TUMOR NECROSIS FACTOR β (TNF-β) INDUCES BINDING OF THE NF-κB TRANSCRIPTION FACTOR TO A HIGH-AFFINITY κB ELEMENT IN THE TNF-β PROMOTER

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The expression of the gene encoding tumor necrosis factor β (TNF-β) (lymphotoxin) is induced in T cells by various extracellular stimuli. We noticed that most such stimuli also activate the NF-κB transcription factor. Here we demonstrate binding of purified human NF-κB to a sequence within positions −98 to −88 (5′-GGGGCTTCCCC-3′) of the TNF-β promoter, which is conserved between the human and mouse genes. Also the NF-κB from the human T-cell line Jurkat, activated upon phytohemagglutinin (PHA)/phorbol 12-myristate 13-acetate (PMA/TPA) treatment in vivo or upon deoxycholate treatment in vitro, binds with high affinity to the sequence in the TNF-β promoter. Apart from a single mismatch, the site is identical to a cis-activating element that is involved in the inducible expression of the MHC class I gene H-2K^b and which interacts with both the inducible NF-κB transcription factor and the constitutive factor KBF1/H2TF1, as we demonstrate here for the site in the TNF-β promoter. The high homology of the well characterized H-2K^b enhancer sequence with the TNF-β site with regard to sequence and factor binding strongly supports a physiological role for NF-κB in the inducible expression of the TNF-β gene. Our observation that the TNF-β protein can rapidly induce the DNA-binding activity of NF-κB in Jurkat T cells and transiently increase TNF-β mRNA levels suggests that NF-κB can mediate a positive autoregulation of TNF-β synthesis.

Tumor necrosis factor β (TNF-β) is a well characterized autocrine and paracrine immunoregulatory cytokine (for reviews see references 1 and 2). It shares a high-affinity cell surface receptor with TNF-α[3,4] and is responsible for mediating cytostatic and cytotoxic effects of activated T lymphocytes on neoplastic cells.[5,6] The expression of the TNF-β gene is induced in CD4-positive and CD8-positive T cells upon stimulation by antigen, viral infections, T-cell mitogens, and various immunomodulatory agents.[1,2] Elevated TNF-β expression is found in virus-transformed B cells and some B- and T-lymphoma cell lines. Cis-activating sequences of the TNF-β gene that confer the inducible and constitutive modes of transcriptional activation have not yet been characterized. The genes encoding TNF-β and TNF-α are situated in tandem within the MHC complex in mouse[7] and man,[8,9] are related in nucleotide sequence, and share inducibility by various agents.[1,2] TNF-β is preferentially expressed in T lymphocytes and TNF-α in macrophages.

Recently, the upstream promoter of the TNF-α gene was analyzed.[10,11] Multiple binding sites for the NF-κB transcription factor (κB motifs) and so-called conserved cytokine motifs (CK-1), which have sequences related to κB motifs, were shown to confer inducible transcriptional activation to a reporter gene following stimulation of macrophages with lipopolysaccharide. The many parallels in the mode of expression of TNF-α and -β genes[1,2,12] and the observation that most of the conditions that induce TNF-β gene expression also activate the NF-κB transcription factor, prompted us to analyze 5′ flanking sequences of the TNF-β gene for potential NF-κB binding sites.

The ubiquitous NF-κB transcription factor controls the inducible expression of a large variety of genes that are all involved in immune response and inflammatory processes (see Fig. 1B; for reviews see references 13 and 14). Upon extracellular stimulation of cells, NF-κB is rapidly activated by its dissociation from a cytoplas-

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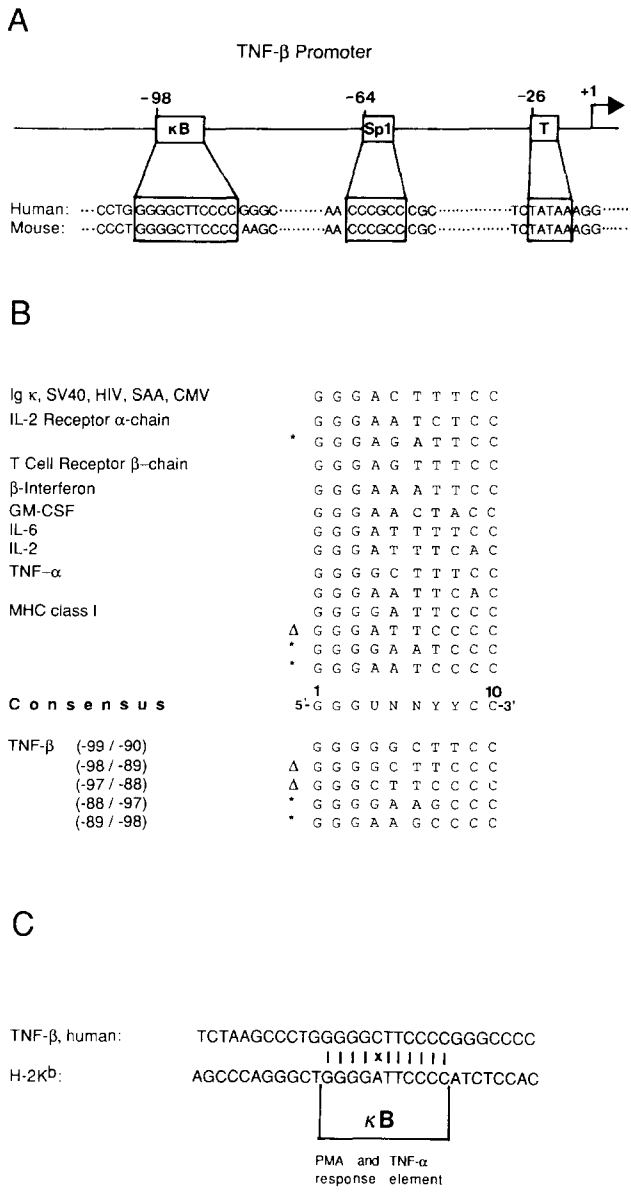

