NF-κB–like Factors Mediate Interleukin 1 Induction of c-*myc* Gene Transcription in Fibroblasts

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Summary

Interleukin 1 (IL-1) is a pluripotent cytokine involved in mediating a variety of physiological processes, including induction of cell proliferation upon wound healing. Treatment of quiescent FS-4 human dermal fibroblast cells with IL-1 activates c-*myc* gene transcription, and nuclear localization of NF-κB. Previously, we have noted that the murine c-*myc* gene contains two functional NF-κB sites located at $^{-}$1101 to $^{-}$1081 bp (upstream regulatory element [URE]) and $^{+}$440 to $^{+}$459 bp (internal regulatory element [IRE]) relative to the P1 promoter. Here we have demonstrated that IL-1 treatment induced binding of NF-κB–like proteins (p50/p65) to these c-*myc* elements. Heterologous promoter-CAT constructs driven by multiple copies of either the URE or IRE were IL-1 inducible when transfected into FS-4 cells. In contrast, constructs harboring elements with two G to C residue conversions, such that they were no longer able to bind NF-κB, were not responsive to IL-1. Mutation of these two base pairs at both NF-κB sites within a c-*myc* promoter/exon I-CAT construct, resulted in loss of inducibility with IL-1 upon transfection into quiescent FS-4 cells. Thus, IL-1 significantly induces c-*myc* expression through positive regulation by NF-κB, suggesting a role for this family of factors in activation of proliferation associated with wound healing.

Increasing evidence indicates that the cytokine family of factors plays a major role in a number of processes involved in host defense mechanisms, tissue remodeling, tumor growth/suppression, and cell proliferation. The action of various cytokines appears cell specific and modulated by the presence of other cytokines or growth factors. One such factor, IL-1, originally designated lymphocyte-activating factor, has been shown to exert diverse biological functions, including wound healing, immune stimulation, cytocidal activity, endogenous pyrogen production, cartilage resorption, and mitogenic stimulation of diverse tissues and cells (1). For example, IL-1 induces proliferation of quiescent cells such as FS-4 human dermal fibroblasts in culture. This growth activation is believed to be a model for the in vivo effects of IL-1 during wound healing (2).

IL-1 has been shown to induce expression of other lymphokines and their receptors, such as IL-2, IL-2 receptor (IL-2R), *gro* and IL-6, the acute phase protein serum amyloid A, and several nuclear protooncogenes such as c-*fos*, c-*jun*, and c-*myc* (2–9). Although some of the activity of IL-1 can be attributed to increased metabolism of arachadonic acid, most of its effects appear to be mediated by activation of factors that control transcription. Thus, in addition to induction of the nuclear protooncogenes, which mediate transcriptional control, activation of the transcription factor NF-κB and related family members has been observed.

Activation of NF-κB has been implicated in the induction by IL-1 of several genes, including IL-2, IL-6, and *gro* (4, 7, 10). NF-κB, originally described as a B cell–specific factor required for transcription of the κ L chain gene (11), has been shown to be a ubiquitous factor capable of stimulating transcription of a variety of genes (10). It appears to function as a dimer composed of 50- and 65-kD subunits, that are highly conserved along species lines (12–15). Binding can involve a homodimer of two 50-kD subunits or a heterodimer composed of a 50- and a 65-kD subunit (12). In mature B cells, NF-κB demonstrates constitutive nuclear activity (11, 16), whereas in other cell types it is sequestered in an inactive form in the cytoplasm, complexed with an inhibitor protein, termed IκB (17). Induction of NF-κB occurs through release from IκB and translocation to the nucleus.

The c-*myc* oncogene has proven to play an integral role in control of cell growth and differentiation (18). A transient induction of the low levels of c-*myc* mRNA follows growth activation of virtually all quiescent untransformed cells examined either in vitro or in vivo (18, 19). This increase in expression is required for cells to enter S phase. Maintenance of a constant elevated level of c-*myc* mRNA is observed in

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cycling cells. A major site of control of these changes in expression is mediated at the level of transcription of the *c-myc* gene (20, 21).

