Genes Encoding Tumor Necrosis Factors: Genome Organization, Polymorphism, and Expression

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Introduction

Tumor necrosis factor (TNF-α) and lymphotoxin (TNF-β) are two related cytokines sharing a broad spectrum of activities [1–4]. In particular, TNF-α is one of the principal mediators of inflammation [2]. TNFs are also involved in the control of hematopoiesis. TNF-α stimulates production of hematopoietic growth factors by fibroblasts, macrophages, and endothelial cells [5, 6]. On the other hand, the same cytokine inhibits colony formation by hematopoietic progenitor cells in vitro [7, 8]. In vivo, TNF-α stimulates formation of spleen colonies and exhibits an overall protective effect in sublethally irradiated mice [9, 10].

We have previously cloned human, mouse, and rabbit genes coding for TNF-α and TNF-β [11–13]. The two TNF genes are tandemly arranged within 7 kb of genomic DNA and map inside major histocompatibility complexes in mice and humans [14, 15]. They apparently evolved from a common ancestor.

TNF-α and -β are differentially expressed. Therefore, TNF loci offer an interesting model system with which to study tissue-specific regulation of gene expression. Here we review our recent findings on the genome organization, polymorphism, and expression of human, mouse, and rabbit TNF genes.

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Materials and Methods

DNA Clones and Probes. Original phage clones 15 and 11 containing human TNF locus were described [11]. DNA of cosmid clone 031 A [16] was generously provided by T. Spies (Harvard University, Cambridge, MA, USA). Further subcloning was done into pGEM3/4 vectors (Promega Biotech, Madison, WI, USA). DNA samples from peripheral blood leukocytes of unrelated blood donors were kindly provided by P. M. Chumakov (Engelhardt Institute of Molecular Biology, Moscow, USSR).

Oligonucleotides. $(TC)_9$ and $(TC)_{13}$ and oligonucleotides used as polymerase chain reaction (PCR) and sequencing primers (see below) were purchased through Nauka, Inc. (Moscow, USSR); $(AC)_{15}$ was a gift from A. Edwards (Institute of Molecular Genetics, Baylor College of Medicine, Houston, TX, USA). Universal sequencing primers specific for pGEM3/4, 5′-TCACTATAGGGAGACCG-3′ and 5′-GTGACACTATAGAATAC-3′ were generously provided by V. N. Dobrynin (Shemyakin Institute of Bioorganic Chemistry, Moscow, USSR).

The following oligonucleotides flanking the $(AC/TG)_k$ and $(TC/AG)_n$ repeats were used as primers for PCR and sequencing:

IR1 5′-
GCACTCCAGC*CTAG*GCCACA
GA-3′ (*Sty*I site is underlined)

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IR2 5′-
GCC*TCTAGA*TTTCATCCAGC
CACA-3′ (*Xba*I site is underlined)
IR4 5′-
CCTCTCTCCCCTGCAACACACA
-3′
IR5 5′-
GTGTGTGTTGCAGGGGAGAG
AG-3′

DNA Sequencing. The dideoxy termination method as modified for supercoiled double stranded templates [17] was used. Universal synthetic oligonucleotides specific for pGEM3/4 as well as gene-specific oligonucleotides (see above) were used as sequencing primers. Most of the sequencing was done using Sequenase (USB Corporation, Cleveland, OH, USA) following the protocol provided by the manufacturer.

Hybridization. Southern hybridizations with kinased oligonucleotides were performed as described [18], except that carrier DNA was excluded from prehybridization and hybridization buffers. Washing was done in 2 × SSC twice at room temperature for 20 min and twice at hybridization temperature for 1–2 min. The latter temperature for these salt conditions was calculated according to Itakura et al. [see 18].

PCR Conditions. PCR [19] was carried out in a volume of 10 µl with 100 ng of genomic DNA template. Conditions for PCR were the following: 5 min at 95 °C and 30 cycles with 30 s at 92 °C (denaturation), 60 s at 55 °C (annealing), and 30 s at 72 °C (elongation). The elongation step in the last cycle was extended to 5 min at 65 °C. Unlabeled PCR products were analyzed on 2% agarose minigels in TBE [18]. Labeling of PCR products was performed either by addition of end-labeled primers to the amplification mixture or by filling the ends of amplified DNA with Klenow fragments of DNA polymerase *E. coli* in the presence of radioactive dNTPs, after digestion with *Sty*I (for primer IR1) or *Xba*I (for primer IR4). Radiolabeled amplification products were analyzed on 5% polyacrylamide sequencing gels. Microsatellites containing polymorphic fragments were sized using sequencing markers, compared to those from cosmid clone 031A, amplified, and processed in parallel (clone 031A microsatellites were sequenced).