Figure 1. Potential NF-κB binding sites in the TNF-β promoter.

(**A**) Position of a highly conserved κB motif in the TNF-β promoter. The positions of potential binding sites for NF-κB (κB), Sp1, and the TATA-factor (T) are shown with regard to the transcription start site (+1; arrow). The number of the first nucleotide in the boxed sequence is given. In the lower part, TNF-β promoter sequences from human[25,26] and mouse[22-24] genes are aligned. In reference 24 (corrected version EMBL/GenBank data banks number Y00467), a fifth G residue is shown in position −99, as was found in the human promoter. (**B**) Alignment of well studied cis-activating binding sites for the NF-κB transcription factor from various genes (for reviews see references 13 and 14). A consensus motif is shown. U, purines; Y, pyrimidines; N, any nucleotide. The gap in the decameric sequences marks the symmetry axis. The triangle marks sequences contained within motifs of 11 or 12 bp. The asterisks marks the antisense strand of a site. Five alignments of decameric sequences from the κB motif of the human TNF-β promoter are shown. Abbreviations: Ig κ, immunoglobulin κ light chain; SV40, simian virus 40; SAA, serum amyloid A; CMV, cytomegalovirus. (**C**) Alignment of the κB motif in the TNF-β promoter with a cis-acting NF-κB binding site from the MHC class I gene H-2K^b.[20] Conserved bases are linked by a dash. The single mismatch is indicated by an x.

mic complex with the inhibitory protein IκB.[15,16] Released NF-κB can adopt an active heterotetrameric composition,[17] migrate into the nucleus, and activate genes upon binding to κB motifs in enhancer and promoter elements. This process is independent of new protein synthesis and involves the NF-κB transcription factor as a cytoplasmic/nuclear messenger.

Here, we report the identification of novel binding motifs for the NF-κB transcription factor within positions −99 to −88 of the TNF-β promoter that are recognized with high affinity by the NF-κB activated in Jurkat T cells following phytohemagglutinin (PHA) phorbol 12-myristate 13-acetate (PMA) or TNF-β treatment. Also, binding to the TNF-β promoter fragment of a second constitutive factor that has characteristics of KBF1/H2TF1 was observed. The NF-κB binding sequence in the TNF-β promoter is highly homologous to an element present in the MHC class I gene H-2K^b that was previously shown to bind the constitutive factor KBF1/H2TF1[18,19] or, upon activation of cells, NF-κB.[19,20] The site in the H-2K^b gene was demonstrated to function as a bona fide enhancer element that confers inducible gene expression following PMA and TNF-α treatment of cells.[20] In view of (1) the high-affinity binding of NF-κB to the site in the TNF-β promoter, (2) the high homology of the TNF-β site to a well characterized cis-activating element of the H-2K^b gene, and (3) the increase of TNF-β mRNA in response to almost all agents reported to activate NF-κB, we suspect that NF-κB is a key regulatory factor in controlling the inducible expression of the TNF-β gene. Considering the finding that TNF-β causes the activation of NF-κB in T cells and increases TNF-β mRNA levels, we discuss a possible role for NF-κB in a transient positive autoregulation of TNF-β synthesis.

RESULTS

Sequence-Specific Binding of Purified Human NF-κB to the TNF-β Promoter

We have sequenced 2 kb of the 5′ portion of the human TNF-β gene and found allelic polymorphism that is in linkage disequilibrium with certain MHC haplotypes.[21] By inspection, we noticed binding motifs for the NF-κB transcription factor between positions −99 to −88 of the human TNF-β promoter that are conserved in the mouse promoter[22-24] with respect to sequence and distance to the transcription start site (Fig. 1A,B). Sequences flanking the putative site were not highly conserved. A comparison of the κB motif from the TNF-β promoter with known functional κB motifs is shown in Fig. 1B. It is intriguing that the TNF-β site mismatches in only one base pair within positions −98 to −89 with a motif found in the MHC class I gene H-2K^b [19,20] (Fig. 1C). The latter allows binding of the NF-κB transcription factor and of factors called KBF1

and H2TF1 and was shown to function in cis-activating a reporter gene upon PMA and TNF-α stimulation of cells, even in the absence of the in situ flanking sequences.[20] Hence, the sequence conservation between the H-2K^b and TNF-β promoters is precisely restricted to a sequence that is sufficient to confer inducible transcriptional activation.

