A 3′ cis-Acting Element Is Involved in Tumor Necrosis Factor-α Gene Expression in Astrocytes

(Received for publication, March 4, 1996, and in revised form, June 25, 1996)

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Tumor necrosis factor-α (TNF-α) contributes to demyelinating diseases in the central nervous system. Astrocytes, the major glial cells in the CNS, do not constitutively express TNF-α, but the TNF-α gene is transcriptionally activated in response to a variety of stimuli, including TNF-α itself. Because of the importance of TNF-α in the CNS, we examined the mechanisms underlying transcriptional regulation of the TNF-α gene in astrocytes. In transient transfection assays, a plasmid construct containing 1.3 kilobase pairs (kb) of 5′ flanking sequence of the rat TNF-α gene showed high basal activity that could not be further enhanced by TNF-α stimulation. A “marked” 10-kb TNF-α gene construct, which contains the whole TNF-β gene with 1.2 kb of 5′ flanking sequence, 1.1 kb of intergenic sequence, and the whole TNF-α gene with 3 kb of 3′ flanking sequence, was able to respond to TNF-α stimulation. Analysis of a series of 5′ and 3′ deletion constructs of the marked TNF-α genes demonstrated that upstream sequence elements such as NF-κB are not required for TNF-α induction and that TNF-α responsive elements are located in the 3′ flanking region of the TNF-α gene. We also found that a TNF-α-inducible DNase I-hypersensitive (DH) site is present in this 3′ region whose deletion abolishes TNF-α inducibility of the marked TNF-α gene. Electrophoresis mobility shift assays showed that TNF-α-inducible nuclear proteins, consisting of p50 and p65 NF-κB proteins, specifically bind to two consecutive NF-κB binding sites within the 3′ DH site. These results indicate that TNF-α-induced TNF-α gene expression in astrocytes involves p50 and p65 NF-κB proteins binding to downstream NF-κB sites and concomitant modulation of the chromatin structure.

Tumor necrosis factor-alpha (TNF-α)† is a proinflammatory cytokine recognized to be an important mediator of immunological and inflammatory responses in a variety of tissues, including the central nervous system (CNS). TNF-α contributes to the pathogenesis of inflammatory demyelinating diseases such as multiple sclerosis by promoting infiltration of inflammatory cells into the CNS, intracerebral immune responses, cytokine production, astrogliosis, and demyelination (for review see Ref. 1). Astrocytes, the major glial cells in the CNS, are capable of producing TNF-α upon exposure to multiple stimuli (2–4) and thus can serve as an endogenous source of TNF-α within the CNS. We have previously shown that rat astrocytes are capable of producing TNF-α at both the mRNA and protein levels in response to lipopolysaccharide (LPS) and TNF-α (2, 5). TNF-α is not constitutively expressed in astrocytes; however, the TNF-α gene is transcriptionally activated upon exposure to these stimuli (5, 6).

Most of the previous studies on transcriptional regulation of the TNF-α gene have focused on the promoter regions of the mouse and human TNF-α genes. Thus far, however, results have not been conclusive regarding the cis-acting elements and transcription factors involved in TNF-α gene expression. The role of the transcription factor NF-κB in the regulation of TNF-α gene expression is controversial. NF-κB is essential for LPS induction of the mouse TNF-α promoter in mouse macrophages (7, 8), whereas the three κB sites in the human TNF-α promoter are neither required nor sufficient for virus or LPS induction of the TNF-α gene in mouse monocytic cell lines (9). Phorbol ester (PMA) activation of the human TNF-α promoter in the U937 macrophage cell line appears to be mediated by the transcription factor AP-1 (10, 11). Analysis of the upstream region of the human TNF-α gene in U937 cells showed that the sequence of −95 from the transcription start site (TSS) is sufficient for PMA induction and that AP-1 plays an important role in both basal and PMA-induced activity of the human TNF-α promoter (10, 11). In contrast, Leitman et al. (12) demonstrated that the proximal human TNF-α promoter containing only 28 nt upstream and 10 nt downstream of the TSS is sufficient for PMA induction in U937 cells; no other upstream sequences were required, and the TATA box structure was important. The minimum region of the human TNF-α promoter required for PMA activation in T and B cells has been localized between −52 and +89 with respect to the TSS, which is different from that in macrophages (13). The TNF-α promoter also can be activated by TNF-α itself, which appears to be mediated by a palindromic sequence present between −125 and −82 that resembles the consensus binding sequences for AP-1, CREB, and ATF (14). In addition to NF-κB and AP-1, other transcription factors such as NFAT (15), Egr-1 (16), C/EBPβ (17), and Ets (18) also appear to be involved in TNF-α gene expression. Furthermore, an involvement of downstream sequences in the TNF-α gene has been suggested (19, 20). Taken together, it appears that the cis-acting elements and trans-acting factors involved in TNF-α gene expression are diverse and that the molecular mechanisms underlying transcriptional activation of the TNF-α gene are species-, tissue-, and stimuli-specific.

Because of the biological importance of TNF-α in the CNS, we have been studying how this gene is regulated in astrocytes.