Recent evidence from this laboratory indicates that the murine c-*myc* oncogene contains two binding sites for NF-κB–like factors. One site lies 1101–1081 bp upstream of the P1 promoter, and is thus termed the upstream regulatory element (URE)[1] (22). The second site is found at $^{+}440$–$^{+}459$ bp within the noncoding portion of the first exon, and is thus termed the internal regulatory element (IRE) (23). These sites are functional in mouse and human cells, as judged by binding and transfection analysis (22, 23). To test the role of NF-κB in the induction of c-*myc* gene transcription via IL-1, we have employed the FS-4 dermal fibroblast cells, discussed above. Here we report that the IL-1–induced stimulation of quiescent FS-4 fibroblasts results in a rapid activation of specific binding of NF-κB–like factors to both the URE and IRE, and that this binding appears to play a direct role in mediating the increase in c-*myc* gene transcription.

Materials and Methods

Cell Culture and Transfection Conditions. Human dermal diploid fibroblast FS-4 cells were kindly provided by J. Vilcek (New York University Medical Center, NY) (2). Cells were maintained in DMEM supplemented with glucose/glutamine, penicillin-streptomycin, nonessential amino acids, and 10% fetal bovine serum (FBS) (DMEM-10% FBS), as described (2). Cells were used at passage 14 and 15 for all experiments.

Transfections were performed by plating 4×10^5 cells in DMEM-10% FBS in 60-cm dishes 24 h before transfection. Cultures were transfected overnight with plasmid DNA (10 μg) by a modified calcium-phosphate method (24), washed in PBS, and replated in DMEM-0.25% FBS for 50 h. After stimulation with 0.27 ng/ml recombinant human IL-1α (generously provided by J. Sipe, Boston University Medical Center, Boston, MA) for 4 h, cells were harvested, lysates prepared, and protein concentration determined with the protein assay reagent (Bio-Rad Laboratories, Richmond, CA) (25). Samples containing equal amounts of proteins were assayed for chloramphenicol acetyltransferase (CAT) activity essentially as described previously (22).

Nuclear Extract Preparation and Gel Shift Analysis. Cells (5×10^6) were plated in 100-cm dishes, allowed to grow to confluence over 5 d, and then made quiescent as above by incubation for 50 h in DMEM-0.25% FBS. Nuclei were either isolated directly or after treatment with IL-1 for 30 min by a modification of the protocol of Strauss and Varshavsky (26). Confluent cells were scraped from dishes, washed in PBS, and incubated in 400 μl of modified Buffer A (10 mM Hepes, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, 10 mg/ml leupeptin) for 15 min at 4°C. Cells were lysed by addition of 25 μl of 0.1% NP-40. Nuclear pellets were extracted in two packed nuclear volumes of 0.35 M NaCl, 5 mM EDTA, 1 mM dithiothreitol, 10 mM Hepes, pH 7.5, 1 mM PMSF, and 10 mg/ml leupeptin for 30 min with intermittent gentle stirring. Nuclei were pelleted and the supernatants were adjusted to 15% glycerol and frozen at −80°C. Mobility shift analysis was performed as previously described (22), except that 3 μg/ml poly(dI-dC) was used as a nonspecific competitor, and 5% polyacrylamide gels were used to separate protein bound versus free DNA. Competitions were performed with 100-fold molar excess of the stated unlabeled oligonucleotide.

Oligonucleotides and Constructs. The oligonucleotides used were as follows: IRE wild-type: 5′-GGCTCCGGGGAGGGAATTTTTGT-3′ (23); URE wild-type: 5′-AAGTCCGGGTTTTCCCCAACC-3′ (22); IL-2 R NF-κB wild-type: 5′-GATCCGGCAGGGGAATCTCC CT-3′ (27); IRE mutant: 5′-GGCTCCGGGGAGCCAATTTTT GT-3′ (23); URE mutant: 5′-AAGTCCGCCTTTTCCCCAACC-3′ (22); and CK: 5′-GGATCCTCATCTTTTAAAAATAACTTTTCAA-AAGGATCC-3′ (25). The core sequence of NF-κB elements are underlined. Oligonucleotides corresponding to the wild-type IRE, URE, and mutant IRE and URE sequences (Amber, Inc., Guilford, CT) were synthesized with directional BamHI linkers for end labeling and cloning. Multiple copies of either the IRE or URE were cloned into the BamHI site of the thymidine kinase (TK) promoter CAT vector (28), as described previously (22, 23). The p1.6 Bgl and p1.2 Acc c-*myc*-CAT constructs span $^{-}1141$ to $^{+}513$ bp and $^{-}615$ to $^{+}513$ bp, respectively, of genomic murine *myc* DNA linked to the CAT gene. Whereas p1.6 Bgl c-*myc*-CAT includes both the URE and IRE elements, p1.2 Acc c-*myc*-CAT construct contains only the IRE. These were kindly supplied by John Cleveland (St. Jude Children's Research Hospital, Memphis, TN). The double oligonucleotide primer method (29) of site-directed mutagenesis was used to introduce the two G to C transversions, illustrated above, at the IRE and URE sites within p1.6 Bgl c-*myc*-CAT.

