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Induction of Mitogen-Inducible Nuclear Orphan Receptor by Interleukin 1 in Human Synovial and Gingival Fibroblasts

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High levels of interleukin 1 (IL-1) found in inflammatory diseases such as rheumatoid arthritis and periodontitis act on the local fibroblasts, resulting in an altered phenotype characterized by hyperplasia and the production of inflammatory mediators and destructive enzymes. The goal of this study was to identify genes induced as an early response to IL-1 in synovial and gingival fibroblasts which might play a regulatory role in the cascade of events leading to their activation. Using the technique of mRNA differential display, we have identified the mitogen-inducible nuclear orphan receptor (MINOR) as a gene up-regulated by IL-1 in human synovial and gingival fibroblasts. The rapid induction of both mRNA and DNA binding activity suggests that MINOR may play an important early role in regulating the response of fibroblasts to inflammation.

Chronic inflammatory diseases such as rheumatoid arthritis (RA) and periodontitis are characterized by high levels of inflammatory cytokines [e.g., interleukin 1 (IL-1) and tumor necrosis factor (TNF)]. These cytokines act on the local fibroblasts, resulting in an altered phenotype characterized by hyperplasia and increased production and release of inflammatory mediators and matrix degrading enzymes (2, 6). Although IL-1 is known to increase the transcription of many genes, the mechanisms involved in the cascade of events leading to fibroblast activation are not well understood.

To learn more about the pattern of gene induction that occurs upon cytokine stimulation of fibroblasts, we used the technique of differential display (9) to identify genes up-regulated by IL-1 in synovial and gingival fibroblasts. Of particular interest were those gene tags induced within one to three hours of stimulation which might play an initiating or regulatory role in the cascade of events leading to fibroblast activation.

Here we report the identification of one such gene as the Mitogen Inducible Nuclear Orphan Receptor (MINOR). MINOR was originally cloned from stimulated T cells (7) and separately as NOR-1 from fetal brain (15). MINOR/NOR-1 is a member of a sub-family of nuclear orphan receptors including two other members: the Nerve Growth Factor Induced-B (NGFI-B, also called Nur77 or NAK-1), and Nurr1/RNR-1/NOT (7, 15). Like other members of this sub-family, MINOR was shown to bind and activate transcription from the NGFI-B response element (NBRE) in co-transfection experiments, although not as strongly as Nur77 (3, 7). We show here that MINOR mRNA and DNA binding activity are both induced by IL-1 in synovial and gingival fibroblasts. Although further research is necessary to determine what genes are activated by MINOR under these conditions, it appears likely that MINOR may play an important role in regulating the response of fibroblasts to chronic inflammation.

METHODS

Cell culture. Human synovial fibroblasts are obtained from synovia of patients with osteoarthritis undergoing reconstructive and restorative surgery; and gingival fibroblasts are obtained from patients undergoing periodontal surgery or surgical removal of third molars. Tissue samples are processed by enzymatic dispersion to produce primary cultures as previously described (11, 12). These cultures are maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (penicillin, streptomycin, amphotericin; Gibco/BRL). Cells in passage five to eight were used in experiments. Cultures were serum deprived for 16 h in serum free EMEM supplemented with 10% ITS (insulin, transferrin, sodium selenite; Sigma) prior to stimulation with 100 ng IL-1β/ml (a generous gift of R. Newton, DuPont-Merck).