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‡ This work was supported by Grant RG-2269-B-5 from the National Multiple Sclerosis Society (to E. N. B.) and Public Health Service Grant MH-50421 from the National Institute of Mental Health (to E. N. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Using a series of marked rat TNF-α constructs, we have determined that upstream sequence elements such as the NF-κB sites are not required for TNF-α induction of the TNF-α gene and that the TNF-α responsive elements are present in the 3′ flanking region of the TNF-α gene. We have also found that a TNF-α-inducible DH site is present in the 3′ flanking region whose deletion abolishes TNF-α inducibility of the marked TNF-α gene. We then identified TNF-α-inducible nuclear proteins consisting of the p50 and p65 members of the NF-κB family that specifically bind to the NF-κB sites within the DH site. These data suggest an involvement of 3′ κB sites and modulation of the chromatin structure in TNF-α gene expression in astrocytes. These findings collectively indicate that regulation of TNF-α gene expression in astrocytes differs from that previously described for monocytes, T-cells, and B-cells, suggesting cell type-specific mechanisms for control of this important cytokine.

MATERIALS AND METHODS

Primary Glial Cell Cultures—Primary glial cell cultures were established from neonatal rat cerebra as described previously (2). Meninges were removed from rat brains prior to glial cell dissociation and culture. Culture medium was Dulbecco’s modified Eagle’s medium, high glucose formula supplemented with glucose to a final concentration of 6 g/liter, 2 mM ornithine, and nonessential amino acid mixture, 0.1% gentamicin, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT). After 14 days in primary culture, oligodendrocytes were separated from the glial cultures by mechanical dislodging, and astrocytes were obtained by trypsinization (0.25% trypsin, 0.02% EDTA). Astrocytes were monitored for purity by immunofluorescence and by nonspecific esterase staining for contaminating microglia. Astrocyte cultures were routinely >97% positive for glial fibrillary acidic protein, an intracellular antigen unique to astrocytes (21), and less than 2% of the cells were microglia based on their positive staining for nonspecific esterase.

Plasmid Constructions—All the TNF constructs originated from the plasmids pBlu, pBlu3, or pGEM3z. pTNF10 was constructed as follows; two unique SacI sites of pGEM3z were destroyed by two steps of cutting, removing the 5′-BglII to 3′-SacI fragment of pGEM3z. This fragment was blunt-ended with the pBluCAT3 vector (23) that was linearized with BglII to generate either pTNF+1229CAT or pTNF−1229CAT. 5′ deletion constructs were generated by exonuclease III digestion of pTNF−1229CAT digested with SplI and BamHI. Deletion points were determined by dideoxy chain-termination sequencing (USB).

pTNF6.5 was constructed by sequential subcloning of the 2.3-kb EcoRI-SacI and the 4.2-kb SacI fragments of the TNF-α gene into EcoRI and SacI sites of pGEM3z. pTNF10 was constructed as follows; two SacI sites of pTNF6.5 were destroyed by two steps of cutting, removing protruding ends, and religating. A 4.9-kb HindIII-SalI (SalI is in the vector) fragment (3′-half of the TNF locus) was ligated with a 2.8-kb HindIII-SalI fragment of pGL2-Basic vector (Promega). The resulting plasmid was cut with SacI and XhoI and ligated with a 5.3-kb SacI-XhoI fragment (5′-half of the TNF locus) to generate pTNF10. A series of marked TNF-α gene deletion constructs were made as follows; a 2.1-kb SalI-XhoI fragment was cloned into the same sites of pGL2-Basic vector to make the BglII site in the 5′ untranslated region of the TNF-α gene unique. An 8-bp synthetic NotI linker (CCGGGGCCGGCC, New England Biolabs) was inserted to the BglII site that had been cut and blunt-ended, which resulted in an extra 12-bp (CCGGGGCCGGCC) introduced to the wild type sequence. The 1.1-kb SacI-XhoI fragment containing the NotI linker sequence was substituted for the same site fragment of pTNF6.5 to generate pTNF5.9*. pTNF10* was generated by substitution of a 5.6-kb SacI-BglII (BglII is in the 3′ flanking region of the TNF-α gene) fragment of pTNF6.5* for the same sequence of pTNF10. pTNF5.9* was generated by cloning the SacI (blunt-ended)-SalI (in the vector) fragment into the SmaI-SalI sites of pGEM3z. The 3′ deletion constructs of the marked TNF-α gene were generated by utilizing the unique restriction enzyme sites in the 3′ flanking region of the TNF-α gene that were also present in the vector. pTNF4.4*–3.8*, −3.4*, and −2.7* were generated by deleting portions of XhoI, SplI, SmaI, and PstI fragments, respectively. The ScaI (blunt-ended)-EcoRI fragment was cloned into SmaI-EcoRI sites of pGEM3z to generate pTNF2.9*. pGL2-Control vector used as the internal control was purchased from Promega.

The template plasmid for the TNF-α antisense RNA probe was prepared as follows. The sequence from −70 to +400 of gene pTNF6.5* (see Fig. 2C) was amplified by PCR. The primers were designed such that EcoRI and BamHI sites were placed at the 5′ and 3′ ends, respectively. The upstream primer was AGTTTCCGGGAATTCGGGTGTGAAAGACCTTTTGC and the downstream primer was CCTTTCTGCGATCCCTCCACCCACTTGGCT. After digestion with EcoRI and BamHI, the 482-bp PCR product was cloned into pGEM4z to generate pTNF5.9*. The plasmid for luciferase antisense RNA probe, pLUC5*, was prepared in the same way. The sequence from 121 to 439 (sequence number according to mammalian 2222) was amplified by PCR and cloned into pGEM4z (see Fig. 2C). The upstream primer was CAATCTTTGGATCCGCGGATCCGACTAAT and the downstream primer was CGGACATTTGGATCCGGTGCTAGCG.