UV Crosslinking. Nuclear proteins were covalently crosslinked to 5-bromodeoxyuridine (BrdU)-substituted oligonucleotides by UV irradiation. For preparation of BrdU-substituted DNA, the opposite strand of the IRE oligonucleotide was synthesized with two additional encoded nucleotides (TG) to enhance annealing with the primer 5′-CAGGCTCCGG-3′. The Klenow fragment of DNA polymerase I was used to extend the primer in the presence of BrdU and ^{32}P-dCTP and dGTP. After purification over a G50 Sephadex column, protein-DNA binding reactions were performed as above except that 5 ng of ^{32}P-labeled BrdU-substituted oligonucleotides were reacted with 20 μg nuclear protein in a total volume of 50 μl. After binding, the mixtures were chilled to 4°C, and then irradiated with 305 nm UV at a distance of 5 cm for 45 min. Laemmli (2×) buffer was added to each reaction, the mixture boiled for 3 min, and protein–oligonucleotide complexes resolved on a denaturing 7.5% polyacrylamide gel. For competition assays, a 100-fold molar excess of double-stranded oligonucleotide was included in the binding reaction. The protein molecular weight markers used were: myosin (200 kD), BSA, (66 kD), OVA (45 kD), and carbonic anhydrase (29 kD).

Results

Effects of IL-1 on Binding to the URE and IRE Elements. To determine whether IL-1 treatment induces binding to the NF-κB–like elements in the c-*myc* locus, mobility shift analysis was performed. A 30-min period of IL-1 treatment was selected since Lin and Vilcek had previously shown that c-*myc* mRNA induction was detected by this time (2). Binding of nuclear extracts from serum-starved FS-4 control cells to ^{32}P-labeled IRE and URE oligonucleotides revealed a single band. IL-1 treatment resulted in a change in the pattern, marked by an increase in intensity of a complex comigrating with the constitutive band and the appearance of a higher molecular weight complex (Fig. 1). This latter complex comigrates with complexes formed with nuclear extracts from the murine WEHI

[1] *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CAT, chloramphenicol acetyl transferase; FBS, fetal bovine serum; IRE, internal regulatory element; TK, thymidine kinase; URE, upstream regulatory element.

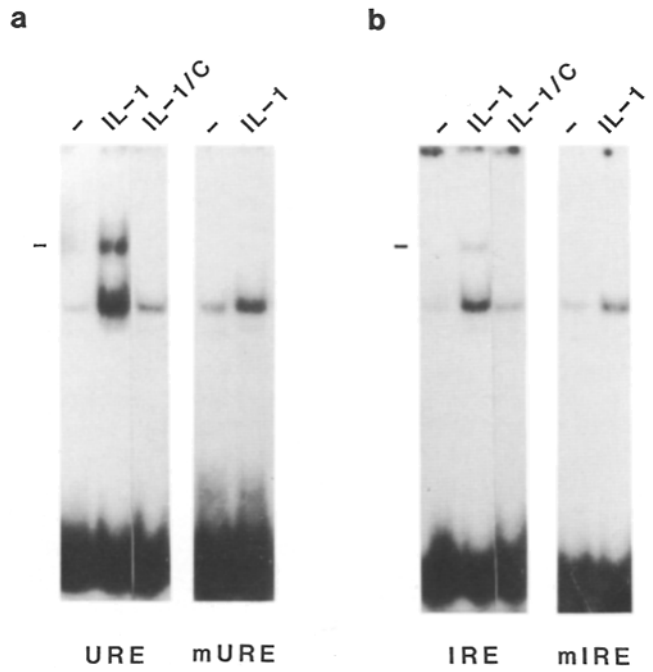

Figure 1. Induction of binding to the URE and IRE elements after IL-1 treatment. Extracts were prepared from serum-deprived untreated (−) FS-4 cells or after 30 min of treatment with IL-1 (*IL1*) and used in mobility shift analysis with wild-type or mutant (*m*) URE or IRE ^{32}P-labeled oligonucleotides. Binding competition was performed with excess unlabeled NF-κB oligonucleotide from the IL-2R promoter using extracts from IL-1 treated cells (*IL1/C*). (*Dash*) Position of induced higher molecular complex. (*a*) URE; (*b*) IRE.