Differential display. Total RNA was isolated from a mixture of gingival fibroblast cells derived from four different individuals and treated for one hour with IL-1β. The RNA was purified by a cesium

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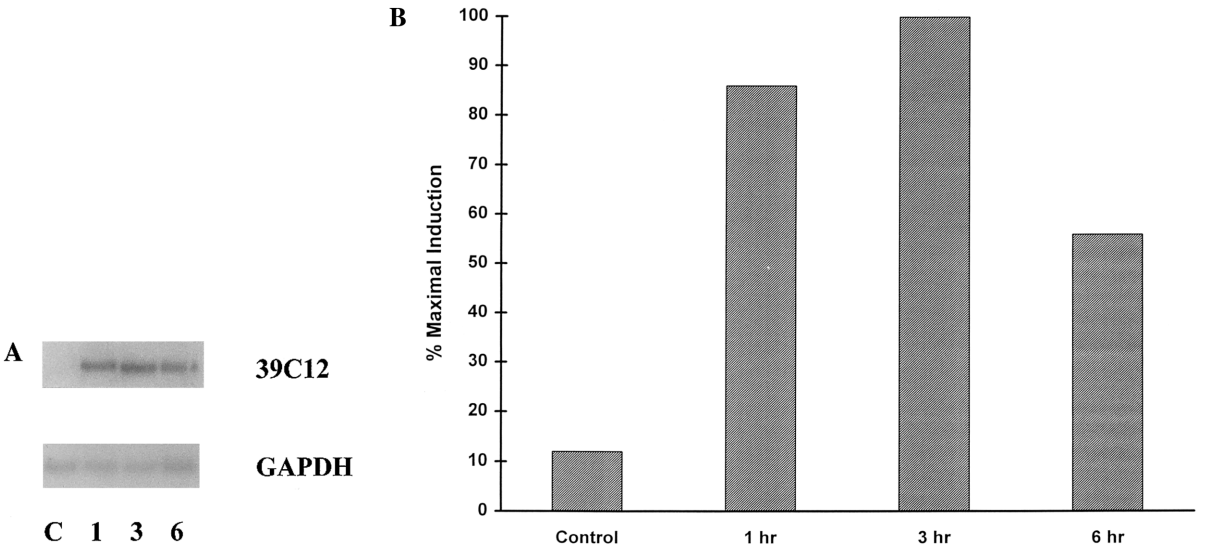

FIG. 1. Northern blot analysis of gene tag 39C12. (A) Twenty micrograms of total RNA was isolated from human gingival fibroblast cultures stimulated for the indicated times with IL-1 (100 ng/ml). The Northern blot was hybridized with the PCR amplified, ^{32}P-labeled gene tag 39C12, and with GAPDH. (B) The relative amounts of hybridization were quantitated by densitometric scanning and levels of 39C12 were normalized to GAPDH to correct for differences in loading. Values are expressed as the percent maximal expression.

chloride gradient (5), followed by DNAse treatment using the MessageClean kit (GenHunter). Differential display was performed using the RNAimager kit from GenHunter, using the $T_{11}C$ and AP-12 primers, according to the manufacturer's instructions. The amplified gene tags were separated on denaturing polyacrylamide gel electrophoresis and detected by autoradiography. The autoradiogram was aligned with the dried gel and the differentially regulated gene tags were excised and eluted from the gel. This DNA was used as probes for Northern blot analysis and cloned using the PCRTrap vector system (GenHunter).

Northern blot analysis. Total RNA was isolated from four pooled strains of synovial or gingival fibroblast cultures according to the method of Chomczynski and Sacchi (4). Twenty μg was separated in agarose-formaldehyde gels and transferred to Hybond-N membrane (Amersham) according to standard procedures. Northern blots were hybridized with the following probes, which were labeled by random priming (Prime-It, Promega): PCR amplified gene tag 39C12 (primers $T_{11}C$ and AP-12, GenHunter); MINOR cDNA, a gift of S. G. Irving, Georgetown University; and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) cDNA, a gift of R. Newton, DuPont/Merck.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were isolated from either synovial or gingival fibroblast cultures at various times after stimulation with IL-1β (100 ng/ml), as well as from control cultures, according to the method of Schreiber *et al.* (16). Complementary oligodeoxynucleotides containing the NBRE binding site (5′GAGTTTTAAAAGGTCATGCTCAATTTGG3′) and a mutated NBRE site (5′GAGTTTTAAGAGGTCAAATTTGG3′) were annealed and end-labeled using polynucleotide kinase and γ^{32}P-dATP. Binding reactions contained 5 or 10 μg protein, 20 mM Hepes-OH, pH 7, 50 mM NaCl, 0.2 M EDTA, 5% glycerol, 4 μg poly-dI-dC and 10,000 cpm probe. Samples were electrophoresed on native 5% PAGE in 0.5X TBE. For the competition experiment, an unlabeled oligodeoxynucleotide probe containing the PEA3 site from the human collagenase promoter (5′ATCAAGAGGATGTTATAAAG3′) was used as a negative control. A rabbit polyclonal antisera directed against MINOR and reported not to cross-react with Nur77 (7) was obtained as a generous gift from S. G. Irving, Georgetown University, and used in supershift analysis.