Transfection and Stimulation of Astrocytes—Transfection of rat astrocytes was performed by electroporation using a Bio-Rad gene pulser as described previously (24). Astrocyte cultures were trypsinized, 7–10 ∗10⁶ cells resuspended in 250–400 µl of complete culture medium containing 20% fetal bovine serum, mixed with 1–20 µg of plasmid DNA (CosCl, purified twice) with the same molar ratios, incubated for at least 10 min on ice, and then pulsed at 250 V and 960 microfarads. Cells transfected with the same plasmids were pooled, resuspended in 100-mm dishes with 10 ml of culture medium containing 10% fetal bovine serum, and allowed to recover for 24–48 h. Transfected astrocytes were stimulated with either medium alone or rat recombinant TNF-α (Biosource Int., Camarillo, CA) (500 units/ml) for 18 h for the CAT assay or 2 h for RNA analysis. The CAT assay was done with the xylene extraction method as described previously (24). The RNAase protection assay (RPA) was performed with the RPA kit according to the manufacturer’s instructions (Ambion) as described previously (6). The antisense RNA probes were generated with an in vitro transcription kit (Ambion, Austin, TX) as described previously (6). pTNF5.9* or pLUC5* linearized with EcoRI was used as template DNA for TNF or luciferase antisense RNA probes, respectively. RNAse protection assay (RPA) was performed with the RPA kit according to the manufacturer’s instructions (Ambion) as described previously (6). 60 µg of total cellular RNA were hybridized overnight at 43–45°C with TNF-α (2 ∗5 ∗10⁶ cpm) and luciferase (2 ∗5 ∗10⁶ cpm) probes in 20 µl of hybridization buffer containing 40 mM PIPES, 80% deionized formamide, and 400 mM sodium acetate. The hybridization reaction was then incubated at room temperature for 1 h in the presence of 5 µg/ml RNase A and 50 units/ml RNase T1. RNA protected from RNase digestion was precipitated and subjected to electrophoresis (5% polyacrylamide, 8 M urea gel) after heating at 90–95°C for 3–4 min. Quantitation of the protected RNA fragments was performed by scanning the gel with the PhosphorImager (Molecular Dynamics, Mountain View, CA).

DNase I Hypersensitive Site Assay—Astrocytes were stimulated with medium alone or TNF-α (500 units/ml) for 20 min, harvested on ice after washing with cold PBS, and pelleted by centrifugation. The cell pellet was resuspended in 1 ml of hypotonic buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 5 mM MgCl₂ incubated on ice for 30 min, and then centrifuged at 1400 ∗ g at 4°C for 4 min. The pellet was resuspended in 1 ml of hypotonic buffer containing 0.1% Nonidet P-40, incubated on ice for at least 10 min, and homogenized by 20 strokes with a type B pestle and 10 strokes with a type A pestle. Nuclei were purified through hypotonic buffer containing 8.5% (w/v) sucrose by centrifugation at 1500 ∗ g at 4°C for 10 min. The nuclei pellet was resuspended in 1 ml of digestion buffer containing 100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 4.2 mM MgCl₂, and 1 mM CaCl₂ and stored on ice until DNAase I digestion. The concentration of the nuclei was adjusted to 4–5 ∗10⁶ nuclei/ml. 500 µl aliquots of the nuclei were incubated either in the absence or presence of varying amounts of DNase I (0.05–2.5 µg/100 µl, Sigma, DN-EP) at 37°C for 5 min. 100 µl of stop solution containing 200 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20 mM EDTA, 2% SDS, and 200 µg/ml protease K (Boehringer Mannheim) was added and then incubated overnight at 37°C. 0.2 volume of 10 mM ammonium acetate was added after phenol/chloroform extraction, and DNA was precipitated by adding 2 volumes of ethanol, followed by centrifugation at 6000 × g (∼8500 cpm for Eppendorf microcentrifuge), and washed twice with 70% ethanol. DNA was dissolved in TE buffer and subjected to restriction enzyme digestion. RNase A (25 µg/ml) was added and incubated at 37°C for 1 h, and DNA purified by a standard protocol of phenol/chloroform extraction and ethanol precipitation. DNA was run on a 1% agarose gel (<1 V/cm, for 16–20 h), transferred to nitrocellulose membrane, and hybridized with labeled probes by standard methods.