231 B cell lymphoma line (data not shown), which contains NF-κB protein (11, 22). Addition of 100-fold molar excess unlabeled oligonucleotide corresponding to the human IL-2 receptor gene promoter NF-κB element reduced formation of complexes induced by IL-1 treatment (Fig. 1). Similarly, the murine κ chain NF-κB oligonucleotide itself, as well as IRE and URE oligonucleotides, competed in the same manner, whereas an oligonucleotide encoding an unrelated binding sequence (CK) from the myosin L chain gene did not alter the binding profile (data not shown). Methylation interference analysis of the IRE indicated the importance of the two internal G residues for binding of IL-1 induced proteins (data not shown), consistent with results with the WEHI 231 line (23). Mutant IRE or URE oligonucleotides, with two base pair transversions of G to C residues, show no induction of the upper complex in mobility shift analysis upon IL-1 treatment (Fig. 1). Thus, IL-1 treatment of FS-4 cells activates factors that bind to the IRE and URE NF-κB elements within the *c-myc* gene.

UV Crosslinking Analysis of Induced Binding Proteins. To begin to characterize the IL-1–induced proteins binding to the *c-myc* NF-κB sites, UV crosslinking analysis was performed. Since migration of the IL-1–induced URE and IRE complexes was similar, we selected the IRE, which has a stretch of T residues for substitution, for analysis. Protein–DNA complexes were covalently crosslinked by UV irradiation to a BrdU-substituted ^{32}P-labeled IRE oligonucleotide. Crosslinking of nuclear protein from control serum starved FS-4 cells to the IRE element resulted in the formation of a specific complex, which could be competed by addition of excess unlabeled IRE oligonucleotide. Under denaturing conditions, the protein migrated at ~67,000 daltons (Fig. 2). Crosslinking of nuclear extracts derived from IL-1 treated cells indicated the formation of several new specific protein–DNA interactions: bands migrating at ~65 and 50 kD were observed (Fig. 2). The unequal labeling of the two subunits is likely due to the preferential BrdU labeling of the 3′ end of the binding site, the region that interacts with the p65 subunit of NF-κB (30). Competition for these proteins by unlabeled IRE indicate the specificity of the crosslinking reactions. These data suggest IL-1 treatment activates interaction of the *c-myc* elements with p50 and p65 proteins, similar to the patterns observed with NF-κB constitutively present in B cells.

Functional Activity of the IRE and URE Elements Linked to a Heterologous Promoter. As an initial test of the functional significance of these protein–DNA interactions, heterologous promoter constructs containing multimerized copies of either the IRE or URE driving the TK promoter linked to the CAT reporter gene (22, 23) were employed. These constructs were transfected into cultures of FS-4 cells, which were then deprived of serum for 50 h and stimulated by IL-1 addition for 4 h. As seen in Fig. 3, constructs containing two or four copies of the IRE were inducible, whereas constructs with only one copy of the IRE or the parental TK-CAT vector failed to be induced. Similarly, a construct containing one

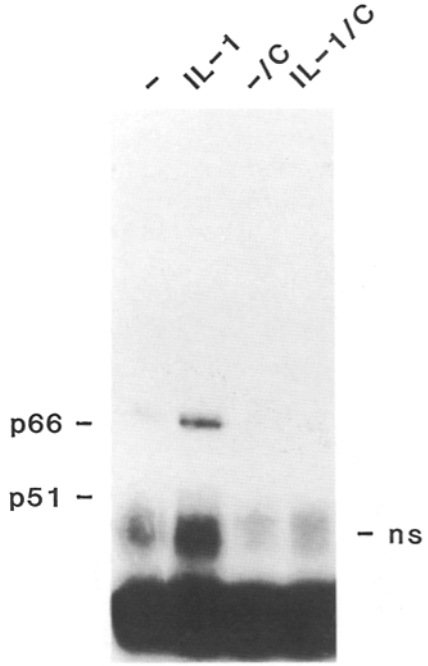

Figure 2. UV crosslinking analysis of induced binding factors. Binding to BrdU substituted ^{32}P-labeled IRE oligonucleotide of extracts from untreated (−) or IL-1 treated (*IL1*) cells was performed in the absence or presence (*c*) of excess unlabeled IRE oligonucleotide. Positions of the p51 and p66 bands, determined based on mobility of the protein markers, is consistent with p50 and p65 protein–oligonucleotide complexes.

