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Nuclear Factor-κB Mediates TNF-α Inhibitory Effect on α2(I) Collagen (COL1A2) Gene Transcription in Human Dermal Fibroblasts[1]

David J. Kouba,*†‡ Kee-Yang Chung,*§ Takafumi Nishiyama,*‡ Laurence Vindevoghel,*‡ Atsushi Kon,*‡ John F. Klement,*‡ Jouni Uitto,*†‡ and Alain Mauviel[2]*‡

Among its plethora of activities as an inflammatory mediator, TNF-α has potent regulatory control on extracellular matrix production and degradation. Earlier studies have documented that TNF-α inhibits type I collagen gene (COL1A2) expression at the transcriptional level, but the characterization of the transcription factors involved has been elusive. In the present study, using transient cell transfection of human dermal fibroblasts with a battery of 5′ end deletion/chloramphenicol acetyltransferase (CAT) reporter gene constructs, we have characterized the TNF-α response element of the COL1A2 promoter. The TNF-α response element was attributed to a specific region that comprises noncanonical activator protein-1 (AP-1) (CGAGTCA) and NF-κB (AGAGTTTCCC) binding sites. TNF-α effect was eliminated by a 2-bp substitution mutation in the NF-κB1 binding half site of the NF-κB *cis* element. Electrophoretic mobility shift assays (EMSA) showed that recombinant human NF-κB heterodimers as well as NF-κB1 and RelA homodimers, but not AP-1, were capable of binding this element. Further, EMSA with human fibroblast nuclear extracts demonstrated enhanced binding of a single, specific complex within 5 min of TNF-α stimulation, which reached a plateau by 1 h and was not affected by preincubation of cells with cycloheximide. Gel supershift assays identified the complex as the NF-κB (p50/p65) heterodimer, whereas Abs to nuclear factor of activated T cells (NF-AT) and Jun family members failed to recognize the complex. These data suggest that in fibroblasts TNF-α activates and initiates the nuclear translocation of NF-κB that binds a divergent NF-κB element and plays a critical role in the observed inhibition of α2(I) collagen gene transcription. ***The Journal of Immunology,*** **1999, 162: 4226–4234.**

Wound healing is characterized by an acute inflammatory reaction concomitant with extracellular matrix (ECM)[3] remodeling. The collagens are a key element in this process, and cytokines elaborated by local inflammatory cells significantly alter collagen gene expression. In general, TGF-β stimulates, whereas TNF-α inhibits, the transcription of collagens in a coordinated fashion within the wound bed. Recently, several attempts have been made to elucidate the mechanisms by which inflammatory cytokines, including TGF-β and TNF-α, regulate transcription of the human α2(I) collagen gene (COL1A2).

Initial observations demonstrated that a 135-bp region of the COL1A2 promoter within 330 bp of the transcription start site could confer responsiveness to both TGF-β (1) and TNF-α (2). This rather large region was shown to contain two smaller regions, the first referred to as box A, spanning nucleotides −313 to −286 and containing two distinct nuclear protein binding sites called 5A and 3A, and the other referred to as box B from nucleotides −271 to −255. The upstream element, box A, was shown to bind Sp1 and to confer high basal promoter activity (1, 3, 4). Box B harbors both a putative activator protein-1 (AP-1) binding element and a noncanonical NF-κB binding site (1, 2). Although no specific transcription factor could be identified by Inagaki et al. (1, 2), they postulated that unknown factors interacting with both box B and the upstream Sp1 binding sites present in box A were necessary for both TGF-β and TNF-α responses (1, 2). Recently, we have demonstrated that the region between nucleotides −271 and −235 is critical and sufficient for enhanced COL1A2 transcription upon TGF-β stimulation (4) and does not require the upstream Sp1 binding sites that only play a role in the basal activity of the promoter. Also, we showed that this short promoter segment, containing both AP-1 and NF-κB-like binding sites, permits antagonist activity of TNF-α against TGF-β, but the exact mechanisms and the responsive sequence elements have not been elucidated in further detail.

In this report, we have identified the specific TNF-α response element (TaRE) that allows COL1A2 transcriptional inhibitory response to TNF-α in human dermal fibroblasts. Specifically, the TaRE was found to reside between nucleotides −271 and −235 relative to the transcription initiation site, a region previously shown to confer TGF-β responsiveness. Electrophoretic mobility supershift assays (EMSA) identified NF-κB1 and RelA NF-κB family members as transcription factors binding the TaRE and mediating TNF-α repression of COL1A2 promoter

Departments of *Dermatology and Cutaneous Biology and †Biochemistry and Molecular Pharmacology, Jefferson Medical College, and ‡Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA 19107; and §Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea

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[2] Address correspondence and reprint requests to Dr. Alain Mauviel, Department of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 South 10th Street, BLSB Room 430, Philadelphia, PA 19107. E-mail address: alain.mauviel@mail.tju.edu

[3] Abbreviations used in this paper: ECM, extracellular matrix; TaRE, TNF-α response element; CAT, chloramphenicol acetyltransferase; NF-AT, nuclear factor of activated T cells; EMSA, electrophoretic mobility supershift assays; TK, thymidine kinase; WT, wild type; AP-1, activator protein-1; CTF-1, CCAAT-box binding transcription factor-1.

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activity. Precise nucleotide requirement for NF-κB binding to the TaRE was determined in nucleotide substitution experiments and clearly indicated that TNF-α and TGF-β responses involve closely located, but clearly distinct, elements within the −271/−235 segment of COL1A2 promoter. Furthermore, our results indicate that NF-κB, in the context of the proximal COL1A2 promoter, may act as a potent transcription inhibitor.

Materials and Methods

Cell cultures

Human dermal fibroblast cultures, established by explanting tissue specimens from neonatal foreskins, were used in passages 3–6. The cultures were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml of penicillin, and 50 μg/ml of streptomycin. Human recombinant TNF-α was purchased from Boehringer Mannheim (Indianapolis, IN).

Plasmid constructs

Transient transfection experiments were performed with several 5′ deletion constructs derived from pMS3.5CAT (kindly provided by Dr. Francesco Ramirez, Mt. Sinai School of Medicine, New York, NY), a plasmid containing ~3.5 kb of human COL1A2 promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene (5). Several additional deletion constructs were generated by PCR as described previously (4). The pRSV-β-galactosidase control vector was used as a standard for control of transfection efficiency (Promega, Madison, WI). pRSV-NF-κB1 (p50) and pRSV-RelA (p65) expression vectors were obtained through the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, National Institutes of Health) from Dr. Gary Nabel and Dr. Neil Perkins (6).