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were prepared from astrocytes as described previously (24). Briefly, astrocytes were incubated with medium alone
Fig. 1. TNF-α induces TNF-α mRNA expression in primary rat astrocytes. Astrocytes were incubated with medium alone (lane 1) and rat recombinant (r) TNF-α at the indicated amounts (lanes 2–4) for 2 h. Total RNA was isolated and analyzed by RNase protection assay. RNA samples were hybridized to both rat TNF-α and cyclophilin probes. Representative of three experiments.

or TNF-α (500 units/ml) for the indicated times, harvested by scraping after washing with cold PBS, and pelleted. Cells (~5 × 10⁷) were then resuspended in 1 ml of hypotonic buffer (10 HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM PMSF), incubated on ice for 10 min, and pelleted at 1,000 × g for 10 min. Pellets were resuspended in 0.5 ml of the hypotonic buffer containing 0.1% Nonidet P-40, incubated on ice for 20 min, and then homogenized with 20 strokes using a type B pestle in a Dounce homogenizer. Nuclei were then pelleted at 40,000 × g for 30 min, resuspended, and homogenized in 1 ml of high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 1 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, and 0.5 mM PMSF), and shaken for 30 min at 4 °C. Nuclei were then pelleted at 40,000 × g for 30 min, and protein extracts were dialyzed against a large volume of dialysis buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF).

EMSA was performed in a volume of 20 μl containing 150 μg KCl, 2.5 mM MgCl₂, 0.25 mM EDTA, 20% glycerol, 0.25 mM DTT, 10 mM Tris-Cl, pH 7.5, 1 μg of polydeoxyinosinic-deoxycytidyl acid, 1 μg of sonicated salmon sperm DNA, 8–10 μg of nuclear extract, and 10,000–20,000 cpm 32P-labeled oligonucleotide probe and incubated on ice for 30 min. Bound and free DNA were then resolved by electrophoresis through a 6% high ionic strength polyacrylamide gel (1 × TGE (Tris/glycine/EDTA) buffer) containing 5% glycerol at 250 V for 1 h. For competition assays, unlabeled probes were incubated with nuclear extracts on ice for 10 min followed by the addition of labeled probe. All the antisera against NF-κB subfamilies (p65, p50, p52, RelB, and c-Rel) were the generous gift of Dr. Nancy Rice (National Cancer Institute, Frederick, MD) (25, 26).

RESULTS

Expression of the TNF-α Gene Can Be Induced by TNF-α in Primary Astrocytes—We have previously shown that rat astrocytes are capable of producing TNF-α at both the mRNA and protein levels in response to LPS (2). The TNF-α gene is not constitutively expressed in astrocytes; however, upon stimulation with LPS, TNF-α mRNA and protein are transiently expressed. Steady state levels of TNF-α mRNA are rapidly (15 min) detected after stimulation, are maximal at 2 h, and then decline (6). It has been shown that TNF-α induces its own expression at both the mRNA and protein levels in HL-60 cells (27). Here, we show that the TNF-α gene can be induced by TNF-α in astrocytes. As shown in Fig. 1, TNF-α mRNA is not expressed in unstimulated cells (lane 1) and can be induced by TNF-α in a dose-dependent manner (lanes 2–4). Further TNF-α dose-response experiments revealed that 500 units/ml TNF-α induced optimal TNF-α mRNA expression (data not shown); thus, this concentration was used for the remainder of the studies.

Functional Analysis of the Upstream Sequence of the Rat TNF-α Gene—To identify cis-acting regulatory element(s) required for TNF-α induction of the TNF-α gene, we made a series of constructs in which serially deleted upstream regions of the rat TNF-α gene were fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Astrocytes were transiently transfected with these constructs and stimulated with medium alone or TNF-α for 18 h, and then CAT activity was measured. Table I summarizes the results of the transfections. The full-length promoter construct, pTNF(-1229)CAT, which contains the sequence from −1229 to +130 with respect to the transcription start site (TSS) of the rat TNF-α gene (the AvaI fragment, see Fig. 2A), was not responsive to TNF-α stimulation, and a high basal level of CAT activity was detected in the absence of stimulation. The rat TNF-α gene has five κB-related sites in its upstream region. The high basal activity was not affected by deletion to −235 bp, which eliminated four NF-κB binding sites. Further deletion to −177, which removed the most proximal NF-κB binding site, resulted in approximately a 3-fold decrease in basal activity compared with the full-length promoter. However, none of the deletion constructs responded to TNF-α stimulation. These data indicate that the 1359-bp upstream region of the rat TNF-α gene is not sufficient for TNF-α-mediated activation of the TNF-α promoter.

Localization of TNF-α Responsive Sequence(s) in the 3’ Flanking Region of the TNF-α Gene—The results described above led us to examine the 10-kb rat TNF locus with respect to the potential TNF-α responsive sequence(s). We prepared a series of “marked” TNF-α genes in which a mutation was introduced to allow discrimination of those mutated genes from the endogenous wild-type TNF-α gene (Fig. 2, A and B). The marked gene has an 8-bp synthetic NotI linker that is inserted to the BspEI site in the 5’-untranslated region of the TNF-α gene (Fig. 2C). An antisense RNA probe was generated such that it is complementary to the sequence of −70 to +400 of the marked TNF-α gene containing the inserted NotI linker. In the RNase protection assay (RPA), this probe protects 359 nt of mRNA that are accurately initiated from the marked TNF-α gene promoter, whereas endogenous TNF-α mRNA is protected as two smaller fragments, 150 and 196 nt (Fig. 2C). This system also allows differentiation of authentic marked TNF-α mRNA from non-
specific transcripts such as readthrough transcripts. pGL2-Control, in which the SV40 promoter drives luciferase gene expression, was used as an internal control for transfection efficiency and RNA integrity. Similarly, the antisense RNA probe for the luciferase gene was generated such that the luciferase mRNA accurately initiated from the SV40 promoter was protected as ~250-nt fragments (Fig. 2C). Thus, it is possible to detect message from three genes in the same gel; the transfected marked TNF-α gene, the transfected luciferase gene, and the endogenous TNF-α gene (see Fig. 2D). A number of preliminary control experiments of transient transfections with pTFN10* and/or vector plasmid showed that the 359-nt message is specifically transcribed from the promoter of the transfected TNF-α gene, that neither the vector nor the co-transfected pGL2-Control affect the regulation of the transfected TNF-α gene, and that the endogenous TNF-α gene was accurately regulated (data not shown).