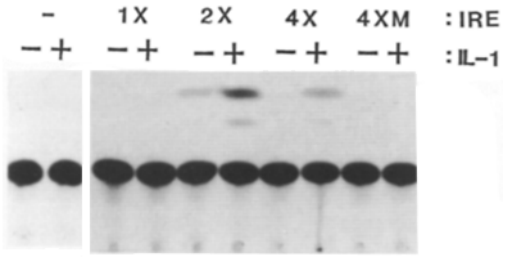

Figure 3. IL-1 inducibility of IRE element driven TK-CAT constructs. CAT enzyme activity of TK-CAT constructs containing the indicated copy number of wild-type or mutant (*M*) IRE elements was analyzed in the absence (−) or presence (+) of IL-1 after transient transfection into FS-4 cells.

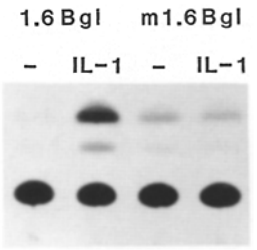

Figure 4. IL-1 inducibility of the c-*myc* promoter. Wild-type and mutant (*m*) p1.6 Bgl-CAT constructs were analyzed for effects of IL-1 after transient transfection, as above.

copy of the URE was also not induced significantly by IL-1 treatment (Table 1). These findings are consistent with the results of other laboratories indicating that two copies of an NF-κB element are required for induction of a heterologous promoter. An average increase in CAT activity of 5.9-fold for the construct containing four copies of the IRE, and 6.6-fold for the construct containing two copies of the URE was measured after IL-1 stimulation for 4 h compared with non-stimulated control cells (Table 1). These values are comparable with those obtained previously upon 48 h of phorbol ester and PHA treatment after transient transfection of Jurkat T cells (22, 23). Furthermore, TK-CAT constructs with mutant IRE (Fig. 3) or URE (data not shown) elements were not responsive to stimulation by IL-1, as expected based on the binding data. Therefore, each wild-type element is capable of behaving as a positive regulator of IL-1–induced transcription.

Function of IRE and URE in Context of the Murine c-myc Promoter. To directly test whether the IRE and URE elements actually play a role in induction of c-*myc* transcription by IL-1, we first assessed the ability of IL-1 to induce transcription of the c-*myc* promoter. The construct p1.6Bgl contains 1.6 kb of murine c-*myc* upstream and exon 1 sequences linked to CAT, including both the URE and IRE elements. After transfection into the FS-4 cultures, cells were serum starved and treated with IL-1 or used as matched untreated starved and treated with IL-1 or used as matched untreated controls as above (Fig. 4). The results of this and two duplicate experiments demonstrated that the p1.6Bgl construct was inducible an average of 8.1-fold by IL-1 treatment (Table 2).

To determine whether the presence of both elements was required, another construct p1.2 Acc c-*myc*-CAT was employed. This construct contains 615 bp of sequences upstream of the P1 promoter, and 513 bp of exon 1 sequences linked to the CAT reporter gene, and thus includes only the IRE. As seen in Table 2, p1.2 Acc c-*myc*-CAT was not significantly induced upon IL-1 treatment of transfected FS-4 cells. This result implies that sequences within the 400-bp region between $^{-}$1141 and $^{-}$615 bp, which includes the URE, is required for activation. Consistent with this finding, a construct containing exon 1 sequences and only the basal c-*myc* promoter ($^{-}$137 to $^{+}$513 bp) also failed to be induced, whereas constructs with exon 1 and upstream sequences that included the URE (up to $^{-}$3660 bp) were all inducible (data not shown).