Gel Retardation Analysis. Nuclear extracts were prepared from bone marrow-derived macrophages before and 4 h after lipopolysaccharide (LPS) activation, from the RAW264 macrophage-like cell line, and from the RPMI-6410t human B lymphoblastoid cell line according to recently published procedure [20]. Synthetic oligonucleotides were kinased, gel purified, and used for binding. Complexes were analyzed on 5% native gels as described [18, 20].

Results and Discussion

Genome Organization and Polymorphism of the TNF Genes

Novel Sequence Polymorphism in the Human TNF Locus

TNF-α and TNF-β genes are tandemly arranged, linked to the major histocompatibility complex (MHC), and map centromeric to HLA-B (or H-2D) and telomeric to class III genes. Since both cytokines encoded by these genes are potent immunomodulators and since some MHC-linked autoimmune diseases are characterized by altered levels of their production or inducibility [21, 22], genetic variability in the TNF locus may be related to the functional polymorphism of MHC.

So far, based on restriction fragment length polymorphism (RFLP) data, low degrees of genetic polymorphism in the human TNF locus have been reported [23, 24]. In the course of hybridization analysis with oligonucleotide probes, we found, and mapped 3.5 kb upstream to the TNF-β gene, tandem AC/TG and TC/AG dinucleotide repeates (otherwise known as microsatellites; Fig. 1).