Site-directed mutagenesis of the TaRE

Point mutations were introduced into the putative AP-1 and NF-κB binding elements of construct −271COL1A2/CAT, containing the region −271 to +58 of the COL1A2 promoter cloned upstream of the CAT gene, using PCR with 5′-end/*Bam*HI overhang mutagenic primers containing two point mutations (bold) in the putative AP-1, NF-κB1, or RelA binding elements (AP-1, CGAGTCA → C**C**AGT**G**A; NF-κB1, AGAGTTTCC → A**TC**GTTTCC; RelA, AGAGTTTCC → AGAGTT**GA**C) and a 3′-end primer close to the transcription initiation site. The PCR products were digested with *Bam*HI/*Xma*I, purified on a 2% agarose gel, and cloned into similarly digested WT−271COL1A2/CAT, generating constructs containing mutated AP-1, NF-κB1 and RelA binding sites, −271 mAP1/CAT, −271 mK1/CAT, and −271 mK2/CAT, respectively. Also, the mK1 mutation was introduced into −376COL1A2/CAT, using the "Quick-Change" site-directed mutagenesis kit according to the manufacturer's protocol (Stratagene, La Jolla, CA). Sequence integrity of all constructs was verified by automated sequencing (Applied Biosystems, Foster City, CA).

Transient transfections and CAT assays

Transient transfections of human foreskin fibroblasts were performed by the calcium phosphate/DNA coprecipitation method, as described previously (7). Briefly, the cells were transfected with 10 μg of reporter DNA mixed with 5 μg of the RSV-β-galactosidase plasmid DNA to monitor transfection efficiencies. After glycerol shock, the cells were placed in DMEM containing 10% FCS for 2 h before the addition of TNF-α. After 40 h of incubation, the cells were rinsed twice with PBS, harvested by scraping, and lysed in reporter lysis buffer (Promega). The β-galactosidase activities were measured according to standard protocols (8). Aliquots corresponding to identical β-galactosidase activity were used for each CAT assay with [^{14}C]chloramphenicol as substrate (9) using thin layer chromatography. After autoradiography, the plates were cut and counted by liquid scintillation to quantify the acetylated [^{14}C]chloramphenicol. Percent acetylation was determined as the average quotient of acetylated products and total (unacetylated and acetylated chloramphenicol) ± SD.

EMSA

Nuclear extracts were prepared from human dermal fibroblast cultures according to the method of Andrews and Faller (10). The following oligonucleotides were used: a 37-bp double-stranded oligonucleotide corresponding to the −271 to −235 region of the COL1A2 promoter, wild-type (WT) TaRE, 5′-GAGGTATGCAGACAACGAGTCAGAGTTTCCCCT TGAA-3′; a similar 37-bp oligonucleotide in which two point mutations were made (bold), mK1 TaRE, 5′-GAGGTATGCAGACAACGAGT CA**TC**GTTTCCCCTTGAA-3′; a 20-bp oligonucleotide spanning the promoter region from residues −258 to −239, WT short TaRE, 5′-ACGAGT CAGAGTTTCCCCT-3′; and a similar oligonucleotide with two (bold) point mutations, mK1 short TaRE, 5′-ACGAGTCA**TC**GTTTCCCCT-3′. In some experiments, a 22-bp oligonucleotide containing the consensus collagenase AP-1 binding site (underlined), 5′-CTAGTGA<u>TGAGTCA</u>GC CGGATC-3′, or a 22-bp oligonucleotide containing a consensus NF-κB binding element (underlined) found in the Ig-κ light chain gene, 5′-GATC GAG<u>GGGACTTTCC</u>CTAGC-3′ (11) was used as either probe or unlabeled competitor. The oligonucleotides were end labeled with [γ-^{32}P]dATP and 4×10^4 cpm were incubated with 6 μg of nuclear extract for 1 h on ice in 20 μl binding reaction buffer (12 mM HEPES/KOH (pH 7.9), 4 mM Tris (pH 7.9), 60 mM KCl, 1 mM EDTA, and 12% glycerol) in the presence of 1 μg poly[dI-dC] (Boehringer Mannheim), as described previously (12). DNA/nucleoprotein complex specificity was determined by coincubation of nuclear extracts with unlabeled homologous or nonhomologous competitor DNA. For competition experiments, 60-fold molar excess of unlabeled oligonucleotide was included in the binding reaction. DNA-protein complexes were separated from unbound oligonucleotide on 4% acrylamide gel in 0.5× TBE. The gels were fixed for 30 min in 30% methanol and 10% acetic acid, vacuum dried, and exposed to x-ray film at −70°C. The following Abs were used in supershift experiments: rabbit polyclonal Abs against NF-κB1 and RelA (13), NF-κB2 (p52) (14), c-Rel (p75) (15), RelB (p68) (our unpublished data) raised against a synthetic 17-amino acid peptide covering the C terminus of human RelB, REAAFGGGLLPG PEAT, a pan-NF-AT (nuclear factor of activated T cells) Ab, raised against an internal peptide of human NF-AT common to all members of the NF-AT family (16), and a rabbit polyclonal anti-c-Jun (Santa Cruz Biotechnology, Santa Clara, CA). All NF-κB and NF-AT Abs were generous gifts from Dr. Nancy Rice (National Cancer Institute, Frederick, MD). Briefly, Abs were added to the nuclear extracts (0.1 μg polyclonal antiserum per 6 μg protein) and incubated on ice for 2 h before the binding reaction. Also, in some experiments, recombinant human NF-κB, NF-κB1, or RelA protein (100 ng/binding reaction; a generous gift of Dr. Timothy Coleman, Human Genome Sciences, Rockville, MD) was used with 1.0 μg of poly[dI-dC] and 10 μg of BSA as a carrier.

Results

Precise delineation of the TaRE within the human COL1A2 promoter

Previous work from our laboratory localized a TNF-α-response region within the COL1A2 promoter between residues −271 and −235 (4). However, because the major focus of that study was to investigate the regulatory regions involved in TGF-β up-regulation of COL1A2 gene expression, the experiments were performed in low serum conditions that kept collagen promoter activity at a low level, unfavorable for a precise evaluation of the inhibitory action of TNF-α. Therefore, to ascertain the localization of the TNF-α-responsive element within the COL1A2 promoter, we repeated the 5′ deletion studies by performing the transfection experiments in medium containing 10% FCS, which we previously showed to provide high basal promoter activity (4). All 5′ deletion constructs containing >271 bp of promoter demonstrate a significantly greater basal activity than the −271COL1A2/CAT construct, previously attributed to three Sp1 elements between −313 and −286 (3, 4). The loss of these elements upstream from nucleotide −271 predictably lowers basal activity (Fig. 1*A*). To maintain CAT activity for larger deletions within the linear range of the assay, small volumes of lysate were used. Therefore, activity of the −271 COL1A2/CAT promoter deletion is hardly detectable on the autoradiogram shown. However, for quantitation purposes, the samples from cells transfected with short constructs were assayed with more extract, providing accurate and reproducible determination of their activity.