RESULTS

Identification of gene tag 39C12 as regulated by IL-1. To begin to identify genes up-regulated as an early response to IL-1, RNA isolated 1 hour after stimulation with IL-1 was analyzed by differential display (9). Using the primers $T_{11}C$ and AP-12, a number of gene tags were found and eluted from the gel and PCR amplified for further analysis. Figure 1 shows results of a Northern blot using one of these gene tags, 39C12, as a probe. A single band was observed hybridizing at approximately 6.8 kb which was induced about 8-fold by IL-1 at 3 h. This amplified gene tag was then ligated to the pCR-TRAP vector and used to transform GH competent cells (GenHunter). A single clone containing a 350-bp insert was isolated and sequenced. Comparison of the sequence with the Genbank database revealed the clone to be nearly identical ($p = 4.2 \times 10^{-63}$) to the Mitogen Inducible Nuclear Orphan Receptor (MINOR) in the 3′ untranslated region, bases 4613 to 4969 (7). The random primer AP12 was found to be identical to the MINOR sequence from 4552 to 4558 bp.

Confirmation of regulation of MINOR by IL-1. To confirm IL-1 regulation of MINOR, a cDNA clone was obtained as a generous gift of S. G Irving and used as a probe in Northern blot analysis of synovial and gingival fibroblasts. Results, shown in Figs. 2 and 3, confirm that MINOR mRNA levels are increased by IL-1 at 3 and 6 h after stimulation. These results are consistent both with earlier Northern analysis using gene tag 39C12 as a probe and with published results for MINOR.