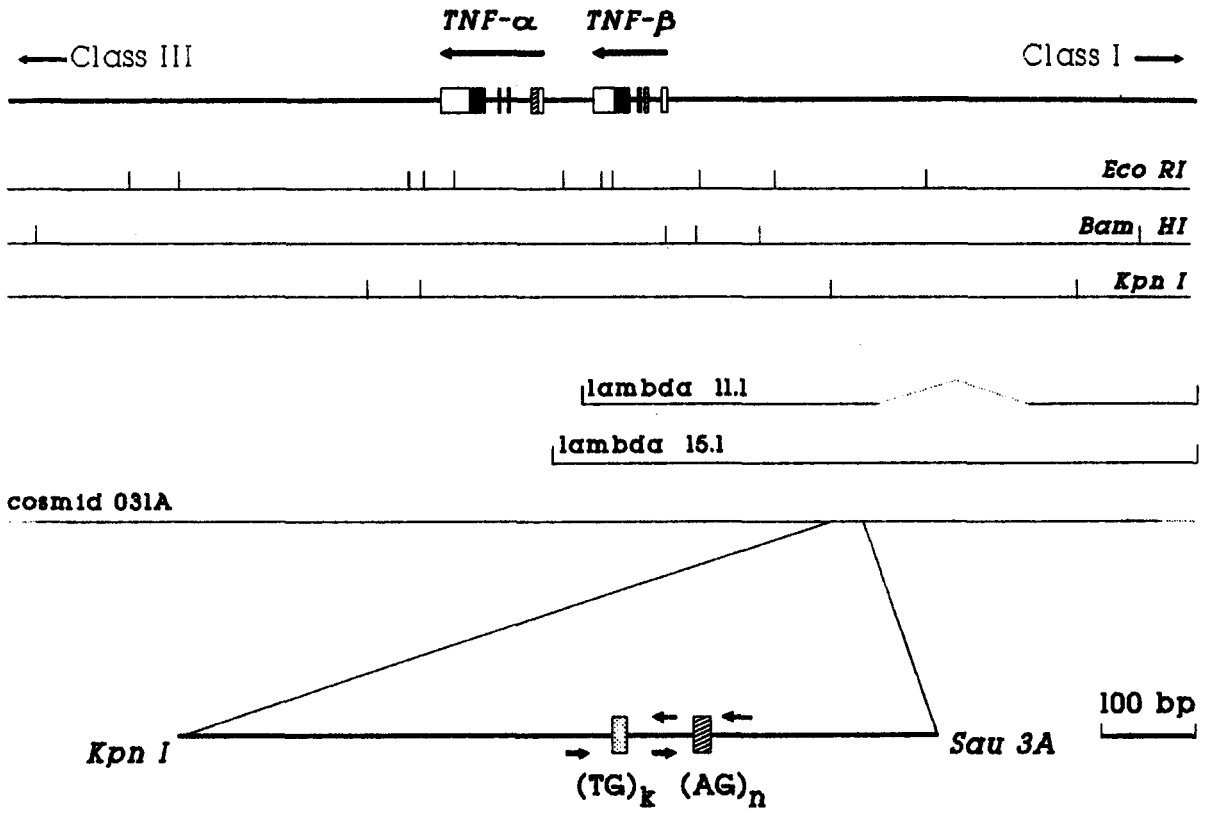

Fig. 1. Localization of polymorphic microsatellites (AC/TG and TC/AG repeats) in the human TNF locus. *Arrows* in the *lower center* correspond to positions of PCR primers. (Map of the TNF locus and restriction maps of phage and cosmid clones are taken from [11, 16])

To obtain DNA sequence information from this putative polymorphic region, the *Kpn*I–*Sau*3A 820-bp fragment derived from cosmid 031A was subcloned to pGEM4 and sequenced (Fig. 1). In addition to microsatellites, the sequence of the *Kpn*I–*Sau*3A fragment contained 200 bp with 73% homology to the human Alu-family interspersed repeat (clone BLUR7).

Since microsatellites found in many genes have been shown to contain various numbers of repeats [25], we measured the size of "TNF-linked" microsatellites in DNA samples from blood donors, using a PCR-based technique. Several oligonucleotide primers based on the sequence of 820-bp *Kpn*I–*Sau*3A fragment were designed to make PCR products across dinucleotide repeats with the expected fragment length of 100–200 nucleotides.

Initial PCR reactions were performed using primers IR2 and IR4 to amplify AC/TG-containing microsatellites from phage clones 11 and 15, cosmid 031A, and human B cell line RPMI-6410t. After cleavage with *Xba*I and labeling with Klenow polymerase, PCR products were analyzed on denaturing gels using an appropriate sequence ladder as a marker (Fig. 2). In this experiment we found that the AC/TG repeat was indeed polymorphic in length: clone 031A (from which *Kpn*I–*Sau*3A fragment was originally cloned) contained 13 AC/TG repeats, whereas both phage clones contained 15 repeats. RPMI-6410t cells were heterozygous and contained two alleles with ten and eight repeats (Table 1).

We then investigated whether the oligonucleotides we designed had unique sequences and could therefore define a novel sequence tagged site (STS) in the human genome. After that, we developed a protocol for genomic PCR analysis of DNA polymorphism in this particular human leukocyte antigen (HLA) region.

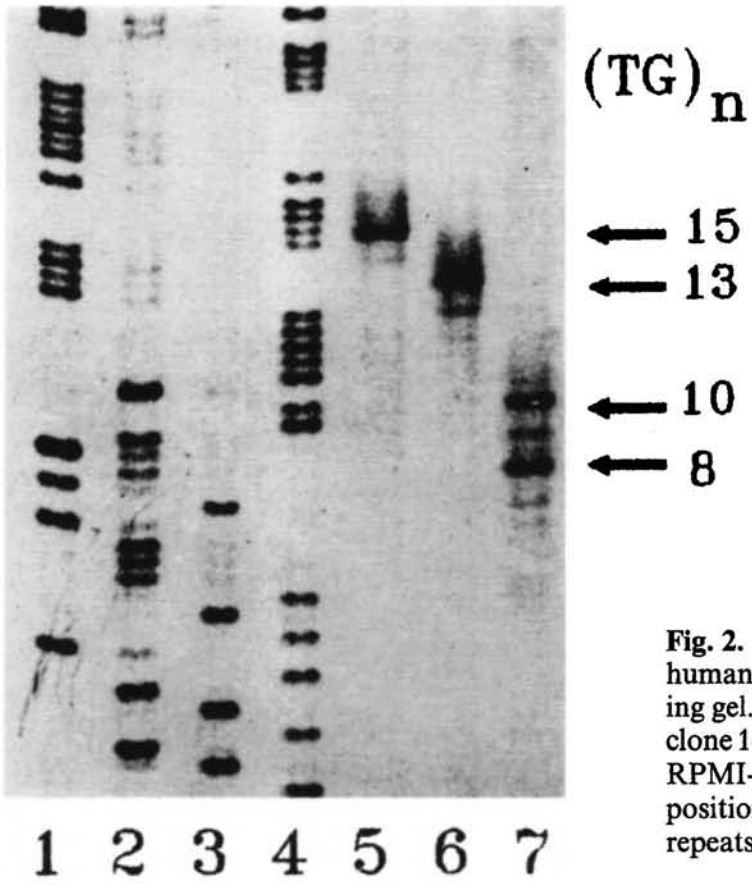

Fig. 2. Microsatellite polymorphism in the human TNF locus as determined on sequencing gel. Lanes *1–4*, sequencing marker; lane *5*, clone 14; lane *6*, clone 031 A; lane *7*, DNA from RPMI-6410t cell line. The *arrows* indicate the positions of specified numbers of AC/TG repeats