Upstream deletion constructs between −3500 and −271 all had a similar inhibitory response to TNF-α challenge (~50–75% inhibition); however, the loss of elements between −271 and −235 entirely abolished the inhibitory response, and even a significant reversal of the effect of TNF-α was noted (Fig. 1). This stimulation

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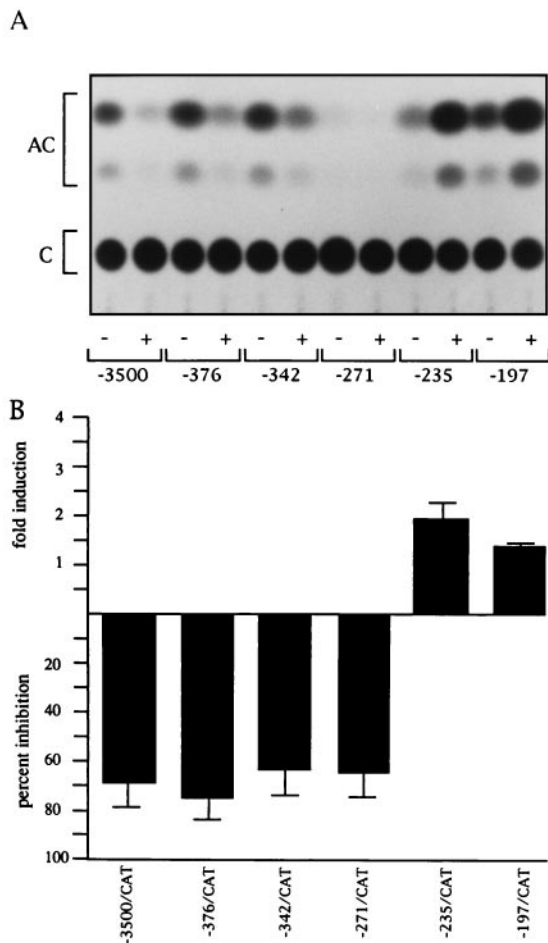

FIGURE 1. Effects of TNF-α on the activity of 5′ deletions of the COL1A2 promoter in transient cell transfection experiments. Confluent fibroblast cultures were transfected with various 5′ deletion/CAT constructs of the human COL1A2 promoter by the calcium phosphate/DNA coprecipitation procedure, as described in *Materials and Methods*. After glycerol shock, the cultures were incubated in fresh medium containing 10% FCS without (−) or with (+) 25 ng/ml TNF-α added to the medium 4 h later. After 40 h of incubation, cell extracts were assayed for CAT activity with [^{14}C]chloramphenicol as a substrate using identical amounts of protein. *A*, Representative autoradiogram of the CAT assay, depicting the separation of acetylated (AC) and unacetylated (C) forms of [^{14}C]chloramphenicol by a thin layer chromatography. *B*, Quantitation of CAT activity, expressed as the mean ± SD of 12 experiments using overlapping sets of 5′ deletion constructs.

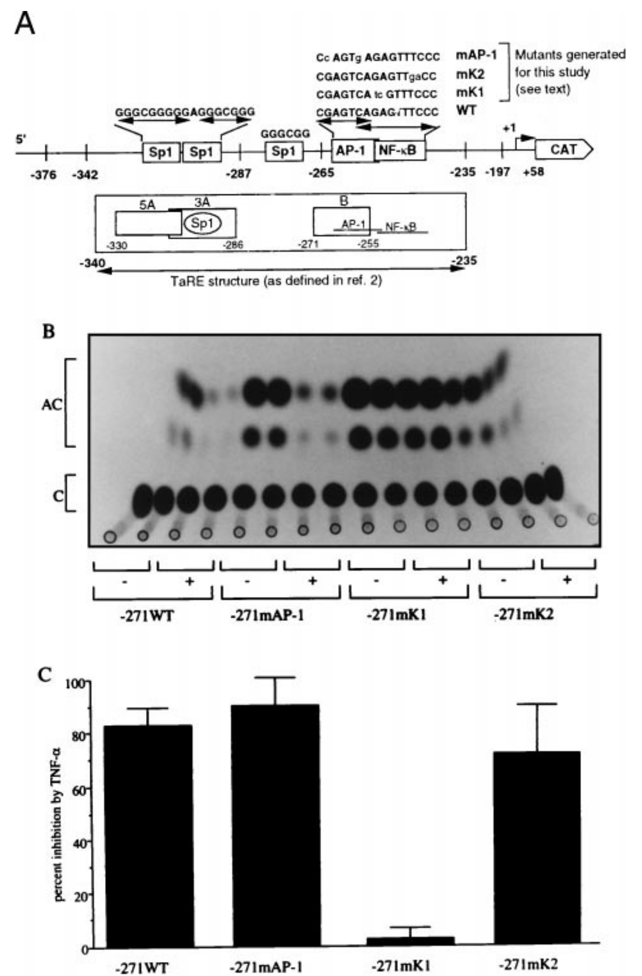

FIGURE 2. Effects of point mutations within putative *cis* elements between nucleotides −271 and −235 of the COL1A2 promoter. *A*, Schematic of the human COL1A2 promoter indicating the positions of the 5′ ends of the promoter/CAT reporter gene constructs used in transient cell transfection experiments. Mutations generated within the TNF-α responsive region as defined in Fig. 1 are shown above the corresponding putative AP-1, NF-κB1 (p50), or RelA (p65) elements, as described in *Materials and Methods*. The TaRE, as defined by Inagaki et al. (2), is shown below. *B*, The WT −271COL1A2/CAT was subjected to several substitution mutations (see *A*). The parent and mutated constructs were used in parallel transfections of fibroblasts. After glycerol shock, the cultures were incubated for 4 h in medium containing 10% FCS and incubated for another 40 h without (−) or with (+) 25 ng/ml TNF-α. Processing of samples was conducted as described in Fig. 1. The autoradiogram of a representative experiment is shown. *C*, The results of three independent experiments, each using duplicate samples quantitated as described in Fig. 1, are expressed as percent inhibition of promoter activity by TNF-α.

in response to TNF-α was maintained in further deletions to −197. However, further promoter deletions past −186 resulted in very low basal promoter activity accompanied with minor, statistically insignificant, inhibitory response to TNF-α (data not shown). These data narrow down the essential TaRE, originally described by Inagaki et al. (4) between residues −378 and −235 of the human COL1A2 promoter to a 36-bp fragment located between nucleotides −271 and −235.

