Human Tumor Necrosis Factor-α Gene 3' Untranslated Region
Confers Inducible Toxin Responsiveness to Homologous Promoter in Monocytic THP-1 Cells*

Anne Seiler-Tuyns, Nathalie Dufour, and François Spertini‡

From the Division of Immunology and Allergy, Department of Medicine, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland

To better define the role of 3' untranslated region (3'UTR) on transcriptional regulation of the human tumor necrosis factor (TNF)-α gene, monocytic human THP-1 cells were transfected with two TNF-α promoter constructs spanning base pairs −1897/−1 and −1214/−1, respectively, and linked to the rabbit β-globin gene. Quantitative globin gene expression of chimeras was measured by reverse transcription-polymerase chain reaction. A construct linking the chicken β-actin promoter and a deleted portion of the β-globin gene was cotransfected and used as internal standard. Unexpectedly, when THP-1 cells were stimulated with lipopolysaccharide or toxic shock syndrome toxin-1, gene regulation was hardly detected. In contrast, endogenous TNF-α gene regulation measured by the same reverse transcription-polymerase chain reaction procedure was vigorous. Remarkably, ligation of 3' UTR to chimeric constructs led to a drastic drop in the basal level of chimeric gene expression, resulting in a 15- to 40-fold induction of the reporter gene. Consistently, when the TNF-α promoter was replaced by the cytomegalovirus early immediate promoter, gene expression was also uniformly reduced but was no longer up-regulated upon stimulation with lipopolysaccharide and toxic shock syndrome toxin-1. These data provide the first line of evidence that, in addition to its role in TNF-α transcriptional level in humans as well.

EXPERIMENTAL PROCEDURES

Cell Culture—Human promonocytic cell line THP-1 was purchased from American Tissue Culture Collection (Bethesda, MD) and expanded at 37 °C, 5% CO₂, in RPMI 1640 medium (Seromed; Biochrom KG, Berlin, Germany) supplemented with 5 × 10⁻⁵ M 2-β-mercaptoethanol and 10% heat-inactivated fetal bovine serum (Mycolene; <1 pg/ml LPS; Life Technologies, Inc.). TSST-1 and SEB were purchased from Toxin Technology (Saratosa, FL), and LPS was purchased from Calbiochem (Lucerne, Switzerland).

Plasmids and Chimeric Constructs—Gene expression was measured by quantitative reverse transcription-PCR (15) using the rabbit β-globin gene as a reporter gene (16). Plasmid pGAlacGID, hereafter referred to as Δp (Fig. 1), contains the chicken β-actin promoter driving a truncated rabbit β-globin gene in which 40 nucleotides were deleted in the second exon and generates a shorter PCR amplification product (17). A second plasmid, pGAlacG+(+), containing the rabbit β-globin gene, was used as a vector for TNF-α gene promoter constructs. Human TNF-α gene promoter fragments were generated by PCR using the High Fidelity Taq DNA Polymerase system from Roche Molecular Biochemicals. The DNA template used for PCR contained the entire TNF locus (kindly provided by V. Jongeneel (Ludwig Institute, Lausanne, Switzerland)). Oligonucleotides used as primers were purchased from Microsynth (Balgach, Switzerland) and derived from published sequences of the human TNF locus (18, 19). Sequences were as follows: 5'-1897 oligo, 5'-GCTCGGATCCCTCTGGAATTGAAC-3'; 5'-1214 oligo, 5'-GCTCGGATCCCTCTGGAATTGAAC-3'; and 3'-1 oligo, 5'-GGCGGAGATCTGGTGTTGAAC-3'. Promoter fragments in chimerase 1 and 2 starting respectively at positions −1897 and −1214 from the transcription initiation site (+1) and terminating at position −1 were introduced in pAL1 vector (7) partially digested by BamHI and totally digested by KpnI. Cytomegalovirus enhancer and immedi-
ate early promoter were derived from pRL-CMV vector (catalogue number E2261; Promega). An enhancer-promoter fragment (nucleotides 7–803) was obtained by PCR using oligonucleotides 5' CMV oligo (5'-GCCGCTTCTGACCTGCTGG-3') and 3' CMV oligo (5'-GGCTCTAGATCTGCAGTGG-3'). This fragment was then introduced in the plasmid pNL1 digested by XhoI and Aap-H18, generating the construct called CMV.