Table 1. AC/TG and TC/AG microsatellite length polymorphism in the human TNF locus

Source of DNA	$(AC)_n$	$(TC)_k$
Gosmid 031A	13	10
γ15.1	15	ND
γ11.1	15	ND
1[a]	12	10
2	12/11	11
3	16/11	11
4	18/14	11
5	15/12	10/8.5[b]
6	16/7	10/8.5
7	10/9	9.5[b]
8	17/10	9.5
9	15/11	9.5
10	16/7	10/8.5
RPMI-6410t	10/8	ND

[a] DNA samples 1–10 correspond to unrelated blood donors.

[b] Lengths 8.5 and 9.5 were calculated from the fragment size. Additional sequencing analysis was not performed.

ND: No data.

The protocol is based on two rounds of PCR amplification: first with primers IR1–IR2 across both microsatellites; and second with IR1 and kinased IR5, or with IR2 and kinased IR4 (for separate amplifications across TC/AG or AC/TG, respectively).

PCR products, after the first round of amplification of DNA from blood donors, ran as single (for homozygous donors) or double bands on agarose gels, with an apparent length of 215 bp (Fig. 3). Variations in the length of amplified fragments indicated the polymorphism of "TNF-linked" microsatellites. For the second round, IR4 primer (for AC/TG analysis) or IR5 primer (for TC/AG) labeled by polynucleotide kinase were added. PCR products after the second amplification were directly analyzed on sequencing gel. The results of some of those experiments are summarized in Table 1 and indicate that AC/TG microsatellite length in the human TNF locus can vary from 6 to 18 repeats, while the TC/AG repeat is less polymorphic and occurs from 8 to 11 times. The fact

