and U937 cells. This sequence is nearly identical to the consensus AP-1 site, which was previously shown to mediate the activation of the collagenase promoter by TNF (22). However, the results of the present study indicate that AP-1 is not the factor from U937 cells which binds to the TNF palindrome and mediates the TNF induction of the TNF promoter. Interestingly, we did find that antiserum to c-junA blocked binding of the factor to the TNF palindrome, suggesting that c-junA or another member of the c-jun family or CREB/ATF families may be a component of the factor complex. If c-jun is a component of the complex it apparently dimerizes with a factor other than c-fos, possibly a tissue-specific factor which alters the specificity for c-jun binding to DNA. A model for this idea comes from the observation that the optimal binding of the c-jun-c-fos heterodimer is 5' TGAGCTCA 3', whereas when c-jun is com-

Fig. 2. The -125 to -82 region (TNF-RE) of the TNF promoter confers TNF responsiveness to an heterologous promoter. The DNA fragments or double-stranded oligonucleotides shown in Fig. 3 were cloned upstream of -32 to +45 TKCAT. The plasmids contained three copies of TNF-RE, mutated TNF-RE, or an oligonucleotide containing the metallothionein TRE or two copies of an oligonucleotide containing the somatostatin CRE upstream of TKCAT. K652 or U937 cells were transfected by electroporation using 15 μg TKCAT plasmids. Triplicate cell cultures were treated with 10 ng/ml TNF for 18 h. Cellular lysates were assayed for CAT activity and β-galactosidase activity. TNF promoter activity is expressed as CAT activity divided by β-galactosidase activity. TK, thymidine kinase.
plexed with CREB-binding protein 1 the preferred binding site is CRE sequence, 5'-TGACGTCA 3' (41). The purification and cloning of a cDNA for the factor will be necessary to establish its composition, its relationship to other factors, and the mechanism of regulation by TNF. The elucidation of the transcription factors that are regulated by TNF should provide a greater understanding of how TNF exerts its pleiotropic effects on physiological and pathological processes.

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