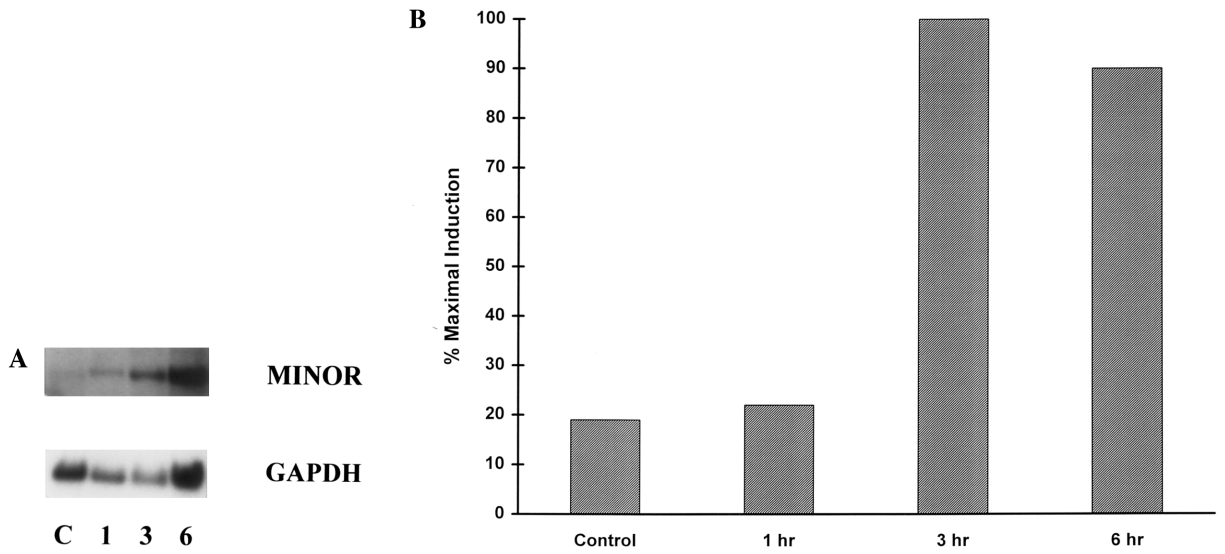

FIG. 2. Expression of MINOR mRNA in human synovial fibroblasts in response to IL-1. (A) Twenty micrograms of total RNA was isolated from human synovial fibroblast cultures stimulated for the indicated times with IL-1 (100 ng/ml). The Northern blot was hybridized with the ^{32}P-labeled MINOR cDNA, and with GAPDH. (B) The relative amounts of hybridization were quantitated by densitometric scanning and levels of MINOR were normalized to GAPDH to correct for differences in loading. Values are expressed as the percent maximal expression of MINOR.

Induction of MINOR DNA binding activity by IL-1. Since MINOR has been shown to be capable of binding to and activating transcription from the NBRE (3, 7), the ability of IL-1 to induce DNA binding to this site in synovial and gingival fibroblasts was tested in gelshift assays. Figure 4 shows results in synovial fibroblasts. Binding to the NBRE was increased after 3 h treatment with IL-1, and was maximal at 6 h. To determine whether this binding was sequence-specific, the same nuclear extracts were incubated with a probe containing a single nucleotide substitution previously shown to be incapable of binding MINOR (7). No binding to this mutated NBRE site was observed (Fig. 4B).

In gingival nuclear extracts, the IL-1 induced binding pattern was very similar, with maximal binding seen at 10 hours (Fig. 5, left). This binding was suc-