To assess whether NF-κB can bind to the putative site(s) in the TNF-β promoter we performed a methylation interference analysis using purified human NF-κB consisting of 50-kDa and 65-kDa protein subunits[17]. As expected, methylation of almost all purines on sense and antisense strands within the dodecameric sequence interfered strongly with binding of NF-κB (Fig. 2A). Methylation of the guanosine residue in position −99 showed a weaker but still significant inhibitory effect on NF-κB binding. The binding study with the TNF-β promoter fragment thus reveals a series of novel κB motifs that vary only within positions 4 to 8 encompassing the less conserved part of the κB consensus motif (Fig. 1B).

High-Affinity Binding of NF-κB Activated in T Cells to the TNF-β Promoter Fragment

To investigate whether the TNF-β promoter fragment can detect the NF-κB activated in a physiological system, we stimulated human Jurkat T cells with PHA/PMA, a condition that mimics T-cell activation,[27] induces binding of NF-κB in T cells to κB motifs of the human immunodeficiency virus 1 (HIV-1),[28] interleukin 2 receptor (IL2R),[29] and granulocyte-macrophage colony-stimulating factor (GM-CSF)[30] upstream promoter elements and increases TNF-β mRNA levels. (See *Activation of NF-κB in Jurkat T Cells by TNF-β.*)

In extracts from T cells, the TNF-β DNA probe detected a DNA-binding activity that showed all the characteristics of NF-κB (Fig. 3). The activity was hardly detectable in unstimulated T cells (Fig. 3A, lane 1) but was strongly induced upon PHA/PMA stimulation (Fig. 3A, lane 3). Its protein-DNA complex comigrated with that of purified human NF-κB (Fig. 3B, lane 1) and its formation was specifically competed for by a 25-fold molar excess of unlabeled oligonucleotides containing κB motifs of the mouse κ light chain enhancer[33] (Fig. 3B, lane 6) and the interleukin 2 enhancer[34] (Fig. 3B, lane 9), but not by an unrelated oligonucleotide (Fig. 3B, lane 11).

The TNF-β promoter fragment was also recognized by NF-κB that was activated in vitro by treatment of cytosolic fractions from Jurkat T cells with deoxycholate (DOC) (Fig. 3C, lanes 2 and 4). This treatment specifically activates a cytosolic form of NF-κB by releasing IκB, an inhibitory protein, that is responsible for the inactivation and cytoplasmic localization of the NF-κB transcription factor.[16] When the T-cell extracts were analyzed in electrophoretic mobility shift assays (EMSAs) with a radioactively labeled DNA probe

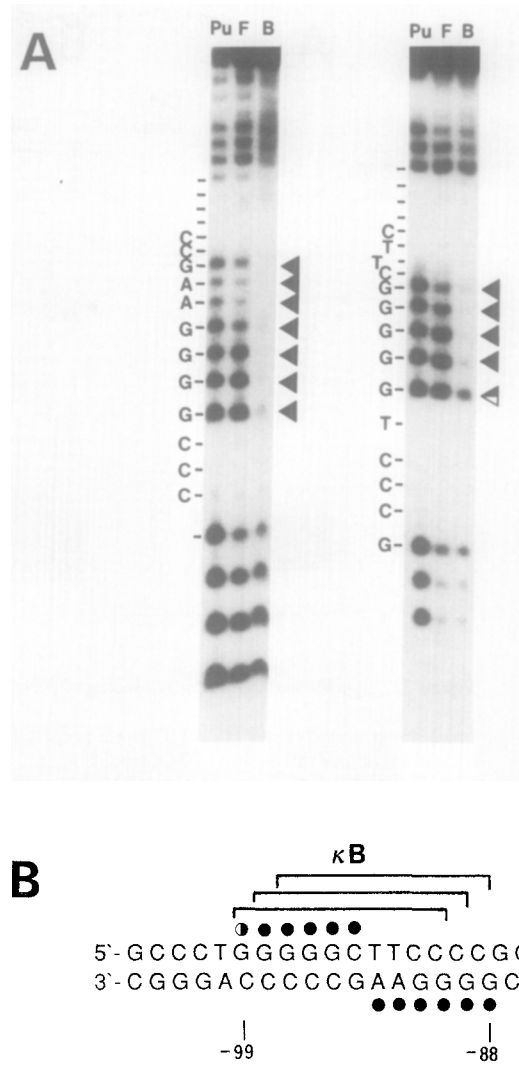

Figure 2. Sequence-specific binding of purified NF-κB to a TNF-β promoter fragment.

(**A**) For the methylation interference analysis, purified NF-κB from human placenta was reacted with partially methylated TNF-β promoter fragments end-labeled on antisense (left panel) or sense strands (right panel), followed by separation of free and complexed DNA on a native gel. A purine ladder (Pu), was coelectrophoresed with the samples. Filled arrowheads indicate purine residues that strongly interfered with NF-κB binding when methylated. The half-filled arrowhead indicates partial interference. (**B**) Summary of the methylation interference data. Purine residues crucial for NF-κB binding are indicated with a filled dot. A guanosine residue less required for protein binding is labeled with a half-filled dot. Three decameric frames representing the minimal NF-κB binding site are shown on top.

encompassing the two κB motifs from the HIV-1 enhancer,[28] a pattern of DNA-binding activities was seen that was almost identical to that obtained with the TNF-β DNA probe (compare Fig. 4A with Fig. 3A and 3C). The only significant difference was the presence of an additional faster-migrating DNA-binding activity detected only with the TNF-β and H-2K^b probes (see Fig. 4).