Further characterization of the TaRE was accomplished by analyzing the effect of various point mutations within the promoter region from −271 to −235 (Fig. 2). In these experiments, CAT activity is significantly higher than that shown in Fig. 1*A* because correction was not relative to longer constructs with higher basal activity. Computer analysis of the sequences comprising this region identified two putative *cis* elements: an AP-1-like element and a noncanonical NF-κB binding site, adjacent and downstream of AP-1, as schematically represented in Fig. 2*A*. The TaRE was previously identified as spanning the region between −340 and −235 (2), and several boxes with regulatory functions were identified (2). We identified the AP-1 element as critical for the TGF-β response (4). The TaRE region also allows antagonism of the TGF-β effect by TNF-α (2); however, the exact mechanisms have not been elucidated before this study. Our results indicate that a double point mutation in the putative NF-κB1 binding site (mK1) completely abolishes the response to TNF-α (5% inhibition with the construct mK1/CAT vs 80% inhibition with WT −271COL1A2/CAT construct; Fig. 2, *B* and *C*). In contrast, mutations in both putative AP-1 and RelA binding regions (construct mAP-1 and mK2, respectively) had little if any effect on the inhibition of COL1A2 promoter activity by TNF-α, as compared with the WT−271COL1A2/CAT construct (Fig. 2, *B* and *C*).

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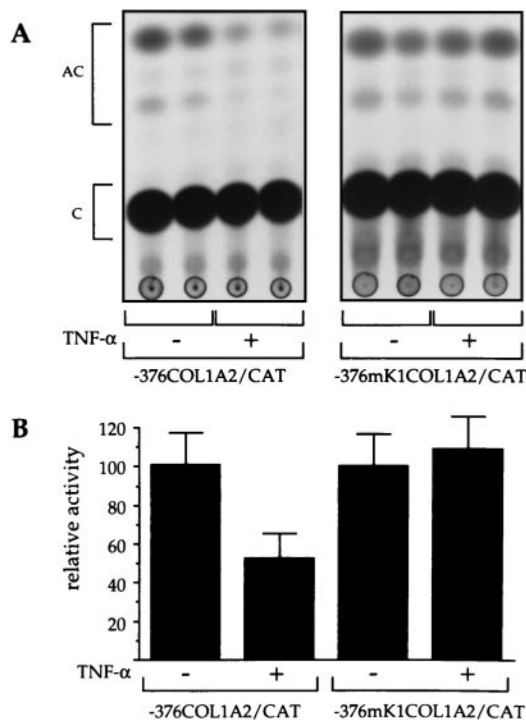

FIGURE 3. Effects of the mK1 mutation on −376COL1A2/CAT response to TNF-α. The −376COL1A2/CAT was subjected to the mK1 substitution mutation, shown to abolish TNF-α response of the −271COL1A2/CAT construct (see Fig. 2). The parent and mutated constructs were used in parallel transfections of fibroblasts. After glycerol shock, the cultures were incubated for 3 h in medium containing 10% FCS and incubated for another 40 h without (−) or with (+) 25 ng/ml TNF-α. Processing of samples was conducted as described in Fig. 1. The autoradiogram of a representative experiment is shown in *A*. The results of three independent experiments, each using duplicate samples quantitated as described in Fig. 1, are expressed as relative activity.

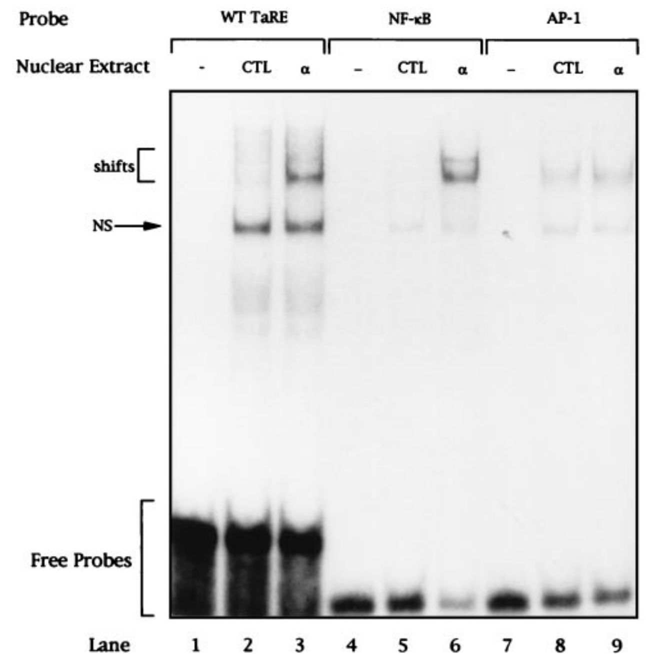

FIGURE 4. Binding of nuclear proteins to the TaRE mimics NF-κB, not AP-1. A 37-bp oligonucleotide spanning the region −271 to −235 of COL1A2 promoter (WT TaRE), as well as NF-κB and AP-1 consensus oligonucleotides were used as probes to study their ability to bind transcription factors. The nuclear extracts used were as follows: −, no protein; CTL, untreated confluent fibroblast cultures; and α, TNF-α-stimulated (24 h) dermal fibroblast cultures. Nonspecific shifts (NS) were differentiated from specific interactions by competition with unlabeled, homologous and nonhomologous competitor DNA (data not shown).

To verify that the element affected by the mK1 mutation was critical for TNF-α inhibitory effect, the −376COL1A2/CAT construct was mutated similarly, and its responsiveness to TNF-α was compared with that of the parent construct. Similar to the data presented above with construct −271, the mK1 mutation essentially abolished TNF-α inhibitory activity (Fig. 3). These results contrast significantly with mutational analysis of the TGF-β response element (4) that identified the AP-1 binding site as essential for TGF-β-induced up-regulation of COL1A2 promoter activity and indicate that the two cytokines use different, although very closely located, regulatory elements.

TNF-α induces nuclear proteins that bind both TaRE and NF-κB elements

Previous studies have documented that numerous cytokines, including TNF-α, can rapidly activate latent NF-κB in the cytoplasm, leading to its rapid nuclear translocation and subsequent gene activation (17). In addition, several members of the AP-1 gene family, including c-*jun*, *jun*-B and c-*fos*, are activated in fibroblasts by TNF-α (18). Therefore, given the putative *cis* elements recognized within the TNF-α responsive region, it was important to establish whether TNF-α activation of either AP-1 or NF-κB was involved as transcription factors binding to the TaRE. For this purpose, confluent dermal fibroblast cultures were incubated for 24 h with or without TNF-α, and EMSAs were performed to compare the binding of nuclear extracts to WT TaRE with their binding pattern to consensus NF-κB and AP-1 probes. As shown in Fig. 4, TNF-α dramatically enhanced the binding of nuclear proteins to both TaRE and NF-κB probes. The relative migration rate of the shifted TaRE complexes (Fig. 4, *lane 3*) was similar to that of NF-κB complexes (*lane 6*). No differential complex formation was observed with the consensus AP-1 probe. This lack of enhancement of AP-1 binding is likely to be due to the fact that AP-1 activation by TNF-α, and subsequent DNA binding activity, are a transient phenomenon that occurs within a few h after stimulation of the cells and can no longer be observed at the 24 h time point studied.

