MOLECULAR AND CELLULAR BIOLOGY, June 1993, p. 3714–3721
0270-7306/93/063714-08$02.00/0
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Vol. 13, No. 6

Characterization of a Tumor Necrosis Factor-Responsive Element Which Down-Regulates the Human Osteocalcin Gene

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Received 28 September 1992/Returned for modification 31 October 1992/Accepted 8 March 1993

Tumor necrosis factor (TNF) down-regulates the production of bone matrix proteins by osteoblasts, thereby inhibiting bone formation. Osteocalcin, the major noncollagenous protein in bone, is inhibited by TNF at the transcriptional level. Mapping studies were undertaken to characterize the TNF-responsive element (TNFRE) in the osteocalcin promoter. Deletion analysis localized the TNFRE to the −522/−511 region, which contains a 9-bp palindromic motif (AGGCTGCCT). Promoter segments containing this sequence down-regulated a heterologous simian virus 40 promoter. Site-specific mutagenesis of the TNFRE eliminated TNF down-regulation. Mobility shift assays demonstrated that a constitutively expressed nuclear factor bound to the TNFRE; this factor was tentatively identified as the p50 homodimer of NF-κB. TNF stimulation induced a second TNFRE-binding protein which displaced the constitutive factor. The TNF-induced protein was not inhibitable by the NF-κB consensus sequence and was unreactive with anti-NF-κB antiserum. DNase footprinting demonstrated that both factors protected the −522/−501 portion of the promoter, consistent with the results of mapping studies and competitive mobility shift assays. It is hypothesized that the generalized catabolic activities of TNF in infectious and malignant diseases may be regulated via this novel element.

Tumor necrosis factor (TNF) is a highly pleiotropic cytokine which induces many proinflammatory effects, including fever, shock, cytokine and prostaglandin synthesis, and bone and cartilage resorption (3, 4). TNF-stimulated up-regulation of many genes, including cytokines, cytokine receptors, immunoglobulins, major histocompatibility antigens, and modulation of human immunodeficiency virus 1 replication, involves induction of the nuclear transcription factor κB (NF-κB) (9, 13, 19, 20, 27, 34, 36, 39). TNF receptor binding results in the phosphorylation and subsequent dissociation of the inhibitor IκB from cytoplasmic NF-κB, freeing NF-κB to bind to specific enhancer sites on DNA (2, 39, 47).

TNF also has significant inhibitory effects on the expression of other genes. TNF plays a key role in the cachexia (wasting) secondary to malignancy, extensive burns, and chronic infections, including AIDS (28, 33, 43). Cachexia involves the consumption of whole-body lipid reserves, a consequence of the down-regulation of lipoprotein lipase and other lipogenic enzymes (5), as well as the decreased synthesis of many structural proteins of muscle and bone, including actin, myosin, collagen, and osteocalcin (3, 15). In contrast to inductive phenomena, the mechanism(s) by which TNF negatively regulates these various proteins has not yet been elucidated.

Osteocalcin is an osteoblast-specific polypeptide which constitutes the major noncollagenous bone matrix protein (18). Osteocalcin production correlates with histomorphometric parameters of bone formation, and levels of this protein in serum have been used clinically to evaluate whole-body bone formation rates (11). Osteocalcin has therefore been widely used as a model protein for studies of the regulation of bone by various agonists, including TNF and other cytokines. TNF down-regulates osteocalcin expression in osteoblasts (30). More recently, using an osteocalcin promoter-chloramphenicol acetyltransferase (CAT) fusion gene, we have found that TNF down-regulation is largely exerted at the level of transcription (25).

In the present work, we have undertaken mapping studies to characterize the *cis*-acting element(s) by which TNF exerts its inhibitory effect on osteocalcin gene expression. Our results demonstrate that TNF inhibition of osteocalcin expression is exerted via a TNF-responsive element (TNFRE) which is homologous to but distinct from NF-κB motifs. The TNFRE motif is also present in the regulatory regions of numerous other genes known to be down-regulated by TNF.

MATERIALS AND METHODS

Osteocalcin gene constructs. An osteocalcin promoter-CAT fusion gene (pHOCCAT) containing a 2.0-kb *Aval* fragment (−1700 to +299, numbered relative to the cap site) of the human osteocalcin 5′-flanking region was used as a starting point for deletion analysis (25). Following partial digestion of pHOCCAT with *Hin*dIII, an *Xho*I linker was added to the *Sma*I site to produce pOCAT-10. pOCAT-10 was digested with *Kpn*I and *Xho*I, and the product was subjected to controlled 5′→3′ digestion with exonuclease III, as described elsewhere (16). The series of 5′ deletions generated were religated with an *Xho*I linker, and their sizes were approximated by electrophoresis in a 3:1 NuSieve-agarose gel or, in selected cases, by DNA sequence analysis with the dideoxy chain termination method (38).

For the generation of promoter region-heterologous simian virus 40 (SV40) promoter constructs, the *Pvu*II-*Xho*I fragments (3′ end, −413) of the most informative osteocalcin promoter deletions (5′ ends: −511, −522, −580, and −707) were cloned into the *Xho*I and *Sma*I sites of pSV40-CAT. pSV40-CAT contains an SV40 promoter but lacks the SV40 enhancer and was produced by insertion of a polylinker into the pCATM (Promega, Madison, Wis.) promoter *Bgl*II site.