To estimate a relative affinity of NF-κB for the TNF-β site(s) in comparison to two physiologically

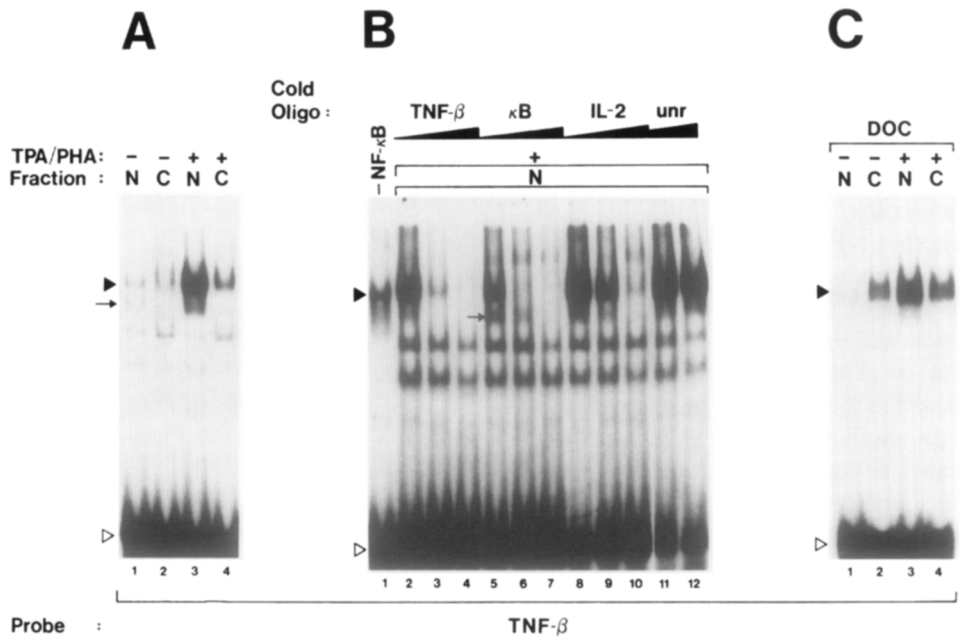

Figure 3. Specific and high-affinity binding of NF-κB activated in Jurkat T cells to a TNF-β promoter fragment.

Nuclear extracts (N) and cytosol (C) were prepared from control cells (−) and cells that were treated for 4 hr with a combination of 50 ng/mL PMA and 5 μg/mL PHA (+). (**A**) Detection of NF-κB activated by mitogen-stimulation of T cells using a labeled TNF-β promoter fragment. The filled arrowhead indicates the position of a NF-κB/DNA complex and the arrow that of a complex formed by KBF1/H2TF1. The open arrowhead indicates the position of uncomplexed DNA probe. (**B**) Competition analysis. In lane 1, a comigration of purified NF-κB (50 pg) from human placenta is shown. A nuclear extract (N) containing mitogen-activated NF-κB (+) was used for the competition analysis (lanes 2-12). Molar excesses of 2.5- (lanes 2, 5, and 8), 25- (lanes 3, 6, 9, and 11) and 250-fold (lanes 4, 7, 10, and 12) of unlabeled oligonucleotide were included in binding reactions with the labeled TNF-β oligonucleotide. Lanes 2-4, competition with increasing amounts (filled slopes) of an homologous TNF-β oligonucleotide (see Fig. 1B); lanes 5-7, a 34-mer[30] encompassing sequences from the mouse κ light chain enhancer (κB) with the κB motif 5′-GGGACTTTCC-3′[33]; lanes 8-10, a 34-mer[30] encompassing sequences from the human IL 2 enhancer (IL-2) with the κB motif 5′-GGGATTTCAC-3′[34], lanes 11 and 12, competition with an unrelated oligonucleotide. (**C**) Binding of DOC-activated NF-κB to the TNF-β probe.

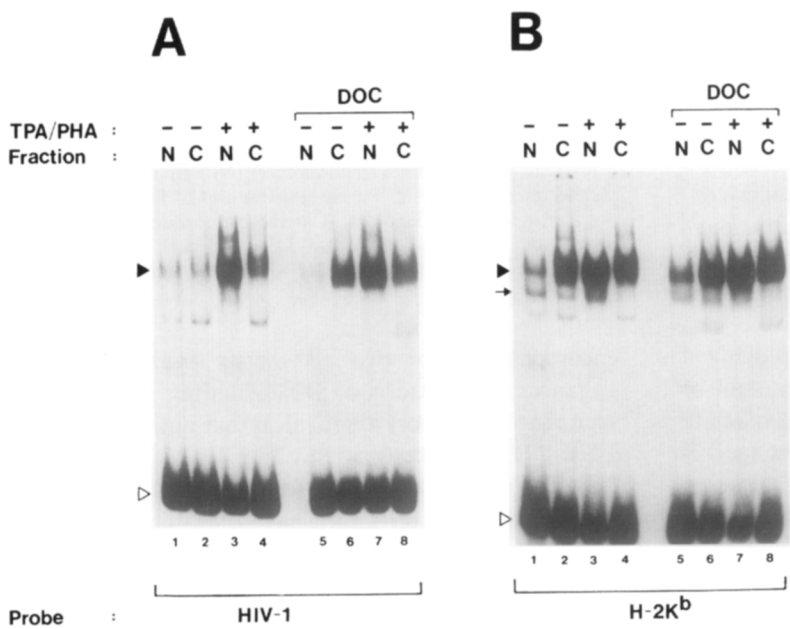

Figure 4. Detection of a KBF1/H2TF1-like binding activity with a fragment from the H-2K^b promoter.