To localize the TNF-α responsive sequence(s), we analyzed a series of 5’ and 3’ deletion constructs of the marked TNF-α genes (Fig. 2B) in transient transfection assays. The results are shown in Fig. 2D. The full-length construct, pTFN10*, which contains the entire TNF-β gene with 1.2 kb of 5’ flanking sequence, the 1.1-kb intergenic space, and the entire TNF-α gene with 3 kb of 3’ flanking sequence, showed basal activity and was able to respond to TNF-α with ~6-fold induction. Two 5’ deletion constructs, pTFN6.5* and pTFN5.9*, were also capable of responding to TNF-α with ~5-fold induction, similar to what was observed with pTFN10*. pTFN6.5* (5’ deletion at ~634), lacking the TNF-β gene, retains three NF-κB sites in the TNF-α promoter region. pTFN5.9* (5’ deletion at ~70) contains in the promoter region an Sp-1 binding site, an AP-2 binding site, and the TATA box (Fig. 2, A and B). These results indicate that, for TNF-α induction of the TNF-α gene, the xβ sites of the upstream region are not necessary, and the sequence of ~70 is sufficient in the presence of downstream sequences.

Deletion points of the 3’ deletion constructs, pTFN4.4*, pTFN3.6*, pTFN3.4*, pTFN2.9*, and pTFN2.7* are ~1.9 and ~1.1 kb, 746, 240, and 56 bp downstream of the polyadenylation signal sequence of the TNF-α gene, respectively (Fig. 2, A and B). As shown in Fig. 2D, the constructs with deletions up to 746 bp (pTFN4.4*, pTFN3.6*, and pTFN3.4*) are capable of responding to TNF-α. The more extensive deletion constructs, pTFN2.9* or pTFN2.7*, do not respond to TNF-α stimulation. As in the 5’ deletion constructs, basal levels of marked TNF-α mRNA were detected in all the 3’ deletion constructs. None of the deletions up to pTFN3.4* significantly affected the magnitude of TNF-α induction (~4–6-fold) of marked TNF-α gene expression, indicating that no other important regulatory sequences are present in regions outside of TNF3.4*.

The regions pointed by EcoRI and BamHI indicate primer binding sites for PCR for the template DNA of the antisense RNA probes. The predicted, protected sizes of mRNA are indicated under the corresponding genes; probe, unprotected free probe (thick line indicates vector sequence). RT, readthrough transcript (5’ TNF/77), mRNA accurately initiated from transfected marked TNF-α gene promoter, TNF(E), endogenous TNF-α mRNA, 5’ LUC, mRNA accurately initiated from the multiple transcription start sites of SV40 promoter of pGL2-Control. In this report, we describe the protected sizes of the TNF-α antisense RNA probe according to the previously described putative transcription start site (22). D, astrocytes were transfected with pGL2-Control (6 μg of transfection) and the marked TNF-α gene constructs at the same molar ratios of μg of DNA/transfection: pTFN10*, 2.76 μg; pTFN6.5*, 2.00 μg; pTFN3.9*, 1.87 μg; pTFN4.4*, 1.52 μg; pTFN3.6*, 1.87 μg; pTFN3.4*, 1.53 μg; pTFN2.9*, 1.22 μg; and pTFN2.7*, 1.17 μg). Cells transfected with the same constructs were combined, replated in two 100-mm dishes, and allowed to recover for 44 h. Transfected astrocytes were then stimulated with medium alone (−) or 500 units/ml rat TNF-α (+). 90 μg of total cellular RNA were used for RPA. Representative of three experiments.
TNF-α gene expression was accurately regulated in all the transfections (≈15-fold induction), indicating that abolishment of TNF-α inducibility of pTNF2.9* or pTNF2.7* is specifically due to deletions of the sequences rather than possible impairment in the TNF-α-mediated signaling pathway leading to activation of the TNF-α gene. These data demonstrate that the 500-bp downstream region of the TNF-α gene, i.e., the sequence between the EcoRI and Smal sites (Fig. 2A), is critical for TNF-α induction of the TNF-α gene in astrocytes.