In a similar fashion, a DNA fragment corresponding to the TNF-α 3' UTR, spanning nucleotides +1977 to +3007 from the transcription initiation site, was generated using oligonucleotides derived from published sequences of the human TNF locus (18, 19) and the corresponding DNA. Sequences were as follows: 5' 1977 oligo, 5'-GCCGCTTCTGACCTGCTGG-3' and 3' 3007 oligo, 5'-GGCTCTAGATCTGCAGTGG-3'.

The generated fragment was then introduced into the BG12 site of vectors 1, 2, and CMV, in a sense orientation in chimerae 1/3's, 2/3's, Δβ's, and CMV/3's and in an antisense orientation in chimerae 1/3's, 2/3's, Δβ's, and CMV/3's. All plasmids were amplified in Escherichia coli DH5α strain and purified by double banding on CsCl gradients before transfection.

The transfection—THP-1 cells were transiently transfected with a predetermined ratio of TNF-α promoter/rabbit β-globin gene chimera and of the reference plasmid Δβ (10 µg/ml:0.25 µg/ml, respectively, in 1 ml of fetal calf serum-free medium). Cells were grown at a density of 5–8 × 10^6 cells/ml and washed thoroughly with fetal calf serum-free medium. After a 4-h incubation (5–8 × 10^6 cells in 1 ml) in the presence of 300 µg/ml DEAE-dextran and the appropriate plasmid DNA, the transfected, resuspended in fresh medium, were incubated for 20–22 h at 37 °C in a 5% CO₂ incubator. Transfected cells were then stimulated with either 2 µg/ml LPS or 30 µg/ml TSST-1 or SEB for 90 min.

Preparation of Total Cellular RNA—Total RNA was extracted as described previously (20). Briefly, after phenol/chloroform extraction, total RNA was concentrated by precipitation and resuspended in DNase-free water. After a 4-h incubation (5–8 × 10^6 cells in 1 ml) in the presence of 300 µg/ml DEAE-dextran and the appropriate plasmid DNA, the transfected, resuspended in fresh medium, were incubated for 20–22 h at 37 °C in a 5% CO₂ incubator. Transfected cells were then stimulated with either 2 µg/ml LPS or 30 µg/ml TSST-1 or SEB for 90 min.

Reporter Gene Assay by Quantitative Reverse Transcription-PCR Assay—A predetermined quantity of RNA was used for each sample of a given experiment. The assay was carried out as described previously (7, 21), with minor modifications. Reverse transcription was performed in 30 µl of Perkin-Elmer PCR buffer containing 0.3–0.5 µg of total RNA supplemented with 0.2 mM deoxynucleotide triphosphate, 1 mM dithiothreitol, 12.5 µl of 10X oligo deoxynucleotidyl acid (Amersham Pharmacia Biotech), and 1 unit of avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech). Reverse transcription was carried out at 42 °C for 1 h. The resulting cDNA was used as an internal standard (22). The PCR product from pAW109 RNA amplified with TFN-α primers was 301 base pairs (bp) long and was designed to be shorter than the PCR product from target TNF-α mRNA (325 bp). After extension, a reverse transcription reaction aliquot (1 µl) was diluted 4-fold in PCR buffer mix (Perkin-Elmer) supplemented with 0.2 mM deoxynucleotide triphosphate, 1–2 µCi of [32P]dCTP (Amersham Pharmacia Biotech; > 3000Ci/mmol), and a pair of oligonucleotides (2 µm) specific for TNF-α (5' oligonucleotide, 5'-CAGAGGGAAGGAGTTCCCACG-3'; 3' oligonucleotide, 5'-CCCTTGGCTCTGAGCTTGGAGGCG-3') and amplified for 15 cycles using 1.5 units of Taq DNA polymerase (Perkin-Elmer). Another reverse transcription reaction aliquot (7 µl) diluted in PCR buffer was amplified as described above and then incubated for 15 min with a mixture of 5 µl of cDNA, 0.5 µg of specific probes (Table I), and 10 µm NS1 oligonucleotide, 5'-CCCTTGGCTCTGAGCTTGGAGGCG-3'.

The amplified cDNA was visualized by autoradiography. The amplified product was quantified using an Instant Imager® (Packard Instruments, Meriden, CT).

RESULTS

TNF-α Promoter Is Not Sufficient to Confer Toxin Responsiveness to Rabbit β-globin Reporter Gene—Stimulation of THP-1 cells transfected with control DNA Δβ, with chimera 1 or 2 (Fig. 1) alone, or with a combination of either Δβ or one of the two chimeras resulted in a strong stimulation of endogenous TNF-α transcripts (TSST-1, 25- to 70-fold induction; SEB, 10- to 55-fold induction; LPS, 30- to 97-fold induction) (Fig. 2A).