For details of illustration see legend to Fig. 3. (**A**) Analysis of Jurkat T cell extracts with a labeled HIV-1 enhancer fragment. A 50-mer oligonucleotide encompassing the two κB motifs of the HIV-1 enhancer[28] was used. (**B**) Analysis of Jurkat T-cell extracts with a labeled H-2K^b enhancer fragment. A 39-mer oligonucleotide was used encompassing the κB motif of the MHC class I H-2K^b gene[19,20] with 14 (5′ end) and 10 (3′ end) bp of original flanking sequence and HindIII and SalI linker sites the on 5′ and 3′ ends, respectively.

relevant cis-acting κB motifs we performed a titration with 2.5-, 25-, and 250-fold molar excesses of unlabeled competitor oligonucleotides (Fig. 3B). Strongest inhibition of NF-κB binding to the TNF-β probe was obtained with the κB site 5′-GGGACTTTCC-3′ from the murine κ light chain enhancer[33] (Fig. 3B, lanes 5-7). Homologous competition with unlabeled TNF-β oligonucleotide was about two- to three-fold less efficient (Fig. 3B, lanes 2-4). It was, however, more effective than competition with an oligonucleotide containing the κB motif 5′-GGGATTTCAC-3′ from the interleukin 2 (IL 2) enhancer, which is functional in conferring transcriptional activation in response to T-cell–activating signals.[34] An unrelated oligonucleotide could not compete, even at a 250-fold molar excess over the radioactive probe (Fig. 3B, lane 12). The finding that the site(s) from the TNF-β promoter competed better for the DNA binding of NF-κB than did the IL 2 enhancer site suggests that the affinity with which NF-κB binds to the TNF-β promoter is sufficiently high to be of significance for the inducible expression of the TNF-β gene.

Binding of a KBF1/H2TF1-Like Factor to the TNF-β Promoter

The TNF-β oligonucleotide detected a second specific DNA-binding activity in extracts from unstimulated and stimulated Jurkat T cells (Fig. 3A and B). Because the TNF-β site is highly homologous to a site found in the H-2K^b class I gene (Fig. 1C), we suspected that the additional DNA-binding activity was KBF1/H2TF1. As has been reported for KBF1,[35] this DNA-binding activity was constitutively present and gave rise to a protein-DNA complex that migrated faster in native polyacrylamide gels than the one formed by NF-κB (Fig. 3A, lane 1). The DNA-binding activity was specific for the TNF-β probe, since a 25-fold molar excess of unlabeled TNF-β oligonucleotide completely inhibited binding (Fig. 3B, lane 3) but a 250-fold molar excess of the unrelated oligonucleotide did not (Fig. 3B, lane 12).

The oligonucleotides containing κB motifs from the mouse κ light chain and IL 2 enhancer also inhibited binding of the factor to the TNF-β probe, but less efficiently by at least one order of magnitude (Fig. 3B, lanes 5-10). A lower affinity for the mouse κ enhancer motif compared to the H-2K^b motif was also observed for the KBF1/H2TF1 factor.[19,36] The KBF1/H2TF1-like DNA-binding activity was not detected when T-cell extracts were examined with a DNA probe encompassing the two κB motifs from the HIV-1 enhancer, which gave an otherwise identical pattern of bands (compare Figs. 3A and 4A). The κB motifs from the HIV-1 enhancer are identical with that from the mouse κ light chain enhancer and should not strongly bind KBF1/H2TF1.[19,36]

To substantiate that the additional DNA-binding activity detected by the TNF-β promoter fragment was indeed KBF1/H2TF1 we performed EMSAs with an oligonucleotide that contained the sequence from the H-2K^b enhancer encompassing the κB/KBF1/H2TF1 binding motif. In contrast to the HIV-1 enhancer (Fig. 4A), a radioactively labeled H-2K^b DNA probe indeed gave rise to a faster-migrating protein-DNA complex with the same mobility as that seen with the TNF-β probe (Fig. 4B, lane 1). Moreover, the pattern of DNA-binding activities detected with the H-2K^b probe in nuclear extracts of T cells was very similar to that detected with the TNF-β probe (compare Figs. 3A,C and Fig. 4B), although with the H-2K^b probe two DNA-binding activities in cytosolic fractions were observed that had mobilities slightly distinct from those of NF-κB and KBF1/H2TF1 (Fig. 4B, lane 2).

Our data suggest that the κB elements from the H-2K^b enhancer and the TNF-β promoter are identical with regard to binding of NF-κB and KBF1/H2TF1. Therefore, they are most likely also similar or identical with respect to their cis-activating potential. In numerous instances it has been shown that binding of NF-κB to κB motifs confers a typical transcriptional responsiveness to genes.[13,14] In that sense, we have noticed that most, if not all, of the various agents that activate NF-κB DNA binding in T cells also induce TNF-β gene expression (or increase TNF-β mRNA levels) (Table 1).