Site-directed mutagenesis. A 1.2-kb *Eco*RI fragment from pOCAT-10.10 containing the −580/+299 region of the osteocalcin gene plus the CAT gene was cloned into the *Eco*RI

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site of M13mp18 to produce M13mp18-OC-1. This construct produced the sense strand, as confirmed by restriction mapping with *Hin*dIII. M13mp18-OC-1 was subjected to oligonucleotide-directed mutagenesis as described before (22), with a 31-mer oligomer (5'-GTGAGTCACCAAAAAG CTTTGCTGTGGGTGC) (Midland Certified Reagent Co., Midland, Tex.). A *Hin*dIII restriction site was thereby introduced into the TNFRE in the mutant fusion gene as indicated in Fig. 4. The 1.2-kb *Eco*RI fragment from the mutant gene was isolated and cloned into the identical site in pOCAT-10.10 to produce pOCAT-10.10m. To test heterologous promoter regulation, an *Xho*I-*Pvu*II fragment (−580 to −413) of the mutagenized TNFRE (pOCAT-10.10m) was also introduced into pSV40-CAT.

Cell transfection and CAT assays. For determination of 5'-flanking regulatory activity, constructs were transfected into the rat osteosarcoma cell line ROS 17/2.8 as described elsewhere (25). In brief, ROS 17/2.8 rat osteosarcoma cells were plated at 3×10^5 cells per 60-mm culture dish in F-12 medium supplemented with 4 mM L-glutamine, 1% penicillin-streptomycin, and 5% fetal bovine serum. After 24 h, transient transfections were carried out by the calcium phosphate method. After a 3-min glycerol shock, cells were exposed to 10 μg of precipitated DNA per dish for 4 to 6 h. ROS 17/2.8 cells were also stably transfected with pHOCCAT by standard methods (36a) and were kindly provided by P. K. DenBesten, Eastman Dental Center, Rochester, N.Y. Transfected cells were stimulated with vitamin D (10^{-8} M) (Hoffmann-La Roche, Nutley, N.J.) alone and in combination with recombinant human TNFα (10^{-9} M) (Genzyme, Boston, Mass.) for 60 h and were cultured thereafter in F-12 medium containing 0.5% fetal bovine serum. Cells were harvested into a buffer consisting of 150 mM NaCl, 40 mM Tris-HCl, and 1 mM EDTA, pH 7.5, and were lysed by five cycles of freezing and thawing. CAT activity was determined in a scintillation assay as described by Neumann et al. (31). CAT assay results were standardized to the protein concentration of cell layer extracts by using a protein assay kit (Bio-Rad, Richmond, Calif.). Relative CAT activity was calculated as disintegrations per minute (dpm) for vitamin D plus TNF (stimulated) divided by dpm for vitamin D alone.

DNase I footprint analysis. The construct pOCAT-10.10 was digested with *Xho*I, generating a 3'-recessed end 67 bp from the site. The 3' recess was filled in with ^{32}P-labeled nucleotides. The DNA was precipitated and digested with *Pvu*II to generate a 170-bp restriction fragment (−580/−413) labeled at the 3' end on the antisense strand. The labeled fragment was separated on agarose gels and electroeluted. Footprinting was carried out with a commercially available kit (SureTrack; Pharmacia). Nuclear extract (12 μg) from unstimulated or stimulated (TNF or vitamin D) ROS 17/2.8 cells was incubated with 1 μg of poly(dI-dC) as the nonspecific competitor and labeled fragment (15,000 cpm) for 30 min at 0°C. DNase I was added (2 U per reaction), and digestion was allowed to proceed for 1 min at 20°C. Digestion was halted by the addition of 200 μl of 0.3% sodium dodecyl sulfate (SDS)–150 mM NaCl. Proteins were extracted with phenol-chloroform, and the DNA was precipitated, heated for 3 min at 90°C, and loaded onto a 6% polyacrylamide sequencing gel to determine the protected portions of the DNA.

Electrophoretic mobility shift assays. ROS 17/2.8 cells were plated at 3×10^5 cells per 60-mm culture dish and left unstimulated or stimulated with TNF (10^{-9} M). Nuclear protein extracts were prepared from cell cultures as described before (12). A 35-mer synthetic oligonucleotide containing the TNFRE (−539/−505) was end labeled with ^{32}P and used as a probe. Binding reactions were carried out in the absence or presence (100-fold excess) of the following double-stranded DNA inhibitors: 35-mer homologous osteocalcin probe (−539/−505); 34-mer osteocalcin probe with the TNFRE core replaced with a *Hin*dIII restriction site, as in Fig. 4; 22-mer NF-κB consensus sequence (AGTTGAGGG GACTTTCCCAGGC) (Promega); and 22-mer Sp1 consensus sequence (ATTCGATCGGGGCGGGGCGAGC) (Promega). Following the binding reaction, complexes were electrophoresed in a 4% polyacrylamide gel under low-salt conditions (8).