To test directly the involvement of binding of NF-κB–like factors to the IRE and URE elements, site-directed mutagenesis was used to introduce the two G to C transversions into the p1.6Bgl construct at both the IRE and URE sites. The activity of the mutant (m1.6Bgl) was measured after transfection (Fig. 4). The introduced mutations had little apparent significant affect on the basal activity of the construct. However, the m1.6Bgl construct was no longer responsive to IL-1 induction (Table 2). These results indicate a direct role for the IRE and URE elements in transcriptional activation of the c-*myc* gene by IL-1.

Table 1. *Transactivation of IRE/URE-TK-CAT Constructs by IL-1*

Construct	Fold-induction*
1 × IRE	1.0 (1)
2 × IRE	3.2 (1)
4 × IRE	5.9 (2)
1 × URE	1.7 (1)
2 × URE	6.6 (2)

* Values for fold-induction represent the mean of the number of experiments indicated in parenthesis.

Discussion

The results of this study indicate the involvement of NF-κB–like factors in the IL-1 induction of c-*myc* gene transcrip-

Table 2. *Transactivation of c-myc-CAT Constructs by IL-1*

Construct	Fold-induction*
1.6 Bgl	8.1‡ (3)
1.2 Acc	1.2 (2)
m1.6 Bgl	0.9 (2)

* Values for fold-induction represent the mean of the number of experiments inducated in parenthesis.
‡ SD ± 1.6.

tion. Mobility-shift and UV crosslinking analysis demonstrated that IL-1 treatment of quiescent FS-4 fibroblasts induces binding to the URE and IRE elements of the c-*myc* gene that is competable by known NF-κB elements from other genes. The induced proteins have molecular weights of ~50 and 65 kD, consistent with the sizes of the previously identified NF-κB subunits (13–15), as well as the more recently detected p50 alternative subunit (31, 32). Commensurate with the previously observed rapid induction of c-*myc* mRNA, i.e., by 20–30 min after IL-1 addition to FS-4 cells (2), the increase in NF-κB–like binding to the IRE and URE elements mediated by IL-1 has occurred within 20 min, the earliest time point we tested (data not shown). Transfection analysis indicated this binding plays a major role in mediating activation of transcription of the murine c-*myc* gene by IL-1. Given the observed importance of c-*myc* gene expression in signaling of quiescent untransformed cells to proliferate (18), IL-1–mediated signaling of growth activation, such as that associated with wound healing, is likely to be mediated at least in part by the induction of the NF-κB family of factors by IL-1.

This is one of the first reports demonstrating the direct role of elements within the c-*myc* gene in the response of this gene to physiologically relevant activators of its expression. Given our current knowledge of NF-κB, this factor has the potential to play a major role in the regulation of c-*myc* gene transcription. In non-B cells, NF-κB is in an inactive cytoplasmic form complexed with an inhibitor protein (17). Activation of NF-kB involves release from the inhibitor and translocation to the nucleus. In addition to IL-1 and TNF-α, activation can be mediated by such diverse agents as phorbol ester, serum, LPS, and infection with such viruses as HTLV-1 and herpes (10, 16, 33, 34). These agents are also known to induce c-*myc* expression in lymphocytes, fibroblasts, and many other cell types, Recently, we have demonstrated transactivation of the c-*myc* promoter by the *tax* gene product of the HTLV-1 virus, implicating these NF-κB sites in T cell activation and viral-induced immortalization (34a).

Cytokine production by infiltrating mononuclear cells at wound sites and in situ production of these cytokines by fibroblasts and stromal cells is complex. For example, in addition to IL-1, production of TNF-α and IL-6 has been demonstrated. TNF-α, originally called cachectin, was discovered as an endotoxin-induced monocyte cell product that caused necrosis in certain tumors in vivo (35). Significant overlap of actions of TNF-α and IL-1 has been noted. For example, TNF-α also activates NF-κB (10). TNF-α has also been found to modulate c-*myc* expression in FS-4 fibroblasts with essentially the same kinetics as IL-1 (2). We have recently found that TNF-α results in similar induction in binding profiles for the IRE and URE sites in this cell line (data not shown). However, evidence for differential activity of IL-1 and TNF-α has also recently been obtained. These two cytokines act in synergy to greatly enhance fibroblast expression of the IL-6 gene, through its NF-κB and serum response elements (36). Thus, the response of the c-*myc* oncogene during wound healing may result from the interaction of a complex array of cytokines mediating multiple stimulatory events.

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