Identification of TNF-α-inducible DNase I-hypersensitive Sites in the 3' Flanking Region of the TNF-α Gene—We examined the chromatin structure of the endogenous TNF-α gene by looking at DNase I-hypersensitive (DH) sites, which are known, in essentially every case, to be associated with cis-acting regulatory sequence elements such as enhancers (28, 29). The 4.2-kb ScaI region, containing the 3' region of the TNF-α gene, was examined for DH sites with a labeled 420-bp ScaI-BamHI fragment abutting the 5' end of the ScaI fragment (see Figs. 2A and 3C for map). As shown in Fig. 2A, there are no detectable DH sites in this ScaI region under basal conditions; only the 4.2-kb parent band is detected. Two DH sites are rapidly (within 20 min) induced upon TNF-α stimulation. One (3' DH1) is mapped around the 3' deletion point of pTNF2.9* (the EcoRI site); the subfragment migrates along with the 1.2-kb band of the TNF-α plasmid marker. The fact that TNF-α-inducible DH site overlaps with the region of the deletion, which abolishes TNF-α responsiveness of pTNF2.9*, strongly suggests that the TNF-α responsive sequence(s) is located in this region (around the EcoRI site). These results also suggest that the chromatin of that region undergoes structural changes from a closed to open configuration. The other DH site (3' DH2), which is much weaker than the 3' DH1, is located at 800 bp downstream of the 3' DH1 (Fig. 3C). This 3' DH2 is not likely involved in TNF-α gene regulation, since deletion of this site (pTNF3.4*) had no effect on TNF-α inducibility of the TNF-α gene (Fig. 2D).

TNF-α Induced NF-κB (p50 and p65) Proteins Specifically Bind to the Two Consecutive κB Sites in the 3' DH1 Region—Sequence analysis showed that the region of the EcoRI site (3' deletion point of pTNF2.9* or the 3' DH1) contains consensus sequences of binding sites for NF-κB or AP-1, which are known to be inducible by TNF-α (30–32). The canonical NF-κB binding site (NF-κB-IC-li in Fig. 4) is present 8 bp upstream of the EcoRI site. This κB site has been shown to be bound by TNF-α-induced NF-κB-like protein and mediates TNF-α induction of the class II-associated invariant chain (ii) gene in rat fibroblast cells (33). The PRDI sequence in the reverse orientation overlaps with the NF-κB-IC-li (one T deletion, indicated by κB-like in Fig. 4) overlaps with the EcoRI site. The consensus sequence for the AP-1 binding site is found 84 bp downstream of the EcoRI site (35).

We wanted to examine the potential involvement of the two consecutive κB-(like) sequences in TNF-α-induced TNF-α gene expression in astrocytes by investigating nuclear proteins binding to these sites. Electrophoretic mobility shift assays were performed using the synthetic oligonucleotides described in Fig. 5A. For convenience, we designate the NF-κB-IC-li and κB-like as κB and κB', respectively. We found that nuclear extracts from unstimulated astrocytes showed two weak com-
**FIG. 5.** TNF-α induces p50 and p65 NF-κB proteins that specifically bind to the κB sites in the 3' DH1 site. A, double strand DNA oligonucleotides used for EMSA. For all the sequences listed, directions are from 5' to 3', and only sense strands are shown. The consensus sequences are underlined, and mutated nucleotides are indicated in lowercase letters. B, labeled κB/κB probe was incubated on ice for 30 min without (lane 1) or with nuclear extracts from primary astrocytes treated with medium alone (lane 2) or TNF-α (500 units/ml) for 15, 30, 60, 120, or 240 min (lanes 3–7). C, nuclear extracts from astrocytes treated with medium alone (lane 1) or TNF-α for 15 min (lanes 2–10) were incubated with labeled κB/κB probe on ice for 30 min, following preincubation on ice for 10 min without (lane 2) or with a 250-fold excess amount of unlabeled κB/κB (lane 3), κB (lane 4), κB' (lane 6), κB/IL-6 (lane 8), or AP-1/TNF-α (lane 10) oligonucleotides. Two hundred and fifty-fold excess amounts of κB (lane 5), κB' (lane 7), and κB/IL-6 (lane 9) oligonucleotides were also used. The dried gel was exposed to film for 24 h at −70 °C. D, nuclear extracts from astrocytes treated with medium alone (lanes 1 and 8) or TNF-α for 15 min (lanes 2–7 and 9–14) were incubated on ice for 10 min with unlabeled 250-fold excess amounts of κB (lanes 3 and 12), κB (lanes 4 and 13), κB' (lanes 5 and 10), κB' (lanes 6 and 11), and κB/IL-6 (lanes 7 and 14) and then incubated on ice for 30 min with labeled κB (lanes 1–7) or κB' (lanes 8–14) probes. The dried gel was exposed to film for 48 h at −70 °C. E, nuclear extracts from astrocytes treated with medium alone (lane 1) or TNF-α for 15 min (lanes 2–14) were incubated at room
plexes on the κB/κB’ probe containing two κB-(like) sequences and that the slower migrating complex (I) was enhanced by TNF-α stimulation, whereas the faster migrating complex (II) remained unchanged (compare lane 2 and lane 3 in Fig. 5B). Kinetic analysis with nuclear extracts from astrocytes that had been treated with TNF-α for different periods showed that complex I was immediately induced (−12-fold induction) after 15 min of TNF-α treatment (lane 3 in Fig. 5B) and gradually decreased after 4 h of stimulation (lane 7 in Fig. 5B). Complex II was not changed at any point after TNF-α stimulation. The kinetics of TNF-α induction of complex I is functionally relevant to TNF-α-mediated activation of the TNF-α gene, since the steady state level of TNF-α mRNA accumulation is maximally induced at 1–2 h after TNF-α treatment and subsequently diminishes.