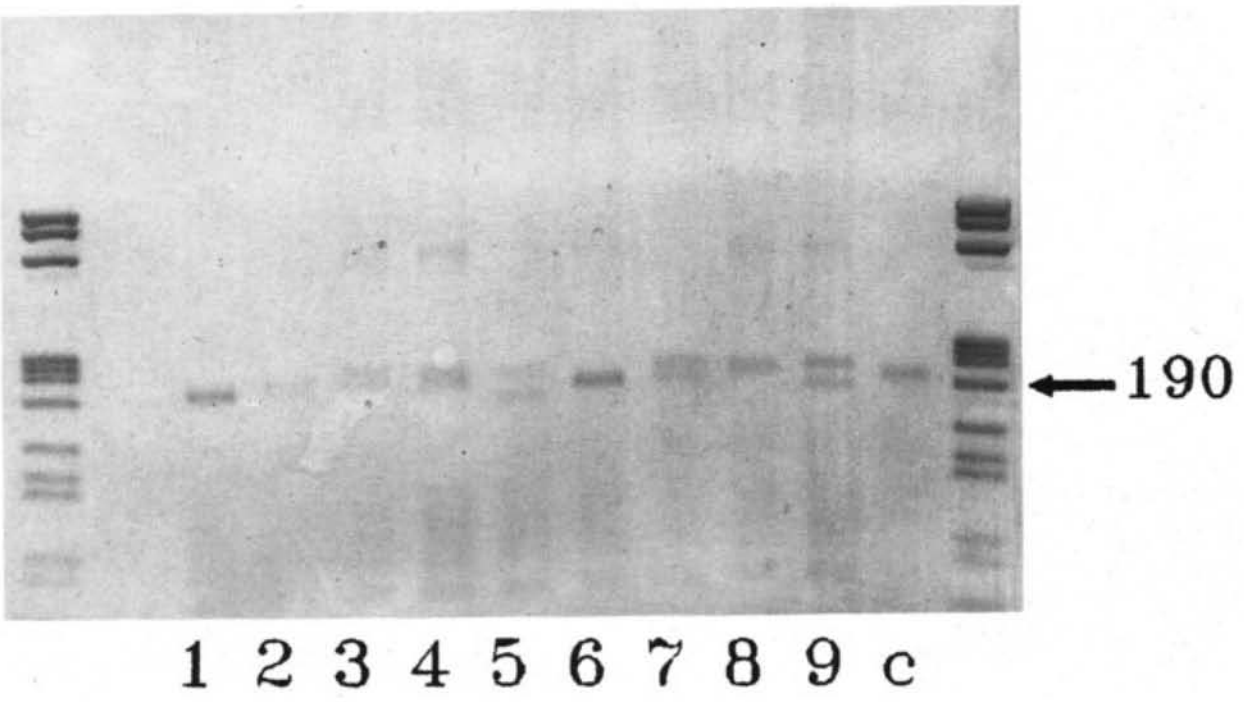

Fig. 3. Detection of microsatellite polymorphism in the human TNF locus by agarose gel electrophoresis. DNA samples were obtained from blood donors (lanes *1–9*) and from cosmid clone 031A (lane *c*)

that both microsatellites are polymorphic (with their combination giving additional variability) indicated that PCR assay of microsatellite lengths in the human TNF locus can be informative for disease-association studies. Similar analysis has been recently performed on the mouse TNF-α gene, where the AC/TG microsatellite located in the promoter region (lacking in homologous position in the human TNF locus) has shown five distinct alleles [26].

Our characterization of novel polymorphic DNA sequences closely linked to the human TNF genes might provide a new experimental tool for the analysis of genetic variability of TNF genes. In view of close linkage between TNF and HLA, it would be of interest now to investigate associations between distinct "TNF alleles," MHC haplotypes, and certain HLA-linked diseases.

Structure of the Rabbit TNF Locus and Homology to Human and Mouse TNF Loci

We have previously isolated a rabbit genomic clone containing TNF genes [13]. We recently sequenced 3.5 kb of DNA including the entire lymphotoxin (TNF-β) gene and the upstream region of TNF-α gene [27]. Our sequence overlaps with that published earlier by Ito et al. [28] and makes 6.5 kb of the rabbit TNF locus available for computer analysis. Several findings are due to this comparative study of human, mouse, and rabbit TNF loci.

First of all, by comparing gene structures for human, mouse, and rabbit TNF-β genes we were able to derive amino acid sequences of rabbit lymphotoxin for which protein or cDNA sequences are lacking [27].

Second, we noted that putative polymorphic microsatellites are lacking in homologous positions in the three TNF loci (see above). We also find that the enhancer sequence which apparently plays an important role in transcriptional regulation of the mouse TNF-α gene is not conserved (site 3, according to [29]). This observation prompted us to search for additional putative regulatory sequences in the human and rabbit TNF loci (see below).

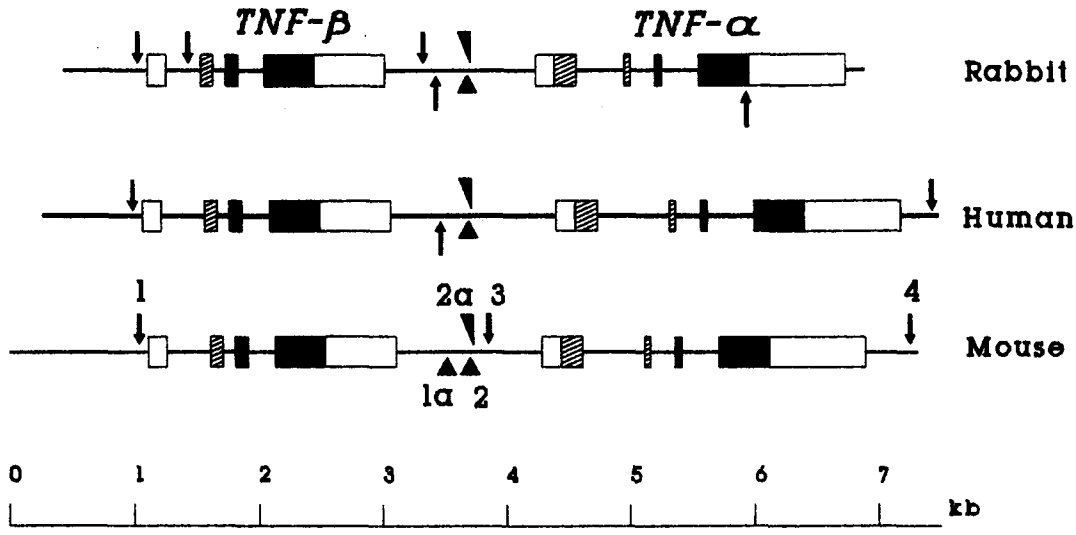

Fig. 4. Localization of κB and κB-related sites in the aligned sequences of the rabbit, human, and mouse TNF loci. *Downward arrows* correspond to sequences in direct orientations, *upward arrows* indicate κB and related sites in reverse orientations