Activation of NF-κB in Jurkat T Cells by TNF-β

In Jurkat T cells, TNF-α induces activation of NF-κB and transiently increases TNF-β mRNA levels.

TABLE 1. Agents that increase TNF-β mRNA levels and that activate the DNA-binding activity of NF-κB

Agents tested for stimulating TNF-β transcription[1,2] and agents activating NF-kB[13,14] have recently been reviewed. Not discussed were the effects of TNF-β (this paper), and the following references have also been used: activation of TNF-β transcription by anti-CD28/CD3,[37,38] and HTLV-I;[39,40] activation of NF-kB by anti-CD28/CD3.[41] n.t., not tested.

Stimulus	Induction of TNF-β messenger RNA	Activation of NF-κB DNA binding
TNF-α	+	+
TNF-β	+	+
IL 1	+	+
antigen	+	+
anti-CD28,-CD3	+	+
Lectins	+	+
Phorbol esters	+	+
Calcium ionophore	+	+
UV light	n.t.	+
Poly(rI)·Poly(rC)	+	+
HTLV-I	+	+
HIV-1	+	n.t.
Herpes simplex virus-2	+	+

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Recent reports have shown that TNF-α can induce the activation of NF-κB[42-44] but similar studies with TNF-β have not yet been reported. Jurkat T cells were incubated for 30 min at a concentration of 60 pM human TNF-β or 60 pM human TNF-α. Nuclear extracts and cytosolic fractions were then assayed for the DNA-binding activity of NF-κB by EMSA using a labeled TNF-β promoter fragment (Fig. 5, lanes 1-7). Compared to control cells (Fig. 5, lane 2), both cytokines induced a strong accumulation of newly activated DNA-binding activities in the nucleus (Fig. 5, lanes 4 and 6). One complex induced by TNF-β comigrated with that induced by TNF-α (Fig. 5, lane 4), the complex activated in cytosolic fractions by DOC (data not shown), and with that of purified human NF-κB (Fig. 5, lane 1). The activation was specific for TNF-β, since a treatment of Jurkat cells with the same volume of buffer containing ten times less TNF-β had no effect (data not shown). The same DNA-binding activity was detected by a DNA-probe encompassing sequences from the IL2R α-chain enhancer containing the κB element (Fig. 5, lanes 8-14). In fractions from TNF-treated cells, a second, faster-migrating band was seen that presumably corresponded to a proteolytically truncated form of NF-κB described earlier.[17] By all these criteria, it was the NF-κB transcription factor that was rapidly activated in T lymphocytes upon TNF-β treatment.

We tested by Northern blot analysis whether TNF-β treatment can induce TNF-β mRNA in the Jurkat T-cell line (Fig. 6, Table 1). While TNF-β mRNA was barely detectable in unstimulated cells (Fig. 6, lane 1), 1.33 nM TNF-β in the medium rapidly induced a maximal increase of its RNA within 4 hr. After 12 and 24 hr (Fig. 6, lanes 3 and 4) the signal declined.

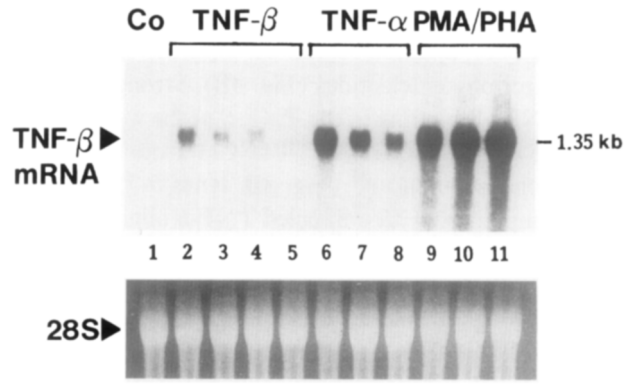

Figure 6. Induction of TNF-β mRNA by TNF-β stimulation of Jurkat T cells.

Jurkat cells were treated with 800 U/mL human recombinant TNF-β (lanes 2-5), or 800 U/mL TNF-α (lanes 6-8) (both Genzyme, USA) and with 50 ng PMA/5 μg PHA per mL (lanes 9-11) for 4 hr (lanes 2, 6 and 9), 12 hr (lanes 3, 7 and 10), and 24 hr (lanes 4, 8 and 11). Lane 1, control. Lane 5, TNF-β stimulation of 12 hr, preincubated with monoclonal antibody 9B9, neutralizing TNF-β activities (BASF, Ludwigshafen). The upper panel shows an autoradiogram of a nylon Hybond-N (Amersham, USA) filter. The filled arrowhead indicates the position of a 1.35-kb TNF-β–specific mRNA signal. The lower panel shows a section of the agarose gel containing the 28S RNA after ethidium bromide staining.

Preincubation of TNF-β with the TNF-β–specific monoclonal antibody 9B9 prevented TNF-β mRNA induction (Fig. 6, lane 5). Stimulation of Jurkat cells with 1.0 nM TNF-α resulted in a stronger increase of TNF-β mRNA with the same kinetics of a decrease after 12 and 24 hr (Fig. 6, lanes 6-8). In contrast to this transient stimulation with both cytokines, PMA/PHA treatment resulted in a steady accumulation of mRNA over the test period (Fig. 6, lanes 9-11).