To test whether the response to TNF-α on the COL1A2 promoter is mediated through a similar rapid nuclear translocation as is the case of NF-κB, the time course of nuclear factor binding to the TaRE was examined in EMSA experiments. Appearance of the TNF-specific complex was observed as early as 5 min after TNF-α addition. A plateau was reached by 15 min and maintained for at least 24 h of stimulation (data not shown).

Activation of TaRE binding is independent of de novo protein synthesis

Although the appearance of TaRE-specific binding proteins in nuclear fractions was very rapid, we wanted to ascertain the involvement, if any, of de novo protein synthesis in this event. For this purpose, dermal fibroblast cultures were incubated with or without 100 μg/ml of cycloheximide, a concentration that effectively blocks fibroblast protein synthesis, for 1 h before TNF-α addition, and nuclear extracts were subsequently prepared. As shown in Fig. 5, and as expected from the above data, TNF-α alone induced strong nuclear protein binding to the TaRE as compared with that of unstimulated fibroblast extracts (Fig. 5, *lane 3* vs *lane 2*). Addition of cycloheximide alone had no effect on TaRE binding and

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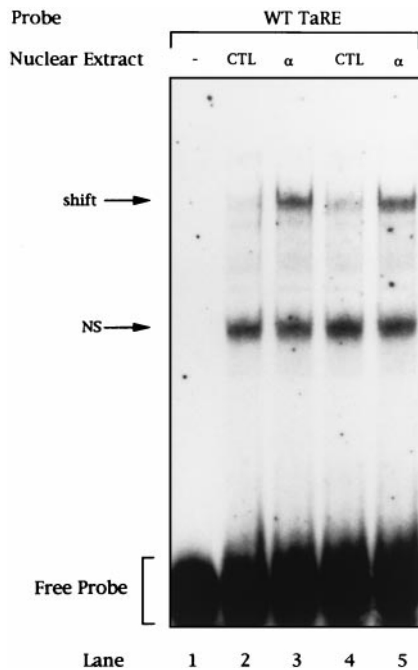

FIGURE 5. Activation of TaRE binding protein does not require de novo protein synthesis. Confluent dermal fibroblast cultures were preincubated with either vehicle (0.1% DMSO, *lanes 2* and *3*) or 100 μg/ml cycloheximide (*lanes 4* and *5*) for 1 h before stimulation with TNF-α for 1 h (*lanes 3* and *5*). The WT TaRE oligonucleotide was used as probe for EMSA. Specific retarded complexes induced by TNF-α (shift) are indicated. NS, nonspecific complexes.

did not alter TNF-α-induced binding (Fig. 5, *lanes 4* and *5*, respectively), indicating that de novo protein synthesis is not required for TNF-α effect.

Identification of the TaRE binding proteins

EMSA results presented in Fig. 4 demonstrated a strong correlation between TNF-α-induced TaRE binding protein and NF-κB binding to its consensus sequence. Kinetic studies also correlate strongly with the rapid induction of NF-κB by TNF-α (19, 20). To determine whether a complex of NF-κB family members was, in fact, the TaRE binding protein, EMSAs were performed to demonstrate complex specificity through comparative competitions (Fig. 6*A*). Nuclear extracts were incubated without (Fig. 6*A*, *lanes 1–3*) or with (*lanes 4–7*) unlabeled competitor oligonucleotides. A 20- and 60-fold molar excess of homologous competitor DNA strongly reduced the TNF-α-induced complex (Fig. 6*A*, *lane 3*) in a dose-dependent manner (*lanes 4* and *5* vs *lane 3*). However, competition with 60-fold molar excess of unlabeled AP-1 oligonucleotide failed to eliminate complex formation (*lane 6*). In contrast, 60-fold molar excess of NF-κB oligonucleotide competed successfully for the shifted complex (*lane 7*), indicating that complexes that bind the TaRE also recognize the consensus NF-κB site.

To further investigate the possibility that NF-κB may indeed be the TaRE binding factor and to characterize the possible Rel subunit combinations that could comprise this factor, we used Abs directed against five of the known Rel/NF-κB family members: NF-κB1, NF-κB2, RelA, RelB, and c-*rel* in gel supershift assays. Also, antisera cross-reactive with either all NF-AT family members and against c-Jun were included in the experiments. Results are shown in Fig. 6*B*. Notably, only Abs against either NF-κB1 and RelA recognized the complex (Fig. 6*B*, *lanes 4* and *6*, respectively), whereas no supershift was observed with Abs directed against the other members of the Rel family (*lanes 5*, *7*, and *8*) or NF-AT (*lane 9*) or c-Jun (*lane 10*). These results suggest that the protein binding the TaRE in the COL1A2 promoter is the NF-κB heterodimer.

To determine whether the Rel family members binding to the TaRE were representative of all Rel members induced by TNF-α, or instead, represent a COL1A2 promoter TaRE-specific subset, the NF-κB element of the Ig-κ light chain enhancer was used as a probe in similar gel supershift assays (Fig. 6*C*). The Rel heterodimer complex binding the Ig-κ NF-κB consensus sequence upon TNF-α stimulation was supershifted by NF-κB1 and RelA Abs only (Fig. 6*C*, *lanes 4* and *6*, respectively), whereas antisera directed to NF-κB2, RelB, and c-Rel failed to recognize the complex (*lanes 5, 7*, and *8*, respectively). These data demonstrate that sequence variation between the NF-κB binding sites of the two genes, COL1A2 and Ig-κ light chain, does not lead to differential binding of Rel subunits.

Mutations within the TaRE eliminate its ability to bind NF-κB

Our next step consisted of establishing the causal relationship between NF-κB binding to the TaRE and the inhibition of COL1A2 promoter activity by TNF-α. For this purpose, the binding ability of a 37-bp oligonucleotide, mK1 TaRE, containing the same mutation as the one generated in the −271 mK1/CAT construct (rendering it unresponsive to TNF-α; see above, Fig. 2), to baculovirus-produced recombinant NF-κB1 and RelA, was compared with that of the WT TaRE probe. As predicted, the TaRE was able to bind both NF-κB1 and RelA homodimers, as well as NF-κB heterodimers (Fig. 7, *lanes 3–5*). It should be noted that RelA homodimers (*) were partially degraded and lacked a portion of their C terminus region. Although it had no effect on their binding ability, it resulted in a faster migration rate than that of NF-κB heterodimers in EMSA (21). Mutation of the TaRE probe in the NF-κB1 binding half site (mK1 TaRE) totally eliminated recombinant NF-κB/DNA complex formation (Fig. 7, *lanes 8–10*), in agreement with results of transient transfection experiments in which similarly mutated COL1A2 promoter constructs, −376 mK1/CAT and −271 mK1/CAT, showed no TNF-α responsiveness (see Figs. 2 and 3). In addition, mutations in the RelA half-site (mK2 TaRE) had no effect on recombinant NF-κB binding (data not shown).