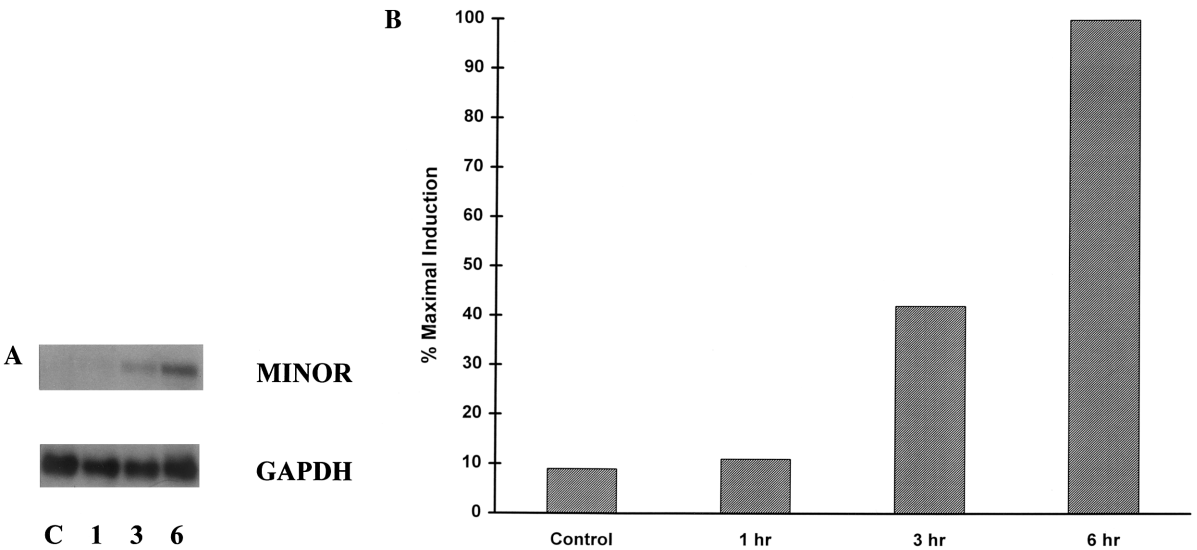

FIG. 3. Expression of MINOR mRNA in human gingival fibroblasts in response to IL-1. (A) Twenty micrograms of total RNA was isolated from human gingival fibroblast cultures stimulated for the indicated times with IL-1 (100 ng/ml). The Northern blot was hybridized with the ^{32}P-labeled MINOR cDNA, and with GAPDH. (B) The relative amounts of hybridization were quantitated by densitometric scanning and levels of MINOR were normalized to GAPDH to correct for differences in loading. Values are expressed as the percent maximal expression of MINOR.

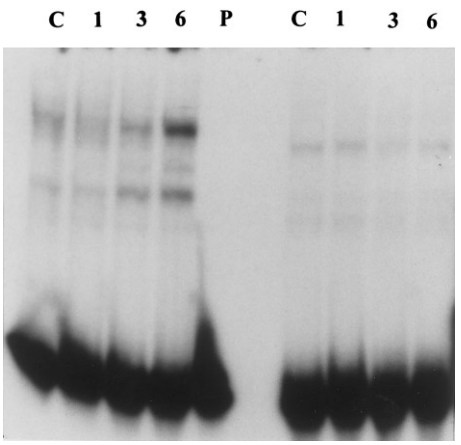

FIG. 4. Induction of binding to the NGFI-B response element (NBRE) by IL-1 in human synovial fibroblasts. Nuclear extracts were isolated from human synovial fibroblasts at the indicated hours after addition of IL-1 (100 ng/ml). Ten micrograms of extract was incubated with ^{32}P-labeled oligodeoxynucleotides containing either the wild-type NBRE (5′GAGTTTTAAAAGGTCATGCTCAATTTGG3′) or a mutated NBRE probe (5′′GAGTTTTAAGAGGTCAAATTTGG3′). P, probe alone, no extract; C, control extract, no IL-1.

cessfully competed with as little as ten-fold excess unlabeled NBRE, but even 100-fold excess of a heterologous site, the collagenase PEA3 site, was unable to compete (Fig. 5B). Thus, IL-1 induced binding to the NBRE is sequence specific in synovial and gingival fibroblasts.

Since other members of the NGFI-B family (e.g., Nur77) are also capable of binding to the NBRE (3, 7, 14), antisera directed against MINOR and shown previously not to cross-react with Nur77 (7), was obtained as a generous gift from S. G. Irving and used in a supershift assay to establish which of the binding complexes actually contained MINOR. Results of this experiment are shown in Fig. 6. In both 6- and 10-h samples, addition of the antisera resulted in inhibition of two of the three complexes, and the appearance of a more slowly migrating, supershifted complex. These results strongly suggest that two of the three IL-l-induced complexes contain MINOR protein.

DISCUSSION

Results shown here have demonstrated that both MINOR mRNA and DNA binding activity are induced by IL-1 in human synovial and gingival fibroblasts. This is the first report of induction of this transcription factor by an inflammatory cytokine. The kinetics of this response, with IL-1 induction of the mRNA occurring within three hours of treatment, are consistent with the possibility that MINOR may play an important regulatory role in the response of fibroblasts to inflammation.

The nature of this role is yet to be determined. To date, there are no genes known to be regulated by MINOR in any cell type. However, the fact that MINOR has been shown to bind and activate transcription from the NBRE (3, 7) suggests that it is probably capable of activating the same genes activated by other members of this orphan receptor sub-family. MINOR and Nur77 are expressed in similar cell types and in response to similar signals, but MINOR expression is somewhat less transient than that of Nur77 (8, 19) and it activates transcription from the NBRE to a lesser extent (3, 7). It has therefore been suggested that MINOR may activate the same set of genes activated by Nur77 *in vivo* in order to maintain a lower level of gene expression for a longer period of time (7). However, since the genes so far demonstrated to be activated by Nur77 are of neuroendocrine origin (10, 13, 17, 18), it is difficult to speculate which genes might be activated by MINOR in fibroblasts responding to inflammation.