To examine the specificity of these complexes, we performed competition assays with excess amounts of unlabeled probes. Complex I formed on the κB/κB’ probe could be specifically competed away by a 250-fold excess amount of unlabeled κB/κB’ probe but not with the same amount of an unrelated sequence such as AP-1/TNF-α (this is the AP-1 sequence located in the 3’ DH1) (Fig. 5C, lanes 3 and 10). Inhibition by the competitors was dose-dependent; we show only the 250-fold excess amount for all the competition data. To determine which κB site of the κB/κB’ probe is responsible for the formation of complex I, we carried out competition experiments with unlabeled κB or κB’ probes. As shown in Fig. 5C, the κB competitor can efficiently compete away complex I formed on the κB/κB’ probe (lane 4), whereas the κB’ competitor is able to partially compete away complex I (lane 6). The binding of complex I is κB site-specific since mutation of the guanine nucleotides GGA, which are critical for NF-κB binding, of both κB and κB’ probes abolishes the inhibition of complex I (lanes 5 and 7). To further ascertain the κB site specificity of complex I, we utilized a κB site from the IL-6 gene promoter (κB/IL-6) as a competitor, which is similar to the κB and κB’ sites but with different flanking sequences (36) (see Fig. 5A). Wild type κB/IL-6 competitor could completely inhibit complex I binding to the κB/κB’ probe, but mutant κB/IL-6 did not (Fig. 5C, lanes 8 and 9). Complex II, which is not modulated by TNF-α treatment, was modestly inhibited by κB/κB’, κB, and κB’ oligonucleotides (lanes 3, 4, and 6) and completely inhibited by the κB/IL-6 oligonucleotide (lane 8).

We next performed similar competition experiments using κB or κB’ as probes. Either κB or κB’ alone is sufficient for formation of complex I, although the affinity of complex I for the κB probe is −6-fold higher than for κB’ (Fig. 5D, lanes 2 and 9). Complex I formation on the κB or κB’ probes is κB site-specific since unlabelled κB and κB’ are strong competitors (lanes 3 and 10), whereas mutants κB and κB’ are not (lanes 4 and 11). The κB/IL-6 oligonucleotide completely inhibits complex I formation on the κB or κB’ probes (lanes 7 and 14), indicating that for both probes the flanking sequence is not important for complex I formation. The κB competitor completely inhibited complex I formation on the κB’ probe (lane 12); however, the κB’ competitor moderately inhibited complex I on the κB probe (lane 5). These results again indicate that the κB site is more efficient than the κB’ site for formation of complex I. Thus, the proteins of complex I can bind to both κB and κB’ sites independently but with different affinities.

The competition data shown in Fig. 5, C and D, strongly suggested that complex I is an NF-κB-like protein. To directly determine the identity of complex I, supershift assays were conducted with antibodies against various members of the Rel protein family (p65, p50, p52, c-Rel, and RelB) (25, 26). As shown in Fig. 5E, antibodies against p65 or p50 supershifted complex I on the κB/κB’ probe (lanes 3 and 4), while antibodies against p52, RelB, and c-Rel were without effect (lanes 5–7). As a control, normal rabbit serum did not affect the migration of complex I (lane 8). Similarly, migration of complex I on the κB or κB’ probes was shifted by both anti-p65 and anti-p50 antibodies (lanes 10, 11, 13, and 14) but not by antibodies against p52, RelB, or c-Rel (data not shown). Complex II migration was not modulated by any of the antibodies tested, indicating that this complex does not consist of Rel protein family members. These results directly demonstrate that complex I consists of the p50 and p65 members of the NF-κB family. It should be mentioned that although antisera against p52, RelB, and c-Rel did not affect the migration of complex I, we are not certain of their ability to recognize rat proteins. Thus, we cannot completely rule out the potential involvement of these proteins at this time.

**DISCUSSION**

We have investigated the mechanism(s) by which transcription of the TNF-α gene is induced by TNF-α in primary astrocytes. Using a series of marked TNF-α gene constructs, we demonstrate that TNF-α responsive sequences are located in the 3’ flanking region of the TNF-α gene. We also find that a TNF-α-inducible DNase I-hypersensitive site (3’ DH1) is associated with the 3’ flanking region that is critical for TNF-α inducibility of the marked TNF-α gene. Gel mobility shift assays identified nuclear proteins consisting of NF-κB members p50 and p65 that specifically bind to NF-κB sites within the 3’ DH1; these proteins are immediately induced (−12-fold induction) after TNF-α stimulation and gradually decrease over time.

Since the role of NF-κB in the regulation of the TNF-α gene is controversial, we wanted to determine whether the upstream κB sequences of the rat TNF-α gene played any role in TNF-α induction of the rat TNF-α gene in astrocytes. We observed that the rat TNF-α 5’ promoter constructs showed high basal activity that could not be further enhanced by TNF-α stimulation (Table I). The marked TNF gene system allowed us to search the 10-kb TNF locus for regulatory sequence elements by analyzing expression of deletion mutants of the TNF-α marked gene that could be differentiated from expression of the endogenous TNF-α gene. We demonstrated that the sequence of −70 from the TSS is sufficient, and a 500-bp region located 260 bp downstream of the polyadenylation sequence of the TNF-α gene is critical for TNF-α induction of the TNF-α gene (Fig. 2D). The five κB-like sequences in the upstream region of the rat TNF-α gene were not required for TNF-α induction of the TNF-α gene.