Regulation of Expression of TNF Genes

Multiple κB and κB-related Sites in TNF Loci and Their Role in the Regulation of Transcription

In collaborative study with the group led by C. V. Jongeneel (Ludwig Institute for Cancer Research, Lausanne, Switzerland) we recently presented evidence that κB-related sequences play an essential role in transcriptional activation of the TNF-α gene in mouse macrophages [29]. The role of necrosis factor (NF)-κB or related factors was also suspected in the control of TNF-β expression in T and B lymphocytes, since the κB enhancer sequence is a highly conserved feature of the upstream region of the TNF-β gene (Figs. 4, 5).

Computer analysis of human, mouse, and rabbit TNF sequences (Fig. 4) revealed a number of κB sites [30] located in direct and reverse orientations in the upstream regions, in the intron (rabbit TNF-β gene), and also downstream to mouse and human TNF-α genes (the corresponding portion of the rabbit sequence is not yet available). In particular, we found the previously overlooked symmetrical NF-κB/H2TF1 binding site [31] closely linked to another κB-related sequence (Figs. 5,6). The latter sequence might be a binding site for recently described NF-GMa [32], although it weekly binds NF-κB (Fig. 6). Interestingly, these two binding sites, which both correspond to conserved features of the three TNF loci (Fig. 4), are separated by three turns of double helix (32 bp), placing the two recognition sequences on the same side of the DNA molecule. Whether factors bind cooperatively and make direct protein-protein contacts or, alternatively, whether they interfere, is currently under study.

Analysis of κB DNA – Protein Interactions: Possible Role of Flanking Regions

In view of the variety of κB enhancer-like sequences found in TNF loci we initiated comparative studies of their functional properties in vitro and in vivo. To this end, a collection of synthetic κB sites containing a consensus [30] of 10–11 nucleotides with various natural or artificial flanking sequences was assayed for in vitro binding to NF-κB containing nuclear extracts from mouse macrophages or the human lymphoblastoid cell line RPMI-6410t. Using gel retardation assay we found that the nonconserved κB site 3 from the mouse TNF gene and the conserved κB site from TNF-β gene, both

1	CTTCTAAGCCCTGGGGGCTTCCCCAAGCCCCAGCCC
1a	CCCCGGTCTTCCAAGGATTCCCCTCCCCCACCCTCC
2	GCTTGTGAGGTCCGTGAATTCCCAGGGCTGAGT
2a	TCATTCCCTCTGGGGCTGCCCCATACTCATCCCA
3	AGAACTCAAACAGGGGGCTTTCCCTCCTCAATATCA
4	TGCCCTGGGGCATGGGAATTTCCCACTCTGGGAATT
Consensus	GGGRNTYYCC

Fig. 5. Nucleotide sequence of several κB-related sites with their natural flanks from the mouse TNF locus (consensus nucleotides are *underlined*). Numbering corresponds to that on Fig. 4. In the case of site *1a*, the complementary sequence is shown. Sequences of sites *2* and *2a* are adjacent (see text for details)