Subsequently, the ability of nuclear extracts from dermal fibroblast cultures, either unstimulated or stimulated with TNF-α for 24 h, to bind the mutated TaRE probes was examined and compared with binding the WT TaRE (Fig. 8). The differential complex formation between unstimulated and stimulated nuclear extracts observed with the WT TaRE probe (Fig. 8, *lane 3 vs. lane 2*) was eliminated by the 2-bp mutation generated in the mK1 probe (*lanes 5* and *6* vs *lanes 2* and *3*). In contrast, the binding pattern observed with the mK2 probe was similar to that observed with the WT TaRE probe (Fig. 8, *lanes 8* and *9* vs *lanes 2* and *3*). Taken together, these results attest to the specificity of the causal relationship between NF-κB binding to the TaRE and TNF-α inhibitory response of the COL1A2 promoter. Also, the ability of point mutations within the NF-κB1 binding region to abrogate not only NF-κB heterodimer but also recombinant RelA homodimer complex formation underscores the strong binding affinity of this half of the *cis* element and further emphasizes the key role of the NF-κB1 site relative to the RelA binding half site in mediating TNF-α effect. These data corroborate the results from transfection experiments in which both the −271 mK2/CAT and −271WT/CAT constructs responded equally to TNF-α, whereas constructs harboring the mK1 mutation were not responsive (see Figs. 2 and 3).

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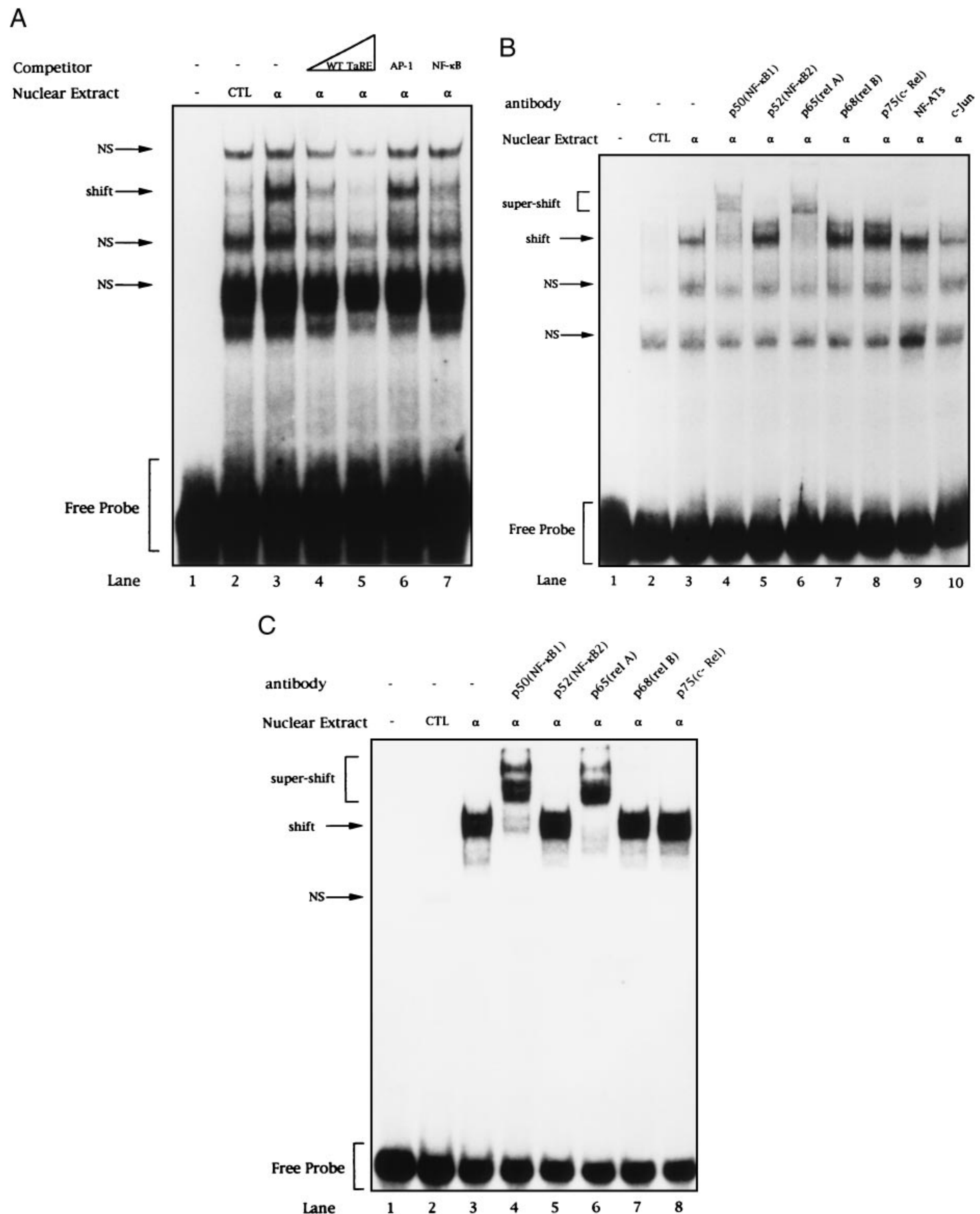

FIGURE 6. NF-κB1 (p50) and RelA (p65) NF-κB family members bind the TaRE. *A*, EMSAs were performed using the WT TaRE probe. −, Probe alone; CTL, extracts from control cultures; and α, extracts from TNF-α-treated fibroblast cultures. Competitions were conducted with 20- and 60-fold molar excess of unlabeled homologous competitor oligonucleotide (*lanes 4* and *5*) or 60-fold molar excess of either AP-1 (*lane 6*) or NF-κB (*lane 7*) consensus oligonucleotides, using nuclear extracts from TNF-α-treated fibroblast cultures. *B*, Supershift experiments were performed with Abs specific for Rel family members (*lanes 4–8*), an Ab cross-reactive with all NF-AT family members (*lane 9*), and an Ab directed against c-Jun (*lane 10*), using WT TaRE as a probe. Shifted NF-κB (p50/p65) is indicated as well as DNA/transcription factor/Ab complexes (supershift) and nonspecific complexes (NS). *C*, EMSA conditions were the same as in *B*, using a consensus NF-κB oligonucleotide as a probe. Note that the faint band between the TNF-α-induced complex and the closest NS band is not consistently present in our experiments and is not likely to represent a TNF-α-regulated complex.

Together, these data indicate that the COL1A2 promoter region between nucleotides −271 and −235 contains two distinct growth factor response elements, one within the upstream AP-1 site and essential for TGF-β effect (4) and the other one slightly downstream within the NF-κB-like site and responsible for TNF-α inhibitory effect.