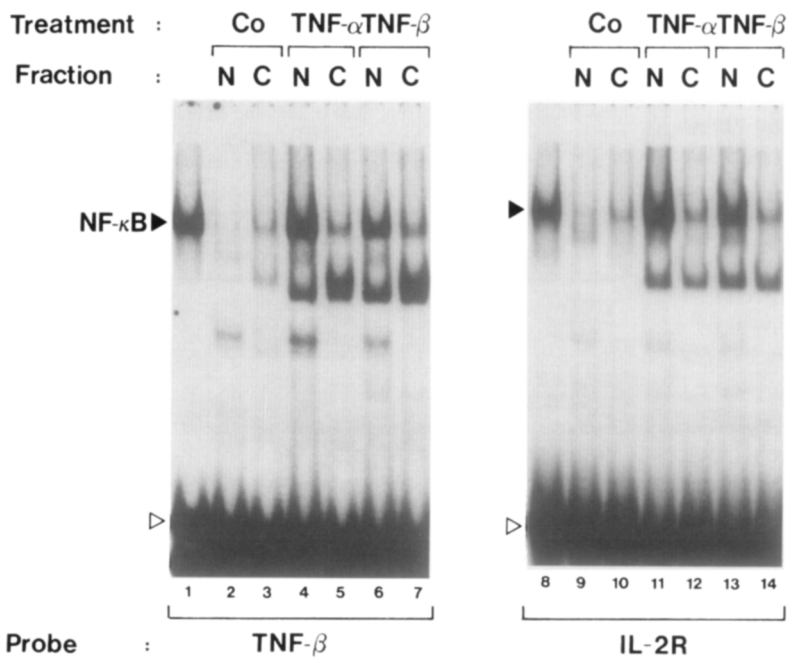

Figure 5. Activation of NF-κB by TNF-β.

Jurkat T cells were treated for 30 min with 60 pM TNF-α (lanes 4, 5, 11 and 12), 60 pM TNF-β (lanes 6, 7, 13 and 14), or left untreated (Co) (lanes 2, 3, 9 and 10). Nuclear extracts (N) and cytosolic fractions (C) were tested for NF-κB activity in EMSAs using a labeled TNF-β promoter fragment (lanes 1-7) and a fragment[30] of the IL2R α-chain enhancer encompassing the κB motif (lanes 8-14). The open arrowheads indicate the position of unbound DNA probe and the filled arrowheads the position of an NF-κB–DNA complex.

DISCUSSION

The finding that in the Jurkat T cell line TNF-β treatment of cells increases both NF-κB DNA binding and TNF-β mRNA levels suggests an involvement of NF-κB in a transient positive autoregulation of TNF-β synthesis. It appears that TNF-β can induce its own synthesis by using NF-κB as a cytoplasmic/nuclear messenger and transcriptional activator.

This study shows that the NF-κB transcription factor can bind with high affinity and specificity to a conserved 12-bp sequence located about 95 bp upstream of the transcription start site of the TNF-β gene. Binding was demonstrated with NF-κB from PMA/PHA-stimulated T cells, NF-κB activated in cytosolic fractions by treatment with DOC, and with highly purified NF-κB from human placenta.

The following observations suggest a role of the interaction of NF-κB with the TNF-β promoter site in the control of transcription initiation of the TNF-β gene: (1) The binding of NF-κB to the TNF-β site(s) is of high affinity. In fact, it was better than binding of NF-κB to a κB motif in the IL 2 enhancer, which was shown previously to be cis-activating.[34] In all cases reported so far, constructs containing isolated high-affinity binding sites for NF-κB were able to confer transcriptional activation to reporter genes when sites were multimerized or placed in single copies close to a promoter.[13,14,45,46] (2) The site identified in the TNF-β gene is, apart from a single mismatch, identical to a well characterized cis-activating element found in the H-2K^b gene. The mismatch lies within the non-conserved center of the κB motif (Fig. 1C) and, as is obvious from our data, has no significant influence on binding of NF-κB or KBF1/H2TF1-like factors. The H-2K^b motif 5′-GGGGATTCCCC-3′ was recently analyzed in the absence of its in situ flanking sequences and shown to confer, in multimerized form, induction of gene expression following stimulation of transfected cells with TNF-α and PMA.[20] (3) NF-κB can be activated by a diverse spectrum of agents and thereby can confer transcriptional activation in response to many different stimuli[13,14] (see Table 1). We observed a striking overlap between agents that induce NF-κB binding activity and those that lead to an increase of TNF-β mRNA levels. Table 1 lists 13 agents that were tested for their effects on NF-κB activation and/or TNF-β transcription. Out of 12 agents found to activate NF-κB in T cells and in a great variety of cell types, 11 were also found to increase TNF-β mRNA levels in T cells, T-cell–derived lines, and B-cell lines.[1,2] The effects of UV light have not yet been tested for TNF-β mRNA induction (Table 1). It is interesting to note that TNF-β is constitutively expressed in HTLV-I–infected T-cells.[39,40] Upon HTLV-I infection of cells, NF-κB becomes constitutively activated through the Tax_1 protein of the virus[47,48] and can thereby provide constitutive transcriptional initiation to genes that are controlled by cis-acting κB motifs. NF-κB is unlikely to be responsible for a cell type–specific expression of the TNF-β gene. Rather NF-κB serves to rapidly initiate transcription following extracellular stimulation of cells. It is likely, however, that NF-κB can synergize with cell type–specific factors. Only a deletion analysis of the TNF-β promoter and other sequences surrounding the gene can clarify this issue.