For competitive mobility shift assays to characterize binding protein specificity, mutant 25-mers of the TNFRE were synthesized with 2-bp substitutions of the wild-type sequence AGGCTGCCT: mutant 1, GAGCTGCCT; mutant 2, AAACTGCCT; mutant 3, AGGTCGCCT; mutant 4, AGGC CACCT; and mutant 5, AGGCTGTTT. For antibody inhibition (supershift) assays, 4 μl of control or specific anti-NF-κB subunit antiserum was preincubated with nuclear extract for 15 min at 20°C prior addition to the binding reaction mixes. Neutralizing antiserum against the p50 subunit was generously provided by Garry Nolan and David Baltimore, Rockefeller University. Antisera against p65 and c-*rel* N- and C-terminal regions were obtained from Santa Cruz BioTechnology, Santa Cruz, Calif.

TABLE 1. CAT activity of ROS 17/2.8 cells transfected with pOCAT-10.1

Stimulant	Mean [^{3}H]acetyl-chloramphenicol (cpm) ± SD[a]
None (basal activity)	4,447 ± 302
Vitamin D (10^{-8} M)	39,330 ± 6,695
Vitamin D (10^{-8} M) + TNFα (10^{-9} M)	19,852 ± 2,985

[a] Three experiments.

RESULTS

Deletion analysis of the 5'-flanking region of the osteocalcin gene. In order to approximate the location of the TNFRE, deletions of the osteocalcin 5'-flanking region were made by exonuclease III digestion. The series of 5' deletions generated were transiently transfected into the rat osteosarcoma cell line ROS 17/2.8 and tested for TNFα-stimulated suppression of 1,25-dihydroxyvitamin D3 (vitamin D)-induced CAT activity. The results demonstrated, in agreement with previous findings (25), that vitamin D stimulates CAT activity by about 10-fold in the long promoter construct pOCAT-10.1 (Table 1). This stimulation was inhibited by 30 to 50% in the presence of recombinant human TNFα (10^{-9} M) (Table 1, Fig. 1). Basal transcription of pOCAT-10.1 was similarly down-regulated by TNFα (TNFα/basal = 0.68 ± 0.11, n = 5). ROS 17/2.8 cells which were stably transfected with this construct gave essentially identical results: down-regulation of vitamin D-stimulated CAT activity of 27, 43, and 55% was observed in the presence of TNF concentrations of 10^{-11}, 10^{-10}, and 10^{-9} M, respectively.

Deletions from −1300 to −522 (constructs 10.2 through 10.12) (Fig. 1) showed a similar pattern of TNF-induced down-regulation, with the single exception of the −659 deletion (pOCAT-10.7), in which inhibition was largely lost.

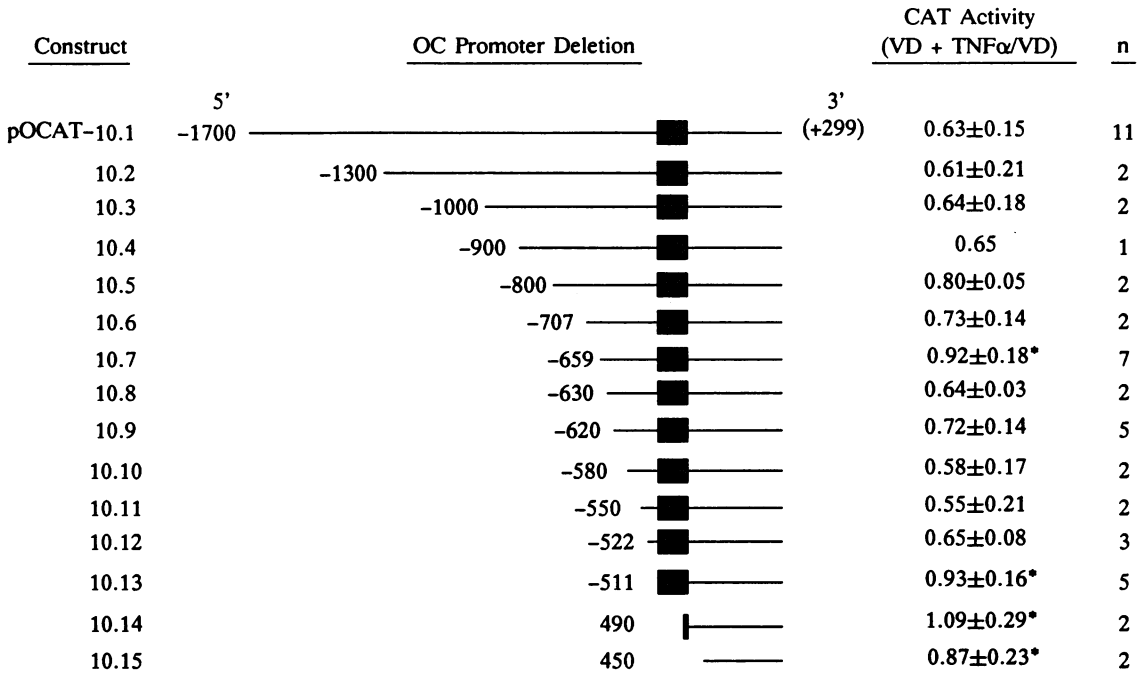

FIG. 1. Deletion analysis of the osteocalcin (OC) 5'-flanking region for localization of the TNFα-responsive element. The solid rectangle indicates the position of the VDRE (−510/−487) (21). Constructs exhibiting loss of TNFα down-regulation of the vitamin D (VD) response are marked with an asterisk. *n*, number of determinations. Transfected cultures were tested for TNFα-stimulated suppression of vitamin D-induced CAT activity.