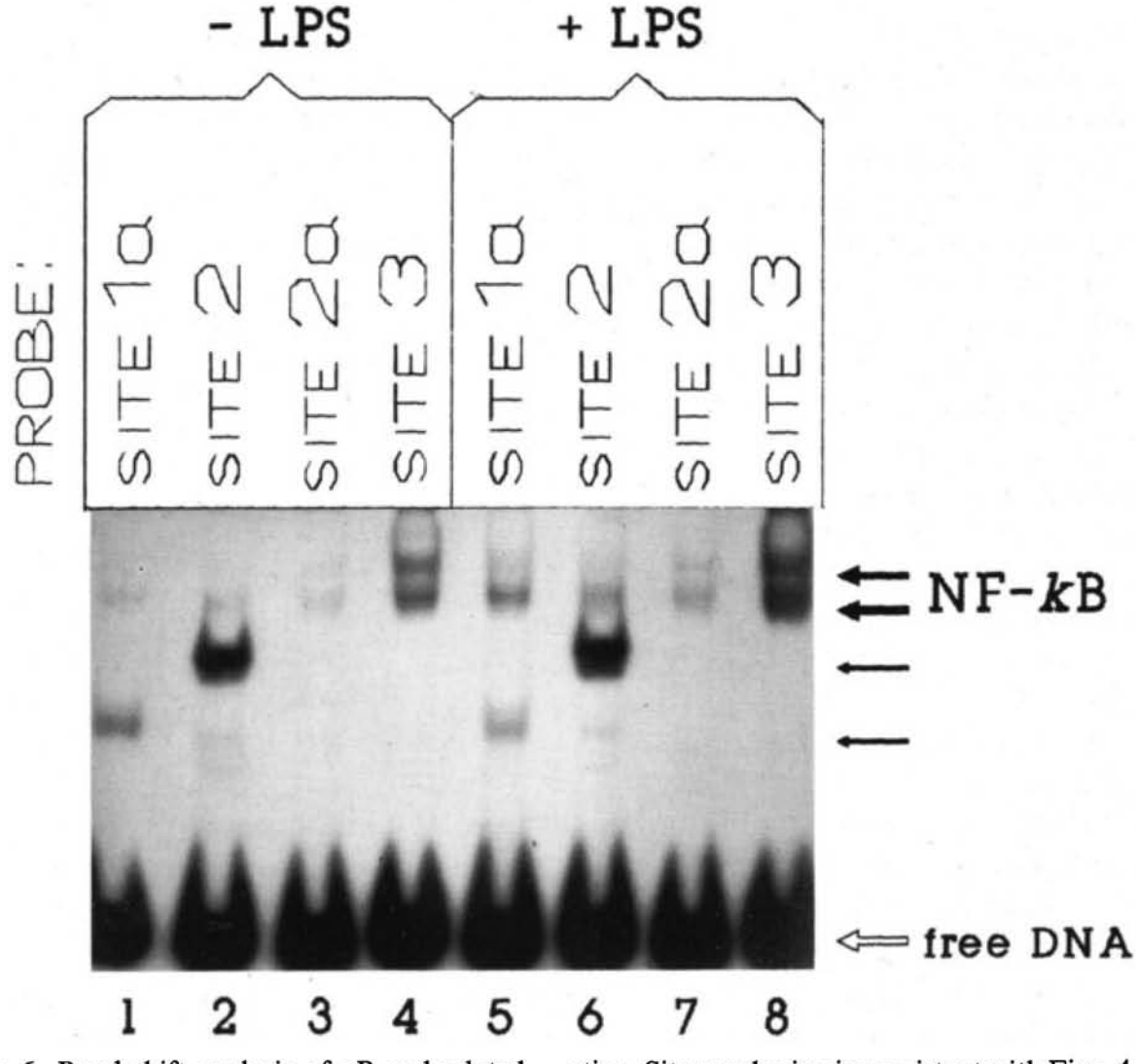

Fig. 6. Band shift analysis of κB and related sites from the mouse TNF-α gene with extracts from mouse bone marrow-derived macrophages before (−) and after (+) *LPS* activation. Site numbering is consistent with Figs. 4 and 5. *Site 1a* (promoter of TNF-β) has affinity similar to that of *site 3*

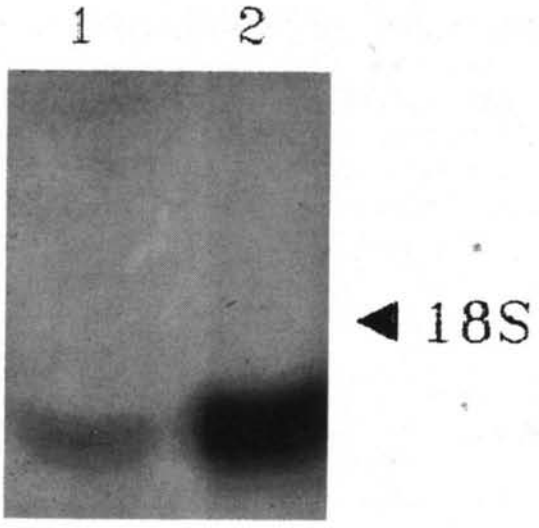

Fig. 7. Northern analysis of TNF-β mRNA in RPMI-6410t cells before (*1*) and after (*2*) PMA activation