In contrast, although chimeric constructs 1 and 2 were strongly expressed, their expression was not regulated or was extremely poorly regulated (1.1- to 1.6-fold induction), irrespective of the stimulus and the presence or absence of the internal standard Δβ (Fig. 2B). As compared with endogenous wild type gene, the absence of reporter gene regulation suggested a lack in regulatory element(s) in the chimera.

huTNF-α 3' UTR Is Necessary to Reconstitute Toxin Responsiveness—Because promoter regions were unable to regulate human β-globin reporter gene expression by themselves, we transfected THP-1 cells with chimeric constructs 1 and 2 linked to human TNF-α 3' UTR gene sequences in a sense (s) or antisense (a) orientation (Fig. 1, chimerae 1/3's, 2/3's, 1/3's, and 2/3's) and examined whether induction could be recovered under these conditions. Endogenous TNF-α expression was strongly enhanced upon stimulation with TSST-1 or LPS (Fig. 3A, Table I), whereas chimeras 1 and 2 were again strongly expressed, but poorly regulated (1- to 2.5-fold induction; Fig. 3, B and C; Table 1). However, the addition of TNF-α 3' UTR drastically affected their level of expression; in contrast to the strong expression of chimera 1 and 2, the expression of chimeras linked to TNF-α 3' UTR (sense or antisense) was 10- to 30-fold lower (Fig. 3, B and C; Table 1). Furthermore, chimerae 1/3's and 2/3's from unstimulated THP-1 cells were hardly expressed. This finally resulted in chimerae 1/3's and 2/3's induction levels comparable to those observed for the endogenous TNF-α gene: a 10- to 45-fold induction for chimera 1/3', and up to a 17- to 57-fold induction for chimera 2/3' (Table I). A stronger constitutive expression of chimerae 1/3'a and 2/3'a was measured in unstimulated THP-1 cells, explaining the poorer induction ratios for the antisense constructs (chimerae 1/3'a had a 1.7- to 3.5-fold induction with TSST-1 and a 2.0- to 3.6-fold induction with LPS, chimera 2/3'a had a 1.7- to 2.6-fold induction with TSST-1 and a 1.9- to 2.3-fold induction with LPS; Table 1). Altogether, these observations indicated that TNF-α 3' UTR ligation introduced crucial elements for promoter regulation (23).

TNF-α 3' UTR Does Not Confer Toxin Inducibility to Unrelated Promoters—To determine whether this regulation occurred at the transcriptional and/or post-transcriptional level, we generated chimeric constructs in which the TNF-α promoter was replaced by other unrelated promoters. Because construct Δβ, driven by the chicken β-actin promoter, was not regulated by LPS or TSST-1, Δβ was linked to TNF-α 3' UTR in the sense and antisense orientations (Δβ/3's and Δβ/3'a) (Fig. 1). RNA levels for Δβ/3's and for Δβ/3'a were extremely weak, and no signal could be detected (Fig. 4B), although TNF-α endogenous induction was strong (Fig. 4A). This strongly suggested that TNF-α 3' UTR, irrespective of its orientation, destabilized chimeric mRNA (12). We then replaced the TNF-α promoter in chimerae 1, 1/3's, and 1/3'a with the cytomegalovirus (CMV) immediate early promoter, which is known to be much stronger than the chicken β-actin promoter. This new series of chimerae, called CMV, CMV/3's, and CMV/3'a (Fig. 1), was designed to generate RNA transcripts almost identical to those produced by chimerae 1, 1/3's, and 1/3'a, but controlled by a different promoter. Only a few nucleotides differed in the very 5' end of the resulting chimeric RNAs. A strong expression of chimera CMV
A. Schematic representation of the different constructs used in the study. First line, the human TNF-β and TNF-α locus (not drawn to scale). □, 5′ and 3′ untranslated regions (UTRs); ■, exons. The TATA box, initiation codon (ATG), stop codon, and polyadenylation sites are indicated. Chimera 1 and 2, two constructs containing 1896 and 1213 bp of the TNF-α promoter cloned upstream of the genomic sequences coding for the rabbit β-globin gene. □, exons. The ATG, stop codon and polyadenylation sites are indicated. 1/3′s, 1/3′a, 2/3′s, and 2/3′a, constructs derived from chimera 1 and 2 carrying the TNF-α 3′UTR in the sense (→) or antisense (←) orientation at the 3′ end of the rabbit β-globin gene. Δβ, modified rabbit β-globin gene carrying a 40-bp deletion in the second exon. □, chicken β-actin promoter. Δβ/3′s and Δβ/3′a, the same constructs with the TNF-α 3′UTR fused at the 3′ end of the last exon. CMV, a construct in which the TNF-α promoter in chimera 1 is substituted with the CMV promoter (□). CMV/3′s and CMV/3′a, the same constructs with the TNF-α 3′UTR hooked to the 3′ end of the rabbit β-globin reporter gene.