Inhibition was regained with the −630 and subsequent deletions to −522, at levels similar to that observed for constructs pOCAT-10.1 through pOCAT-10.6. Down-regulation was also abrogated in deletions −511, −490, and −450. Note that the −511 deletion terminates just 5' to the vitamin D response element (VDRE) (35), whereas the latter deletions removed the VDRE. The differential response of the −522 (inhibited) and −511 (not inhibited) constructs was especially informative and indicated that the TNF response might be controlled through an element located in this 11-bp region. Examination of the osteocalcin promoter sequence (Fig. 2) (29) revealed the presence of a 9-bp palindromic motif located at −523 to −515 (AGGCTGCCT), which possesses dyad symmetry to the complementary DNA strand. Of interest, a 13-bp palindromic motif homologous to this sequence is also present at −668 to −656 (CTGGCTGT GCCAG). This region was interrupted by the −659 deletion, which also lost TNFα-mediated down-regulation, as noted above (Fig. 1).

Heterologous promoter regulation by the TNFRE. The effect of the putative TNFRE on a heterologous SV40 promoter was determined. The *Pvu*II-*Xho*I fragments (3' end, −413) of the most informative osteocalcin promoter deletions were cloned into pSV40-CAT, which lacks the SV40 enhancer and was unaffected by the presence of 10^{-9} M TNFα (not shown). These constructs were tested for TNFα regulation of basal and vitamin D-stimulated CAT activity. As shown in Fig. 3, the −580/−413 fragment conferred TNFα inhibition on the SV40-CAT construct in the absence of vitamin D. In addition, all deletions except

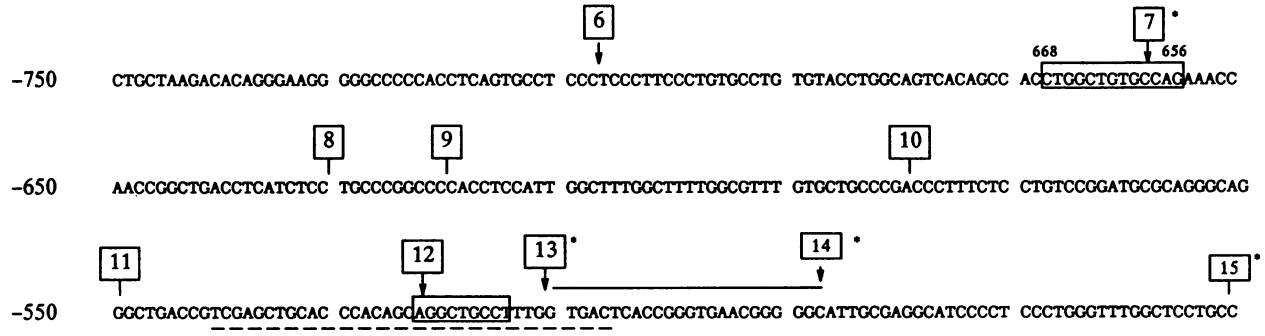

FIG. 2. Putative TNF-responsive sequences in the osteocalcin 5'-flanking region. The sequence is taken from the report by Morrison et al. (29). Homologous motifs are located at −668/−656 and −521/−513, as indicated by the boxed regions. Endpoints of deletions are indicated; those with an arrow were determined by sequence analysis. The VDRE is overlined. The homologous probe used for electrophoretic mobility shift assays (see Fig. 5) is underlined. Deletions which result in loss of TNFα inhibition of the vitamin D response are marked with an asterisk.

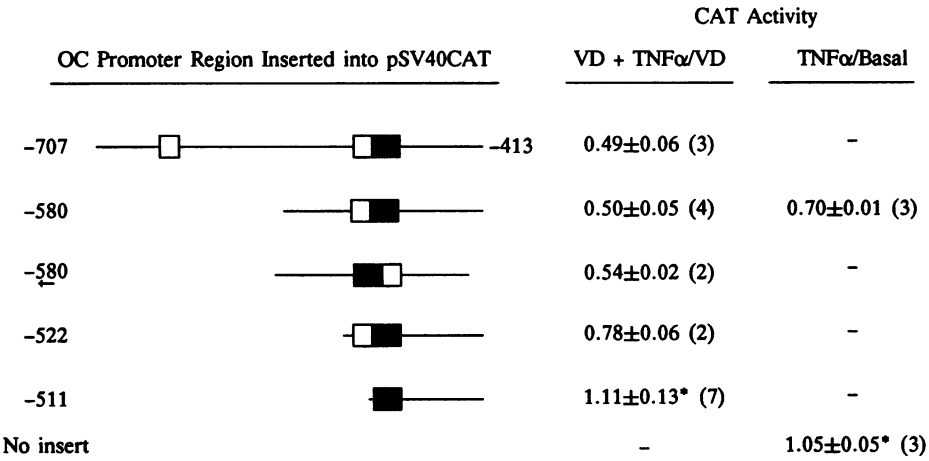

FIG. 3. Effect of osteocalcin (OC) promoter regions containing the putative TNFRE on a heterologous SV40 promoter. Constructs were transiently transfected into ROS 17/2.8 cells and stimulated with vitamin D (VD) and TNFα or TNFα alone for 60 h, and CAT activity was determined. The VDRE is indicated by the shaded rectangle. Constructs not significantly inhibited by TNFα are marked with an asterisk. The number of determinations is shown in parentheses.