The only function thus far demonstrated for MINOR *in vivo* is a role in T cell receptor-mediated apoptosis (3). Although there is no evidence at this time that MINOR plays the same type of role in fibroblasts, it is tempting to speculate that MINOR may serve to counter-act the proliferation induced by inflammatory cytokines by stimulating programmed cell death. However, since hyperplasia of the inflamed synovium is one

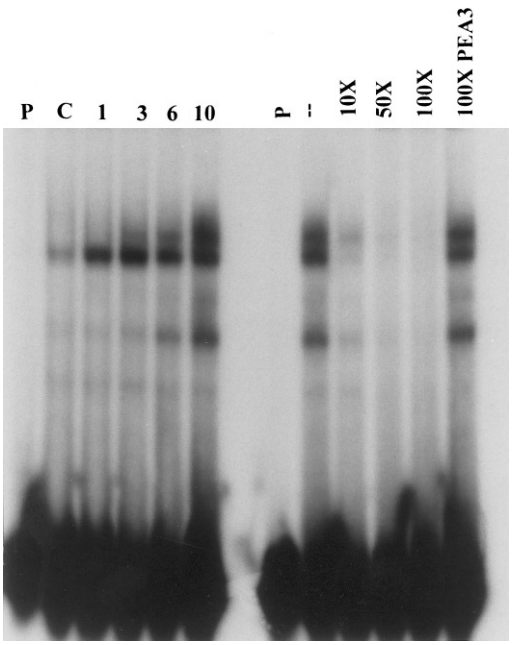

FIG. 5. Induction of sequence-specific binding to the NBRE by IL-1 in human gingival fibroblasts. (Left) Nuclear extracts were isolated from human gingival fibroblasts at the indicated hours after addition of IL-1 (100 ng/ml). Ten micrograms of extract was incubated with ^{32}P-labeled oligodeoxynucleotides containing the wild-type NBRE. (Right) The same 10 hour nuclear extract shown in A was incubated with the ^{32}P labeled NBRE probe in the presence of 10-, 50-, and 100-fold molar excess of the unlabeled NBRE, or a 100-fold excess unlabeled oligodeoxynucleotide containing the collagenase PEA3 site.

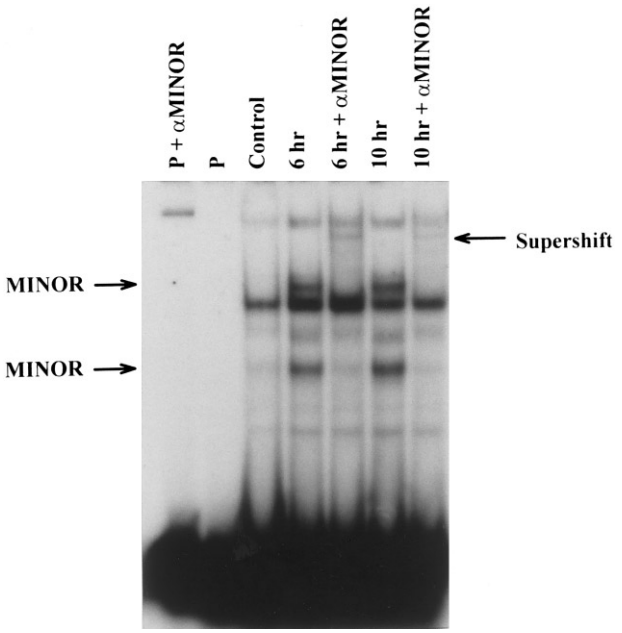

FIG. 6. IL-1 induced binding to the NBRE contains MINOR. Ten micrograms of nuclear extract isolated 6 and 10 h after stimulation with IL-1 was incubated for 5 min in the presence or absence of a polyclonal antisera directed against MINOR protein before addition of the ^{32}P labeled NBRE. As a control, the antiserum was also incubated with the probe in the absence of extract. The arrow indicates a supershifted complex.