B. Figure 2. Chimeric constructs with the promoter region of huTNF-α are strongly expressed in THP-1 cells. A, autoradiogram showing endogenous TNF-α gene expression in THP-1 cells transfected with chimera 1 and 2 in the presence or absence of Δβ and stimulated by TSST-1 (T), SEB (S), and LPS (L) or left unstimulated (–). Int. st., internal standard pAW109 RNA. B, autoradiogram showing the expression of transfected chimera 1 and 2 in the same samples as in A. Two successive rounds of amplification of 20 and 15 cycles, respectively, were performed. Signals corresponding to the β-globin reporter gene and to the transfection control Δβ are marked β and Δβ, respectively. Data are representative of at least three independent experiments

(Fig. 5B) was observed, which was unaffected by cell stimulation (TSST-1 induction ratio, 1.0–1.6; LPS induction ratio; 1.7). Signals from cells transfected with chimerae CMV/3′s and CMV/3′a were about 10- to 30-fold weaker than signals from cells transfected with chimera CMV (Fig. 5B). However, a strong expression was detected in unstimulated cells even when transfected with chimera CMV/3′s, in contrast to our observations with chimerae driven by the TNF-α promoter (Fig. 3B). The presence of the unrelated CMV promoter in chimerae CMV/3′s and CMV/3′a strongly affected reporter gene regulation because the induction level in cells stimulated

with TSST-1 was abrogated or only minimally enhanced upon treatment with LPS (induction ratio, 2.4–3.7; Table I). In any case, this latter increase was significantly lower than the induction level of constructs driven by the TNF-α promoter (chimer CMV/3′s, 30-fold induction) (Fig. 3; Table I). Altogether, these results suggested that the TNF-α 3′UTR not only played a role at a post-transcriptional level by destabilizing RNA in the absence of induction but also played a role at a transcriptional level by modulating the use of the TNF-α promoter. This regulation was not specific for the type of stimulus applied.

DISCUSSION

We have shown here that the TNF-α 3′UTR plays a crucial role in human TNF-α gene transcriptional regulation, an observation that further extends its role in RNA transcript stability and translation as described previously (22). In our approach, we took advantage of a reporter gene system that allows direct analysis of RNA expression, in contrast to CAT or luciferase gene assays, which are less appropriate to tackle this issue. These latter systems involve protein enzymatic assays that not only reflect transcriptional regulation but, depending on the transfected chimera, also reflect the post-transcriptional and/or translational regulations that can affect the final enzymatic activity. Because TNF-α 3′UTR contains sequences that can modulate translation (10), we overcame this pitfall by directly measuring RNA transcripts. Thus, it appeared that chimeric constructs driven by large fragments of the human TNF-α promoter were strongly expressed in the monocytes cell line THP-1 but only weakly regulated by stimuli such as TSST-1 and LPS. These data are consistent with results from Goldfeld et al. (8), who transfected human TNF-α promoter-CAT chimeric constructs in the murine monocytic cell line P388/D1 and found a significant level of expression of the chimeras in uninduced cells. In agreement with our data, only
weak (1.5- to 2-fold) induction by LPS could be detected. Takashiba et al. (9), who also used CAT assays, found similar induction ratios. Very different results were obtained for the mouse gene because a strong induction by LPS was detectable in chimeric constructs containing mouse TNF promoter only (24). Furthermore, kB-type enhancers were involved in the transcription of the murine gene (25) but not the human gene (8). Although the regulation of human and mouse TNF genes differs in many respects, we took advantage of previous observations on the murine gene to design the chimeric constructs studied here. The role of mouse 3' UTR on chimeric gene expression has been well documented (12–14, 23, 26). In particular, mouse TNF-α 3' UTR is able to suppress TNF-α promoter constitutive activity in non-macrophage cell lines (14). Furthermore, it interacts with the mouse TNF-α 5' end to modulate chimeric CAT construct expression (12). We have shown in this study that human TNF-α 3' UTR suppressed the strong basal expression of TNF-α promoter constructs observed in its absence in unstimulated THP-1 cells and partially restored gene regulation in induced cells. This regulation could be due to transcriptional and/or post-transcriptional phenomena. Evidence for transcriptional regulation was provided by the analysis of constructs in which TNF-α promoter fragments were replaced with the CMV immediate early promoter. CMV chi-