−511 also conferred TNFα inhibition on vitamin D-stimulated CAT activity. These findings demonstrate the ability of a TNFRE in the −522 to −511 region to down-regulate vitamin D as well as basal responses in the context of a heterologous promoter. The −580 deletion in the reverse 3′→5′ orientation retained down-regulation, consistent with the activity of a classical repressor element.

Site-specific mutagenesis of the TNFRE. A direct determination of the activity of the putative −521 to −513 TNFRE was provided by site-specific mutagenesis of this element. The −580 deletion (construct pOCAT-10.10, Fig. 1) was modified by replacement of the TNFRE with a *Hin*dIII restriction site by oligonucleotide-directed mutagenesis (22) to yield pOCAT-10.10m (Fig. 4). A *Pvu*II-*Xho*I fragment of pOCAT-10.10m (−580/−413) was also inserted into pSV40-CAT to assess heterologous regulation. The results, summarized in Fig. 4, clearly demonstrated that both mutagenized constructs, pOCAT-10.10m and pSV40-CAT-10.10m, completely lost TNFα-mediated inhibition. Together with findings above, these data strongly suggest that TNFα suppresses the transcription of the osteocalcin gene via this responsive element.

Electrophoretic mobility shift assays. Comparison of the TNFRE sequence with other known transcription factor-binding motifs indicates some homology to the consensus sequence for NF-κB [GGGR(CAT)TYYCC] (23), with 4 of 10 differences, as indicated by the underlined bases (CAG GCTGCCTT), but only 1 of 7 differences in the core region. Electrophoretic mobility shift assays were carried out to attempt to clarify the relationship between the TNFRE and NF-κB binding sites. Nuclear extract from uninduced ROS 17/2.8 cells contained a protein(s) which bound to a 35-mer osteocalcin probe containing the TNFRE (−539/−505) (Fig. 5, lane 2). Binding was inhibited both by homologous probe (Fig. 5, lane 3) and by an NF-κB consensus oligomer (Fig. 5, lane 5), but not by the consensus oligomer of an unrelated nuclear factor, Sp1 (Fig. 5, lane 6). A 34-mer oligomer corresponding to the homologous sequence but with the core of the TNFRE replaced by a *Hin*dIII restriction site partially inhibited binding (Fig. 5, lane 4). These data suggest that a DNA-binding protein which can bind to both the NF-κB and TNFRE motifs is constitutively expressed in osteoblastic cells.

Importantly, TNF-stimulated ROS cells also expressed a second, lower-molecular-weight DNA-binding moiety (Fig. 5, lane 7). The interaction of this protein was inhibited by the

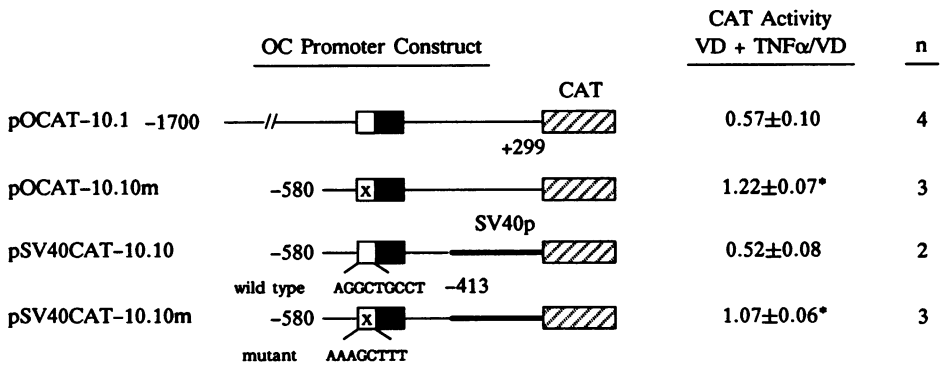

FIG. 4. Response of site-directed TNFRE mutations. Open rectangle, TNFRE; box with X, *Hin*dIII site-specific mutant of TNFRE; shaded box, VDRE. An *Xho*I-*Pvu*II fragment (−580/−413) of the mutagenized TNFRE (pOCAT-10.10m) was also introduced into pSV40-CAT to test heterologous promoter regulation. The transfection, stimulation, and CAT assay protocol were then performed. Deletions which result in loss of TNFα inhibition of the vitamin D (VD) response are marked with an asterisk. *n*, number of determinations.