with their natural flanks, were the best specific in vitro NF-κB binders (see Fig. 6 for examples). Attachment of the flanks from site 3 to some of the poorer binding κB sites resulted in improved binding. These preliminary results implied the role for DNA sequences immediately flanking the consensus 10–11 nucleotides in κB- and NF-κB interactions. Since one feature of site 3 with its natural flanks resembled that found in the "ideal nucleosome" [33], our current hypothesis is that wrapping of DNA around a protein complex facilitates affinity of binding. We recently cloned NF-κB site 3 into pBend2 vector [34] and prepared a set of labeled fragments with various locations of the target sequences. Band shift analysis confirmed that the κB sequence, which itself had no intrinsic bend, could be bent once bound to NF-κB.

Expression of TNF Locus in a Human Lymphoblastoid Cell Line RPMI-6410t

We have shown previously that the human lymphoblastoid cell line RPMI-6410t secretes an autocrine growth activity which can be neutralized by antibodies raised against purified recombinant human TNF-β [35]. Cytotoxicity neutralization assay with antibodies specific for purified human TNF-α or TNF-β showed the secretion of significant amounts of TNF-β, but little or no TNF-α. The constitutive level of TNF-β expression was enhanced up to a hundred fold after phorbol myristate acetate (PMA) treatment, reaching a maximum 3–4 days after the beginning of stimulation [35].

Northern (Fig. 7) and nuclear run-on (Fig. 8) analysis showed that both TNF

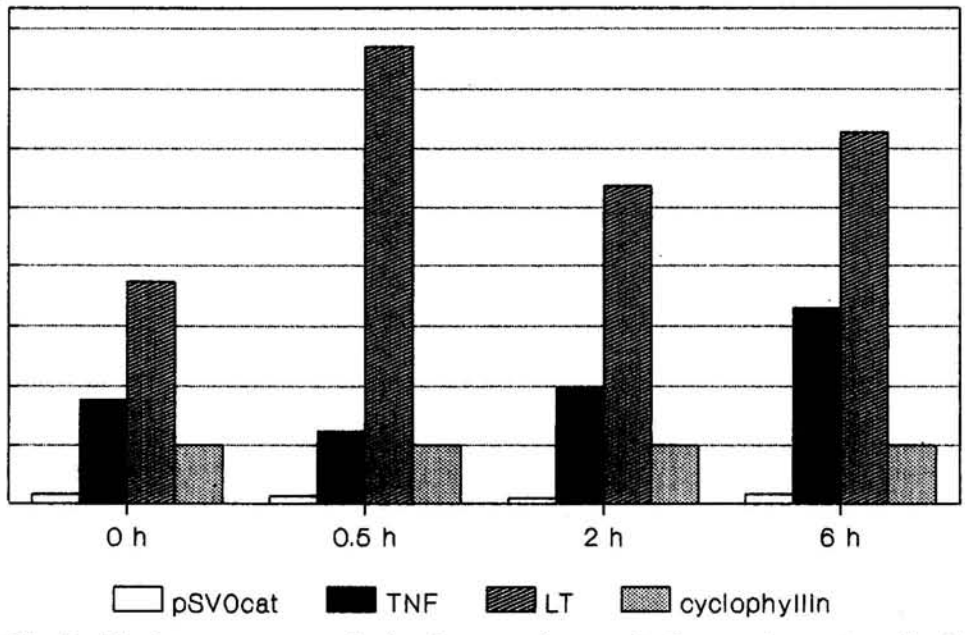

Fig. 8. Nuclear run-on analysis of expression of TNF-α (*TNF*) and TNF-β (*LT*) genes in RPMI-6410t cells. Experimental details are similar to those described in [36]. Signals are normalized against cyclophyllin gene transcription

genes were transcribed, that TNF-β mRNA was more abundant, and that its level was significantly enhanced upon PMA treatment (TNF-α mRNA level remained the same; data not shown). In contrast to mouse CTL clones [36], where the TNF-α gene was always transcribed more actively (in spite of the absence of protein expression of TNF-α), transcription of TNF-β in RPMI-6410t seemed to be more active. Additionally, nuclear run-on assay showed significant constitutive level of TNF-β and TNF-α transcription which was only slightly changed upon PMA activation for various time intervals (Fig. 8). These data implied that regulation of TNF-β expression in the RPMI-6410t cell line occurred primarily on a posttranscriptional level.

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