As mentioned previously, some studies have suggested that the downstream sequence is involved in TNF-α gene expression. Using transgenic mice, Keffer et al. (19) showed that a 3’-modified TNF-α transgene could not be activated by LPS, whereas the wild type TNF-α transgene was LPS-inducible. In this study, however, whether the defect in the transgenic mice carrying the 3’-modified TNF-α gene was due to interference at the transcriptional level, in mRNA stability, or in translation of mRNA was not determined. Kuprash et al. (20) have recently reported that a κB element located downstream of the TNF-α gene partially mediates LPS-induced TNF-α gene expression in murine macrophages. Although this downstream κB element was shown to act as an LPS-responsive enhancer, it appears to
be auxiliary in function since its activity is not significantly pronounced in the presence of three upstream κB elements. However, the rat TNF-α gene in astrocytes seems to be mainly regulated by downstream sequence elements (possibly two consecutive κB sites, see below), since deletion of all the upstream κB sites did not affect the magnitude of TNF-α-induced TNF-α gene expression (Fig. 2D). Similar observations were made for LPS stimulation, i.e. no upstream sequences are required, and LPS-responsive sequences are located in the same region as that for TNF-α.

Two consecutive κB-like sequences (κB and κB') are present within the TNF-α-inducible 3’ DH1 site that is associated with the functionally critical region for TNF-α-induced TNF-α gene expression (Fig. 4). We have identified a protein complex consisting of p50 and p65 components of NF-κB that specifically binds to the κB site (Fig. 5). This complex (complex I) is constitutively expressed at very low levels, immediately enhanced upon TNF-α stimulation, and then gradually decreases (Fig. 5B). The kinetics of TNF-α induction of complex I implies its involvement in TNF-α-induced TNF-α gene expression. Interestingly, complex I also binds to the κB’ site. The 3’ end of the κB’ sequence is destroyed in the pTNF2.9* construct, which is not activated in response to TNF-α, suggesting that the κB’ sequence could be important for TNF-α inducibility. In this regard, it is possible that κB and κB’ cooperatively act to respond to TNF-α stimulation. This may be the reason that pTNF2.9* loses TNF-α inducibility even though it retains the κB site that alone is sufficient for formation of complex I (Figs. 2D and 5D).

Our results suggest that expression of the TNF-α gene is under stringent regulatory control, which involves a transition of gene activity from repression to derepression to activation. This study provides some evidence supporting this idea. First, the transfected TNF-α promoter (both the promoter-CAT and marked gene constructs) shows high basal activity (Table I and Fig. 2D), whereas the endogenous TNF-α gene is transcriptionally silent under basal conditions (6). The chromatin structure of transiently transfected nonreplicating plasmids could be different from that of replicating DNA, which would more closely mimic the endogenous gene (37, 38). The endogenous TNF-α gene thus could be under repression associated with the chromatin structure, which is not reflected in the transiently transfected TNF-α gene constructs. Second, the marked TNF-α genes with TNF-α inducibility showed basal activity but could be further activated upon TNF-α stimulation (Fig. 2D). These results suggest that the TNF-α gene is positively activated following derepression upon TNF-α stimulation. In fact, the lower degree of induction by TNF-α of the marked TNF-α genes (4–6-fold, Fig. 2D) compared with the endogenous TNF-α gene (at least 15-fold, Fig. 1 and Fig. 2D) is due to the high basal activity of the transfected marked TNF-α genes. Third, TNF-α induces the 3’ DH1 which is associated with κB sites to which TNF-α-inducible p50/p65 NF-κB proteins bind (Figs. 3 and 5). The absence of a constitutive 3’ DH1 suggests that κB sites are restricted in accessibility to constitutive transcription factors (complex I in Fig. 5), which maintains the TNF-α gene in a repressed state under basal conditions. Upon TNF-α stimulation, the κB sites become immediately accessible to increased amounts of TNF-α enhanced complex I following chromatin opening. Interestingly, a recent study has provided evidence that NF-κB plays a direct role in modulating of chromatin structure (39).

These regulatory mechanisms would be beneficial for expression of a transient gene such as TNF-α, which should be under tight repression followed by rapid induction upon stimulation. Recently, Probert et al. (40) showed that transgenic mice that overexpressed the TNF-α gene in the CNS spontaneously developed a chronic inflammatory demyelinating disease and that administration of a neutralizing TNF-α antibody prevented development of disease, indicating a direct role for TNF-α in the pathogenesis of this disease. Because of the detrimental effects of TNF-α within the CNS, it would be advantageous to have this gene under strict regulatory control.

Acknowledgments—We thank Dr. Casey Morrow (University of Alabama at Birmingham) for helpful discussions and Sue Wade for secretarial support. We also thank Dr. Nancy Rice (NCI-Frederick) for the generous gift of antisera against NF-κB subfamily proteins (p65, p50, p105, and c-Rel).
A 3′ cis-Acting Element Is Involved in Tumor Necrosis Factor-α Gene Expression in Astrocytes

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J. Biol. Chem. 1996, 271:22383-22390.
doi: 10.1074/jbc.271.37.22383

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