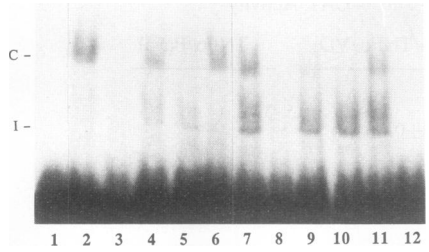

FIG. 5. Electrophoretic mobility shift assay of DNA-binding proteins from TNF-stimulated and unstimulated ROS 17/2.8 cells. A 35-mer synthetic oligonucleotide containing the TNFRE (−539/−505) was end labeled with ^{32}P and used as a probe. Nuclear protein extracts were prepared from unstimulated or TNF-stimulated (10^{-9} M) ROS 17/2.8 cells as described elsewhere (12). Binding reactions were carried out in the absence or presence of the following double-stranded DNA inhibitors: 35-mer homologous osteocalcin probe; 34-mer osteocalcin probe with TNFRE core replaced with a *Hin*dIII restriction site, as in Fig. 4; 22-mer NF-κB consensus sequence (AGTTGAGGGGACTTTCCCAGGC) (22) (Promega); 22-mer Sp1 consensus sequence (ATTCGATCGGGGCGGGGCGAGC) (24) (Promega). Following the binding reaction, complexes were electrophoresed in a 4% polyacrylamide gel under low-salt conditions (28). Constitutive (C) and TNF-induced (I) DNA-binding proteins are indicated. Lanes 1 and 12: probe alone; lanes 2 through 6, uninduced extract (8 μg per lane); lanes 7 through 11, TNF-induced extract (8 μg); lanes 2 and 7, no inhibitor; lanes 3 and 8, homologous probe as inhibitor (100-fold excess); lanes 4 and 9, *Hin*dIII mutant (34-mer synthetic oligomer) (100-fold excess); lanes 5 and 10, NF-κB (100-fold excess); lanes 6 and 11, Sp1 (100-fold excess).

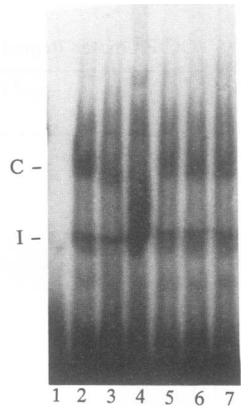

FIG. 6. Supershift mobility assay with NF-κB antiserum. Nuclear protein extracts from ROS 17/2.8 cells were incubated with antiserum against NF-κB subunits prior to binding reactions with ^{32}P-labeled probe (−539/−505). Lane 1, no extract; lanes 2 through 7, TNF-induced extract (8 μg); lanes 2 and 3, control antisera; lane 4, anti-p50; lane 5, anti-p65; lane 6, anti-c-*rel* N terminus; lane 7, anti-c-*rel* C terminus. Constitutive (C) and TNF-induced (I) DNA-binding proteins are indicated.

homologous oligomer (Fig. 5, lane 8) but not by NF-κB (Fig. 5, lane 10), Sp1 (Fig. 5, lane 11), or the *Hin*dIII replacement oligomer (Fig. 5, lane 9). This TNF-inducible protein therefore possesses high affinity for the TNFRE but no or low affinity for the NF-κB motif. The NF-κB motif-binding protein was also present in TNF-induced extracts but in lower amounts than in uninduced cells (Fig. 5, compare lanes 7 and 2), suggesting its displacement by the TNF-induced binding protein.

Antisera directed against NF-κB subunits were used in mobility supershift assays to attempt to identify these DNA-binding proteins. As shown in Fig. 6, an antiserum directed against the p50 subunit retarded the mobility of the constitutive factor (lane 4). In contrast, antisera against the p65 subunit (Fig. 6, lane 5) and the C and N termini of c-*rel* (Fig. 6, lanes 6 and 7) and two control sera (Fig. 6, lanes 2 and 3) had no effect. None of the antisera affected the TNF-induced factor, further indicating that it is distinct from NF-κB. Treatment with the anti-p50 antiserum did result in increased intensity of the TNF-induced complex, suggesting that neutralization of p50 may permit its increased binding. The constitutive factor therefore contains p50 but not p65 or c-*rel* and is presumptively identified as a p50 homodimer of NF-κB.

DNase I footprinting and competitive mobility shift assays. DNase I footprinting was carried out in order to define the region of contact between the DNA-binding proteins and the osteocalcin regulatory region. As shown in Fig. 7, both unstimulated (lane 2) and TNF-stimulated (lane 3) ROS 17/2.8 nuclear proteins protected a region of the probe which included the TNFRE (−522 to −501). The area of protected nucleotides was essentially identical for the unstimulated and TNF-stimulated nuclear extracts, consistent with the inhibition patterns observed in competitive mobility shift assays (below). As a positive control, the protection provided by an extract produced from vitamin D-stimulated cells (Fig. 7, lane 4) included the TNFRE plus a further 3′ extension into the VDRE (−510 to −490) (35). This pattern

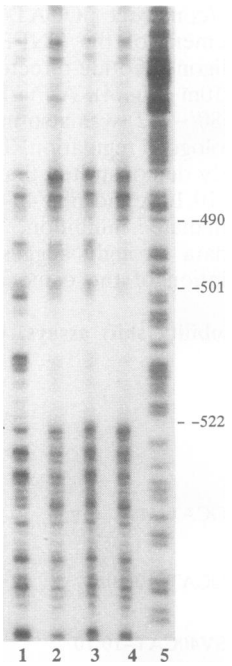

FIG. 7. DNase I footprinting of TNFRE-binding proteins. The *Pvu*II-*Xho*I fragment (−580/−413) was labeled at the 3′ end on the antisense strand. Lane 1, incubation with bovine serum albumin (12 μg); lanes 2 to 4, 12 μg of nuclear extracts from ROS 17/2.8 cells; lane 2, uninduced; lane 3, TNF induced; lane 4, vitamin D (10^{-8} M) induced; lane 5, degradative sequence (C/T) residues. Numbers on the right indicate nucleotide locations in the osteocalcin gene 5′-flanking region.