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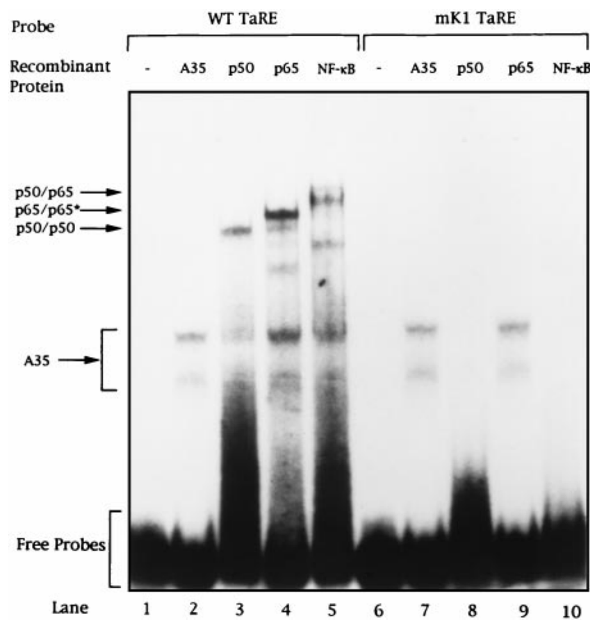

FIGURE 7. The COL1A2 TaRE binds dimers of NF-κB1 and RelA. WT TaRE (*lanes 1–5*) and mK1 TaRE (*lanes 6–10*) were used as probes in EMSAs and incubated with either no protein (*lanes 1* and *6*), cell extract A35 from mock-transfected cells (*lanes 2* and *7*), or equivalent molar amounts of Baculovirus-produced human recombinant NF-κB1/NF-κB1 (*lanes 3* and *8*), RelA/RelA (*lanes 4* and *9*), or NF-κB (*lanes 5* and *10*). Note that RelA homodimer (*) complexes migrate faster than NF-κB heterodimers, a phenomenon due to partial degradation of the RelA protein in the preparation (see explanations in *Results*).

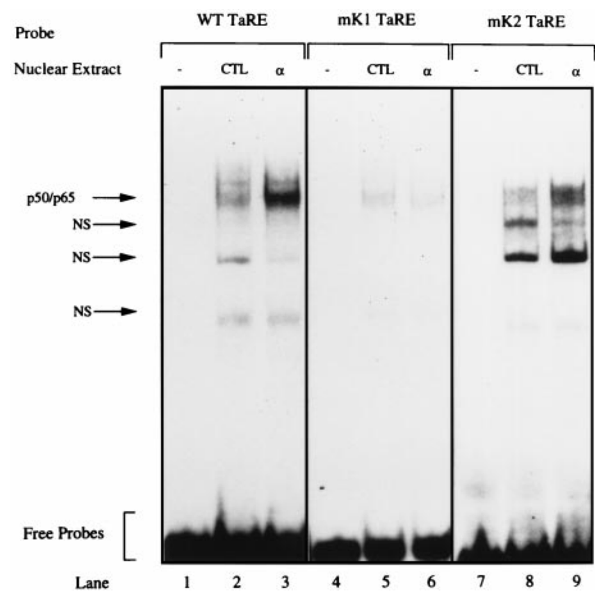

FIGURE 8. Point mutations within the NF-κB1 (p50) binding site of the TaRE abolish NF-κB binding. WT TaRE oligonucleotide (*lanes 1–3*), mK1 TaRE (*lanes 4–6*), and mK2 TaRE (*lanes 7–9*) probes were incubated with either no protein (-), nuclear extracts from untreated fibroblast cultures (CTL), or nuclear extracts from fibroblast cultures incubated for 24 h with TNF-α (α) before EMSA. NF-κB (p50/p65) shift is indicated. NS, nonspecific complexes. Note the absence of NF-κB binding with the mK1 mutated probe, whereas it is present with both WT and mK2 probes.

Overexpression of NF-κB inhibits COL1A2 promoter activity

To establish a functional link between RelA binding to the COL1A2 TaRE and the inhibitory effect of TNF-α on COL1A2 promoter activity, cotransfection experiments were performed in which NF-κB1 and RelA expression vectors were transfected together with several COL1A2 promoter CAT constructs. As shown in Fig. 9, NF-κB overexpression significantly inhibited the activity of both −342COL1A2/CAT and −271COL1A2/CAT constructs. In contrast, the activity of the −235COL1A2/CAT construct was not altered. Similar results were obtained when RelA alone was cotransfected with the CAT constructs (data not shown), indicating that the inhibitory effect of RelA, is exerted through the NF-κB binding element identified as the TaRE and located between nucleotides −271 and −235.

Discussion

Inflammatory cytokines play a critical role in promoting connective tissue remodeling in physiologic and pathologic conditions. Through their ability to modulate the expression of ECM components and ECM degrading enzymes, cytokines such as TNF-α, TGF-β, and IFN-γ orchestrate the balance between ECM destruction and regeneration, and therefore, play an important role in reparative processes as well as in the pathogenesis of fibrotic diseases (22).

Several studies have suggested that TNF-α depresses type I collagen gene expression, acting predominantly at the level of transcription (23–25). In more recent work using footprinting analysis of the COL1A2 promoter, responsiveness to TNF-α was localized to a region between −378 and −235 relative to the transcriptional start site (2). Two protected regions of promoter within this 143-bp element were identified: box A and box B. When placed together in the context of a heterologous promoter, these two boxes conferred responsiveness to TNF-α (2). However, the discrete sequences and the transcription factors involved in TNF-α response were not fully characterized. Indeed, an Ab directed against RelA obtained from Santa Cruz Biotechnology failed to supershift or abolish the TNF-α-induced complex that they observed in EMSA with probes spanning the entire region from −340 to −235 or box 5A (see Fig. 2*A*) in which the authors identified a potential NF-κB binding site (2). The 3′-end of the TaRE that contains the functional NF-κB site identified in our study was not investigated in further detail by these authors. Based on their results, Inagaki et al. (2) excluded NF-κB from being part of the transcription complex involved in the regulation of COL1A2 promoter activity by TNF-α.

Recently, we have shown that regions downstream of nucleotide −271 are sufficient for antagonist activity of TNF-α against TGF-β-mediated up-regulation of COL1A2 promoter activity (4). In the present study, we have expanded upon these results by demonstrating that regions upstream of nucleotide −271 are not required for TNF-α-mediated transcriptional repression of COL1A2 promoter activity, and we have demonstrated that the TaRE is indeed restricted to a short segment of promoter between nucleotides −271 and −235. Further, we have identified two nucleotides critical for transcription factor binding to the TaRE, and we have shown that NF-κB is the TaRE binding protein complex that mediates TNF-α inhibitory effect, as evidenced by the fact that mutations, which abolish NF-κB binding to the TaRE, also prevent TNF-α-mediated repression of COL1A2 promoter activity. These data exclude the involvement of upstream Sp1 binding sites between residues −286 and −330 in growth factor regulation of COL1A2 promoter activity as previously believed (1, 2) but are in total agreement with the previous demonstration of the fundamental role of these sites for high COL1A2 promoter basal activity (3, 4).