TNF-β adds to an increasing number of cytokine genes that are likely to be regulated by the NF-κB transcription factor. Other examples are the β-interferon[49,50] IL 2,[34] IL 6,[51,52] TNF-α,[10,11] and GM-CSF genes.[30] Not only are many cytokine genes activated by NF-κB, but NF-κB itself is activated by at least two distinct cytokines, TNF-α and IL 1.[42,43] Here we show that picomolar amounts of the TNF-β protein, which presumably shares high affinity cell surface receptors with TNF-α,[3,4] can rapidly induce the DNA-binding activity of NF-κB. Thus, in the case of both TNFs, NF-κB appears to be involved in a transient positive autoregulation. The transcription factor emerges as a central signaling molecule in cytokine networks that allows coupling of cytokine stimulation of cells to the new synthesis of cytokines. Because NF-κB is rapidly activated through a simple dissociation reaction and can actively participate in cytoplasmic/nuclear signaling,[15,16,53] it is particularly suitable to translate cytokine stimuli into new patterns of gene expression and, subsequently, into the synthesis of new effector proteins.

MATERIALS AND METHODS

Cell Lines and Culture

The T-cell line Jurkat was grown in RPMI-1640 medium supplemented with 10% fetal calf serum (both Gibco). Cells were tested negative for mycoplasma using bisbenzimid H 33258 (Riedel-de Haen). Stimulation with 5 μg/mL PHA (Sigma) and 50 ng/mL PMA (Sigma) was performed for 4 hr and treatment with 60 pM recombinant human TNF-α or TNF-β (both BASF, Ludwigshafen) for 30 min. Cells were harvested by centrifugation for 10 sec at 1000*g* and washed in ice-cold PBS, and the cell pellets obtained after recentrifugation were lysed in hypotonic buffer A.[54] Protein concentrations were determined with the BioRad Microassay.

Oligonucleotides

Oligonucleotides were synthesised by the phosphoramidite method on an Applied Biosystems DNA synthesizer. The κB motifs were placed such that after annealing of single strands the NF-κB binding site was in the center of the probes flanked by 5 to 10 bp of original sequence and HindIII and SalI linker sites on 5′ and 3′ ends, respectively. For EMSAs, annealed oligonucleotides were labeled with [α-^{32}P]dCTP (Amersham) by the Klenow enzyme (Boehringer). After

labeling, the double-stranded oligonucleotides were extracted from melted agarose by phenol/chloroform, precipitated in 70% ethanol, and used after dissolving in 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA.

To avoid possible annealing artifacts with the GC-rich TNF-β oligonucleotide, labeling was performed in a thermocycler (Hybaid). The two single-stranded TNF-β oligonucleotides were denatured for 2 min at 94°C in 50 μl of a polymerase chain reaction standard mix containing 1 mM $MgCl_2$, 5 pmoles of each of the three unlabeled dNTPs, and 5 pmoles of [α-^{32}P]dCTP (Amersham). 1.6 U of Taq DNA polymerase (United States Biochemical) was added and the reaction cooled to 72°C over a period of 20 min. The DNA probe was purified on a non-denaturing 12% polyacrylamide gel. The unrelated oligonucleotide (unr) used in competition experiments was derived from the plasmid pKK 223-3 (Pharmacia) and encompassed 56 bp upstream of the EcoRI site of the polylinker.

Electrophoretic Mobility Shift Assays

Conditions were essentially as described earlier.[15,30] Activation of the cytoplasmic form of NF-κB was performed by adding 2.5 μl of a 4% solution of DOC (deoxycholate) to cellular fractions followed by addition of a DNA-binding reaction mixture[30] and 2 μl of a 10% (v/v) solution of Nonidet P-40 (Sigma).

Nuclear extracts and cytosolic fractions were prepared from control and stimulated Jurkat cells and equal portions of fractions (2 μl; 1-3 μg of protein) were reacted with a radiolabeled κB oligonucleotide (0.1 ng per reaction) and samples analyzed by EMSA as described.[30] Autoradiograms of native 4% polyacrylamide gels are shown.

Methylation Interference Analysis

The TNF-β promoter oligonucleotides, sense and antisense strands, encompassing a sequence from position -111 to -79 (referred to as TNF-β probe) were end-labeled with ^{32}P using polynucleotide kinase (Boehringer) and reacted with purified human NF-κB[17] (approximately 0.5 ng) in a 10-fold scaled-up EMSA DNA-binding reaction. Complexed and free DNA were separated on a 4% native polyacrylamide gel, transferred onto DEAE paper by semi-dry blotting, and further processed as described elsewhere.[30]

Analysis of mRNA Levels

Ten micrograms of total RNA extracted from stimulated and unstimulated Jurkat T cells according to Chirgwin et al.[31] was electrophoresed on a 1.2% denaturing formaldehyde agarose gel and transferred onto a nylon filter. The TNF-β mRNA was detected by hybridization according to the protocol of Church and Gilbert,[32] using a human genomic 600-bp PvuII/PstI TNF-β fragment containing sequence of exon 4 and the 3′ untranslated region.[21]

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