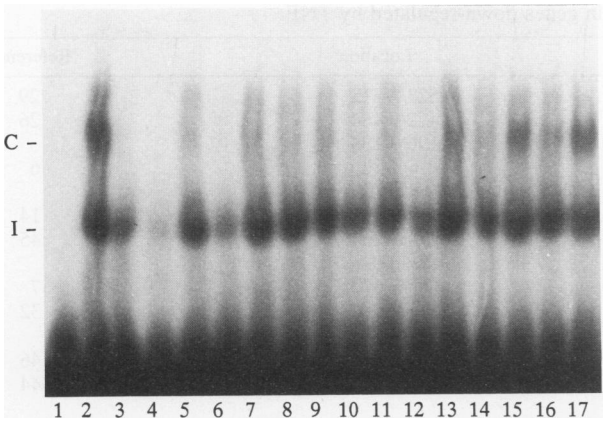

FIG. 8. Competitive mobility shift assays to characterize DNA-binding protein specificity. Five 25-mer oligonucleotides with 2-bp substitutions in the TNFRE were used in competitive inhibition assays with TNF-induced nuclear extracts from ROS 17/2.8 cells. Lane 1, probe; lane 2, no inhibitor; lanes 3 and 4, homologous 35-mer as inhibitor; lanes 5 and 6, mutant 1; lanes 7 and 8, mutant 2; lanes 9 and 10, mutant 3; lanes 11 and 12, mutant 4; lanes 13 and 14, mutant 5; lanes 15 and 16, SP1; lane 17, no inhibitor. For each pair of lanes, the inhibitor was used at a 10-fold (first lane) and 100-fold (second lane) excess. Constitutive (C) and TNF-induced (I) DNA-binding proteins are indicated.

is consistent with the simultaneous binding of the constitutive factor and vitamin D receptor proteins to this area. Taken together, these data indicate that the TNFRE-binding proteins have DNA contact areas which overlap the vitamin D receptor complex. These proteins are thus likely to interact to regulate osteocalcin transcription.

Competitive mobility shift assays were also performed to further characterize the specificity of the two TNFRE-binding factors. Five 25-mer oligonucleotides were synthesized with 2-bp substitutions throughout the TNFRE (see Materials and Methods). These were used in competitive mobility shift assays with nuclear extracts from ROS 17/2.8 cells which had been stimulated with TNF. As shown in Fig. 8, the TNF-induced factor was completely inhibited by a 100-fold excess of homologous unlabeled probe (lane 4). Mutation 1 (Fig. 8, lanes 5 and 6), with a substitution at the 5′ side of the TNFRE consensus sequence, showed only slightly reduced inhibition. In contrast, mutation 2 (Fig. 8, lanes 7 and 8), which replaced the GG, had a nearly complete loss of inhibition, indicating alteration of nucleotides which were essential for binding. Mutation 5 (Fig. 8, lanes 13 and 14), which replaced the CC, had a lesser but still significant effect. Mutations 3 (Fig. 8, lanes 9 and 10) and 4 (Fig. 8, lanes 11 and 12), which replaced nucleotides in the center of the TNFRE, had much less effect. By densitometric analysis, the mutations were ranked 2 > 5 > 3 > 4 > 1. Thus, the GG and CC residues appear to be of greatest importance for TNF-induced factor binding.

A slightly different inhibition pattern was observed for the p50 constitutive factor, which in general was much more easily inhibited by all oligonucleotides. Reduced inhibition capability was observed in the order 5 > 2 > 3 > 4 = 1, suggesting somewhat greater importance of the CC than of the GG residues in p50 versus TNF-induced factor binding. Competitive inhibition of the p50 factor with extracts from unstimulated cells gave an identical result (not shown).

DISCUSSION

TNF stimulates a coordinated array of effects which are tissue and gene specific and which may reflect the utilization of different signal transduction pathways in different tissues (4). For example, TNF up-regulates collagenase while concomitantly down-regulating the expression of collagen (10). Whereas most studies have focused primarily on inductive phenomena, TNF-stimulated inhibition of gene expression may be equally important and may have implications in the pathogenesis of infectious and inflammatory diseases, including arthritis, periodontitis, malignancies, and AIDS. In the present study, we present evidence that TNF down-regulation of the model bone matrix protein osteocalcin is mediated via a TNFRE located at −523 to −515 of the 5′-flanking region. The TNFRE motif is palindromic (AG GCTGCCT) and possesses homology to the consensus NF-κB motif (23). Promoter segments containing this sequence down-regulated a heterologous SV40 promoter. Site-specific mutagenesis of the TNFRE eliminated TNF down-regulation. Competitive mobility shift assays demonstrated the binding of a TNF-inducible nuclear protein to this motif, which was not inhibitable by the consensus sequence for NF-κB; this factor appears to displace a constitutively expressed, NF-κB-inhibitable protein. By reactivity with antisera, the constitutive factor was tentatively identified as a p50 homodimer of NF-κB (KBF1), whereas the TNF-induced protein was unreactive with NF-κB antibodies. DNase I footprinting revealed that both the p50 and TNF-induced TNFRE-binding proteins protected the same region (−518 to −497), and their binding specificity was similar in competitive mobility shift assays. These data strongly suggest that TNF-mediated down-regulation of osteocalcin operates via a novel regulatory pathway which is distinct from that employed for stimulatory responses.