![FIG. 3. Expression of chimeric constructs containing the huTNF-α promoter region hooked to combinations of huTNF-α 3' UTR. A, endogenous TNF-α gene expression in THP-1 cells transfected with chimeras 1/3's, 1/3'a, 2/3's, and 2/3'a and stimulated with TSST-1 (T), SEB (S), and LPS (L) or left unstimulated (-). Int. st., internal standard pAW109 RNA. B, expression of transfected chimeric genes 1/3's, 1/3'a, 2/3's, and 2/3'a in the same samples as in A. Two successive rounds of amplification of 20 and 15 cycles, respectively, were performed. Signals corresponding to the β-globin reporter gene and to the transfection control Δβ are marked β and Δβ, respectively. C, autoradiogram after an additional 2 cycles of amplification (second amplification was 17 cycles instead of 15 cycles) for transfected chimeric genes containing the TNF-α 3' UTR. Data are representative of at least three independent experiments.]

**TABLE I**

Quantitative expression of endogenous TNF-α versus rabbit β-globin reporter gene in THP-1 cells transfected with TNF-α or CMV promoter constructs linked or not to TNF-α 3' UTR

| Experiment no. | Stimuli | Constructs | 1 | 1/3's | 1/3'a |
|----------------|---------|------------|----|-------|-------|
|                |         | TNF-α      | β |       |       |
| 1              | TSST-1  | 40.0       | 1.4| 19.0  | 20.0  |
|                | LPS     | 69.4       | 1.00| 35.3  | 19.1  |
| 2              | TSST-1  | 32.6       | 1.5| 23.3  | 15.8  |
|                | LPS     | 42.1       | 1.6| 30.8  | 45    |
| 3              | TSST-1  | 28.5       | 1.8| 27.5  | 10.3  |
|                | LPS     | 53.1       | 2.3| 71.5  | 44.0  |

| Experiment no. | Stimuli | Constructs | 2 | 2/3's | 2/3'a |
|----------------|---------|------------|----|-------|-------|
|                |         | TNF-α      | β |       |       |
| 1              | TSST-1  | 24.6       | 1.1| 37.5  | 21.0  |
|                | LPS     | 37.3       | 1.1| 55.6  | 57.0  |
| 2              | TSST-1  | 23.8       | 1.7| 29.8  | 17.6  |
|                | LPS     | 35.1       | 2.5| 56.8  | 28.5  |
| 3              | TSST-1  | 20.1       | 1.4| 30.6  | 17.2  |
|                | LPS     | 49.2       | 1.5| 56.6  | 29.2  |

| Experiment no. | Stimuli | Constructs | CMV | CMV/3's | CMV/3'a |
|----------------|---------|------------|-----|---------|---------|
|                |         | TNF-α      | β |       |       |
| 1              | TSST-1  | 23.2       | 1.6| 28.1   | 0.6    |
|                | LPS     | 15.0       | 1.7| 24.6   | 2.4    |
| 2              | TSST-1  | 9.6        | 1.0| 22.2   | 0.5    |
|                | LPS     | 33.1       | 1.7| 13.8   | 3.7    |

*a Results are expressed as the fold induction over unstimulated cells, as determined by reverse transcription-PCR and quantitation on an Instant Imager®. Constructs are depicted in Fig. 1.
these experiments are large, and the identification of potential cis- and trans- regulatory elements in both the 5’ or 3’ regions will require the study of smaller gene fragments. TNF-α transcription may be enhanced by stimulatory mechanisms and/or the release of a pre-existing block. The precise characterization of transcription factors binding to DNA regions essential for regulated expression of the TNF-α gene may open new perspectives in the understanding of human diseases related to abnormal TNF-α gene expression. Interestingly, mutated genomic sequences in regions flanking the 3’ TTATTTAT signal element of TNF-α gene have been described in murine models (27), but not in young patients with autoimmune diseases (28). Because our results demonstrate the presence of other crucial regulatory domains interacting with promoter regions, the precise identification of discrete regulatory elements may help us to better understand the regulation of the TNF-α gene in inflammatory diseases.

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