It should be noted that COL1A2 promoter response to TGF-β requires the AP-1 site slightly upstream of the TaRE described in

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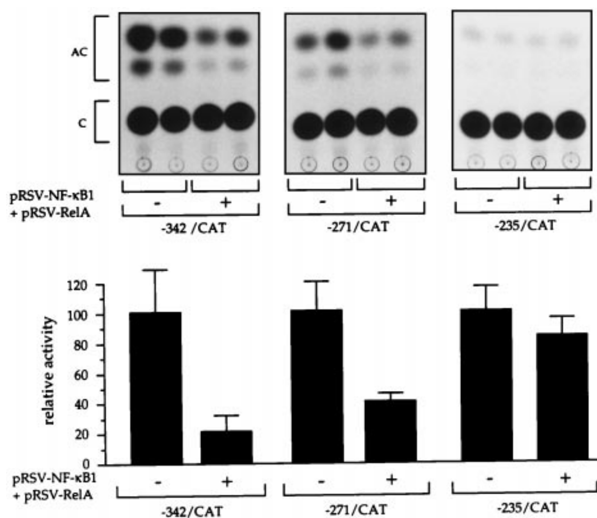

FIGURE 9. Effect of NF-κB overexpression on the activity of various COL1A2 promoter/CAT constructs in transient cell transfection experiments. Confluent fibroblast cultures were transfected with various 5′ deletion/CAT constructs of the human COL1A2 promoter by the calcium phosphate/DNA coprecipitation procedure, together with pRSV-NF-κB1 and pRSV-RelA expression vectors or the corresponding pRSVe empty vector. After glycerol shock, the cultures were incubated in fresh medium containing 10% FCS for 40 h, cell extracts were assayed for CAT activity with [^{14}C]chloramphenicol as a substrate using identical amounts of protein. The figure shows a representative autoradiogram of the CAT assay, depicting the separation of acetylated (AC) and unacetylated (C) forms of [^{14}C]chloramphenicol by thin layer chromatography and quantitation of CAT activity, expressed as the mean ± SD of three separate experiments each using duplicate samples.

this study (4). These two growth factor response elements bind different transcription factors, and mutations in either binding site only prevent the effect of the corresponding cytokine. The close proximity and partial physical overlap of these sites suggest that the antagonist activity of TNF-α and TGF-β may be the result of a steric interaction between the respective transcription factors. Specifically, it is conceivable that a protein complex bound to either site will prevent binding of transcription factor(s) to the adjacent site, or it will be displaced depending on their respective binding affinity.

The mechanism described above differs significantly from the recently described antagonism between TGF-β and TNF-α on NF-1/CTF-1-mediated transcription (26). Specifically, in the latter case, a single element, CTF-1, is the molecular target for mutually antagonistic regulation by TGF-β and TNF-α. CTF-1/NF-1 is involved in the activation of murine COL1A2 promoter by TGF-β (27). However, no data are currently available to support the possibility that TNF-α may exert an antagonistic activity through this element in the case of the murine COL1A2 promoter. Regarding the human COL1A2 promoter, we and others have shown that CTF-1 is not involved in either TGF-β or TNF-α regulation. Instead, Sp1 and AP-1 transcription factors, as well as NF-κB, have been implicated (1–4). In the present study, we have demonstrated the fundamental role played by NF-κB in TNF-α down-regulation of the human COL1A2 promoter. It is tempting to speculate that the antagonism between TGF-β and TNF-α in the context of the human COL1A2 promoter may result from steric interactions between NF-κB and AP-1 proteins binding the corresponding *cis* element immediately upstream of the NF-κB binding site (4).

As described in other NF-κB elements, discrete variations within the κB element are critical in defining NF-κB binding specificity (13, 28). Indeed, a double mutation within the NF-κB1 binding half site of the COL1A2 TaRE *cis* element abrogated both NF-κB complex formation and TNF-α responsiveness, whereas mutation of the downstream or RelA binding half site had little effect on either of these parameters. These findings are not surprising considering the roles played by each subunit in transcriptional regulation and their relative binding affinities to the NF-κB element. Whereas RelA confers transcriptional modulation of the basal promoter machinery, NF-κB1 serves predominantly as a DNA binding anchor for the heterodimer to its cognate DNA element (29–32). These functional differences are reflected in the relative binding affinities of the NF-κB1 and RelA homodimer complexes to the Ig-κ enhancer element (K_d NF-κB1/NF-κB1 = 0.9×10^{-12} M; K_d RelA/RelA = 32.2×10^{-12} M). These differences in K_d explain the relative insensitivity of NF-κB heterodimer binding to sites mutated in their lower affinity RelA half, as shown for the Ig-κ element (33), or the COL1A2 TaRE (present study).

Although NF-κB sites are generally considered enhancer elements, we have found that NF-κB heterodimer binding to the COL1A2 TaRE is responsible for TNF-α-mediated repression of COL1A2 promoter activity. In fact, we have shown that NF-κB overexpression significantly reduces the activity of both −342 and −271COL1A2/CAT constructs, whereas a shorter construct lacking the NF-κB binding site was unresponsive (see Fig. 9). Repression of gene transcription by Rel proteins has been previously described in other systems, and additional levels of control may be imposed upon their ability to transactivate gene transcription (34–40). For example, Rel subunits can pair outside their family with other transcription factors through the Rel homology domain, subsequently modulating transcriptional activity (34). Further, the interaction of activated NF-κB complexes with other nucleoplasmic DNA binding factors as well as nuclear matrix proteins has been shown to modulate the binding characteristics of NF-κB, hence its transactivation properties (35). In the case of the *Drosophila* protein, Dorsal, a member of the Rel family, a high mobility group (HMG)-like protein termed dorsal switch protein (DSP1) can, through its interaction with a negative regulatory element flanking the IFN-β NF-κB element, convert Dorsal from transcriptional activator to repressor (36, 37). DSP1 could also exert the same effect on human NF-κB, suggesting that a mammalian counterpart to DSP1 may exist (38, 39). Together, these data indicate that in the presence of different nuclear factors, NF-κB may act positively or negatively on transcription. This theory is indeed demonstrated in mice inactivated for c-*rel*, in which Rel can act either as a transcriptional activator or as a repressor of TNF-α, IL-6, granulocyte-macrophage CSF, and granulocyte CSF genes in different mature macrophage populations (40). In the present study, although direct evidence for a functional inhibitory role of RelA was demonstrated, it cannot totally be excluded that other factors, including c-*rel*, may be involved in this phenomenon.

In summary, we have clarified the COL1A2 promoter TNF-α-responsive element as a short region immediately downstream and independent from three tandem Sp1 elements that regulate COL1A2 basal activity but are not involved in growth factor modulation. Within this TaRE, a solitary NF-κB site rapidly binds NF-κB in response to TNF-α challenge, resulting in a pronounced down-regulation of α2(I) collagen gene transcription.

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