Nuclear run-on studies by Nanes et al. (30) and evidence from our own laboratory with an osteocalcin promoter-CAT fusion gene have indicated a transcriptional site of TNF action (25), although the responsive element(s) has been previously unidentified. A preliminary report by Harrison et al. (17) has indicated the presence of an inhibitory TNFRE in the collagen α1(I) promoter, although no information has yet appeared on its characterization. Of interest, in mapping studies of the osteocalcin VDRE by Ozono et al. (35), a fortuitous deletion which removed most of the TNFRE (−520/−511) resulted in enhanced vitamin D-stimulated transcription, consistent with our findings. A similar TNFRE motif is also present just 5′ to the VDRE (−472/−464) in the rat osteocalcin gene, the only other osteocalcin regulatory region for which data are currently available (26).

Previous data from this and other laboratories have shown that TNF concomitantly stimulates bone resorption and inhibits bone formation via suppressive effects on the expression of bone matrix proteins by osteoblasts (3, 41, 42). The 5′-flanking sequences of other proteins known to be down-regulated by TNFα also contain sequences identical or closely homologous to the TNFRE (Table 2). These include α1(I) collagen, alkaline phosphatase, osteonectin, myosin H chain, thrombomodulin, and the c-*myc* oncogene. In other genes, TNFRE-like sequences were also found in the 5′-flanking region, with the exception of collagen α1(I), in which two homologs were present in the first intron. Considering all sequences, a consensus core region of seven bases [GGC(A/T)GCC] can be identified. Although none of these sequences have as yet been tested directly for functionality, we might nevertheless speculate that some of the

TABLE 2. Presence of the TNFRE motif in genes down-regulated by TNF

Gene	Sequence	Location[a]	Reference
Human osteocalcin	AGGCTGCCT	−523/−515	29
Rat osteocalcin	GAGCTGCCT	−472/−464	26
	CGGCAGCCT	−196/−188	
Human collagen α1(I)	GGGCAGCCG	661/669 (intron 1)	6
	GGGCAGCCC	879/887 (intron 1)	
Chicken collagen α1(I)	CGGCAGCCG	Intron	14
Human alkaline phosphatase	GGGCAGCCC	−274/−266	45
	GGGCTGCCC	−68/−60	
Rat myosin H chain	TGGCTAGCCA	−686/−677	7
Human thrombomodulin	AGGCTGCCT	−11/−2	32
	GGGCGGCCA	−147/−139	
Bovine osteonectin	GGGCAGCCA	−909/−903	46
Human c-*myc*	CGGCTGCCC	−170/−162	44
Consensus	GGC(A/T)GCC		

[a] Numbered relative to transcription start site unless otherwise indicated.

generalized catabolic activities of TNF in infectious and malignant diseases may be regulated via this novel response element.

At least two osteoblastic nuclear binding proteins were shown to interact with the TNFRE, (i) constitutively expressed, NF-κB-mediated and (ii) TNF-induced, NF-κB noninhibitable. The constitutively expressed NF-κB-inhibitable protein appears to represent the 50-kDa homodimer KBF1 (1, 21). Interestingly, constitutively expressed KBF1 appears to be important in maintaining basal expression of major histocompatibility complex class I antigen, whereas TNF up-regulation results in displacement of KBF1 by NF-κB (1). Given the core region homology between the TNFRE and NF-κB motifs (one of seven differences), the binding of an NF-κB-like factor is not surprising. The NF-κB2b site (GGGGCTGCCCC) in the TNF promoter is closely homologous to the TNFRE (20). NF-κB has only weak affinity for this motif, indicating that it is readily displaced. The role played by the constitutive factor in regulating osteocalcin transcription is not known, but could, by analogy with major histocompatibility complex control, affect basal osteocalcin expression. On the other hand, the unique TNF-induced protein was unreactive with NF-κB antiserum. This may constitute a novel nuclear factor or, alternatively, may be related or identical to NF-GMa (40). NF-GMa is induced by TNF and binds to the CK-1 site (consensus, GRGRTTYCYN) which is important in granulocyte colony-stimulating factor induction. Analogous to the TNFRE-protein interactions described herein, NF-κB binds to the CK-1 site, but NF-GMa fails to bind to NF-κB sites. Arguing against such a relationship is the limited homology between the CK-1 and TNFRE motifs. Further studies are required to characterize this regulatory protein.

The generalized catabolic actions of TNF may have evolved to meet the challenge of acute infections, i.e., to mobilize amino acids from body structural proteins for use by the immune system and the liver. In contrast, the long-term effects of chronic TNF exposure are clearly maladaptive, as illustrated in the cachexia associated with chronic infections and malignancies. Chronic infusion of TNF or the implantation of TNF-secreting tumors causes a wasting syndrome in mice (33, 43). Whereas protein-conserving mechanisms are maximized in starved animals, cachectic hosts catabolize body energy stores (protein and lipid) in the face of diminished caloric intake. Elucidation of the mechanisms underlying TNF down-regulation of gene expression may provide the basis for the rational design of inhibitors of TNF action.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DE-07378 from the National Institute of Dental Research.

We thank W. Chen and P. Fujiyoshi for excellent technical assistance, MaryAnn Cugini for graphics and manuscript preparation, and M. Duncan, I. Nishimura, D. Graves, and F. E. Dewhirst for a critical review of the manuscript.

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