of the hallmarks of rheumatoid arthritis, one is forced to conclude that if the function of MINOR is in apoptosis, there must either be some other defect in the apoptotic process downstream of MINOR [e.g., somatic mutations in p53 or other tumor suppressors as suggested by Firestein *et al.* (1)], or perhaps the signals for proliferation simply exceed the capacity for apoptosis.

Further study is clearly needed to define what genes are activated by MINOR in fibroblasts in response to IL-1, and whether or not it actually does play a role in stimulating apoptosis in these cells. If MINOR does play a role in apoptosis, it would be interesting to determine whether over-expression of this transcription factor might be used therapeutically to decrease the hyperplasia by over-riding the presumed defect in apoptosis, and perhaps thereby decrease the destructive processes associated with inflammation.

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REFERENCES

1. Aupperle, K. R., Boyle, D. L., Hendrix, M., Seftor, E. A., Zvaifler, N. J., Barbosa, M., and Firestein, G. S. (1998) *Am. J. Pathol.* **152,** 1091–1098.
2. Birkedal-Hansen, H. (1993) *J. Periodontal Res.* **28,** 500–510.
3. Cheng, L. E., Chan, F. K., Cado, D., and Winoto, A. (1997) *EMBO J.* **16,** 1865–1875.
4. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
5. Davis, L., Dibner, M., and Battey, J. (1986) *in* Basic Methods in Molecular Biology, pp. 130–135, Elsevier, New York.
6. Harris, E. J. (1981) *in* Pathogenesis of Rheumatoid Arthritis, Textbook of Rheumatology, pp. 896–927, Saunders, Philadelphia.
7. Hedvat, C. V., and Irving, S. G. (1995) *Mol. Endocrinol.* **9,** 1692–1700.
8. Irving, S. G., June, C. H., Zipfel, P. F., Siebenlist, U., and Kelly, K. (1989)*Mol. Cell. Biol.* **9,** 1034–1040.
9. Liang, P., Bauer, D., Averboukh, L., Warthoe, P., Rohrwild, M., Muller, H., Strauss, M., and Pardee, A. B. (1995) *Methods Enzymol.* **254,** 304–321.
10. Lin, H. H., Tu, Z-J., and Ann, D. K. (1996) *J. Biol. Chem.* **271,** 27637–27644.
11. Mochan, E., Uhl, J., and Newton, R. (1986) *Arthritis Rheum.* **29,** 1078–1084.
12. Mochan, E., Uhl, J., and Newton, R. (1986) *J. Rheum.* **13,** 15–19.
13. Murphy, E. P., and Conneely, O. M. (1997) *Mol. Endocrinol.* **16,** 39–47.
14. Murphy, E. P., Dobson, A. W., Keller, C., and Conneely, O. M. (1996) *Gene Expression* **5,** 169–179.
15. Ohkura, N., Ito, M., Tsukada, T., Sasaki, K., Yamaguchi, K., and Miki, K. (1996) *Biochim. Biophys. Acta* **1308,** 205–214.
16. Schreiber, E., Matthais, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419.
17. Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J., and Parker, K. L. (1993) *Mol. Cell. Biol.* **13,** 861–868.
18. Zhang, P., and Mellon, S. (1997) *Mol. Endocrinol.* **11,** 891–904.
19. Zipfel, P. F., Irving, S. G., Kelly, K., and Siebenlist, U. (1989) *Mol. Cell. Biol.* **9,** 1041–1048.