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The Mitogenic Response to Tumor Necrosis Factor Alpha Requires c-Jun/AP-1

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In the present study, we addressed the role of the *c-jun* proto-oncogene in the mitogenic response of human fibroblasts and primary acute myelogenous leukemia blasts to tumor necrosis factor alpha (TNF-α). Our data indicate that TNF-α treatment of these cells is associated with transcriptional activation of *c-jun*, resulting in accumulation of *c-jun* mRNA and protein expression. In order to elucidate the role of c-Jun/AP-1 in TNF-mediated growth stimulation, the antisense (AS) technique was used. Uptake studies of oligonucleotides were performed with fibroblasts, demonstrating that incorporation of oligomers was maximal at 4 h. Oligodeoxynucleotides remained stable in these cells for up to 24 h. Treatment of fibroblasts with the AS oligonucleotide resulted in intracellular duplex formation followed by an efficient translation blockade of c-Jun/AP-1. In contrast, sense (S) and nonsense (NS) oligodeoxynucleotides failed to form intracellular duplexes and also did not interfere with translation of c-Jun/AP-1, suggesting specific elimination of c-Jun/AP-1 by the AS oligomer. Fibroblasts cultured in the presence of the AS oligonucleotide but not those cultured in the presence of the S or NS oligonucleotide failed to respond proliferatively to TNF-α. These findings could be confirmed by experiments with primary acute myelogenous leukemia blasts, which also demonstrated that TNF-induced growth stimulation required c-Jun/AP-1 function. Taken together, our results indicate that activation of c-Jun/AP-1 plays a pivotal role in the signaling cascade initiated by TNF, which leads to a proliferative response of its target cells.

Tumor necrosis factor alpha (TNF-α) is a 17-kDa polypeptide primarily synthesized by activated monocytes (33). Signaling of TNF-α requires the presence of two specific receptors, a high-affinity 75- to 89-kDa receptor and a low-affinity 55- to 60-kDa receptor. TNF-α binding sites have been identified on the surface of most mammalian cells (11). Consistent with its ubiquitous expression, TNF-α exerts a wide range of biologic activities involved in host defense against infection, tissue remodeling, and regulation of blood cell formation (5). Although TNF-α mainly exerts growth-inhibiting effects on various tissues in culture, it has also been shown to act as a mitogen for several cell types. The growth-promoting effect of TNF-α has been most convincingly documented when fibroblasts and normal or malignant myelopoietic progenitor cells have been examined (8, 12, 16). Although it is still unknown whether distinct signaling events initiated by TNF can be linked to defined biologic activities, the diverse biological actions of TNF-α may be related to its capacity to initiate different genetic programs in its target cells. Among the genes known to be activated by TNF-α are the early response genes *c-jun* (10), *c-fos* and *c-myc* (21), the NF-κB transcription factor gene (26), and the genes for many cytokines, including macrophage colony-stimulating factor (27), interleukin-6 (IL-6) (19), IL-1 (25), and IL-8 (23). We have previously shown that the *c-jun* proto-oncogene, which encodes a component of the AP-1 transcription factor complex (1), is transcriptionally activated in proliferating acute myelogenous leukemia (AML) cells upon exposure to TNF-α (8). Other studies have documented that *c-jun* is also involved in the immediate response of fibroblasts to TNF (10, 28) and that *c-jun* is transcriptionally activated in these cells during the G_0/G_1 cell cycle transition (29). Neutralizing antibodies to c-Jun or c-Fos are capable of abolishing cell cycle progression in human fibroblasts (20). Taken together, these findings suggest that *c-jun* expression is linked to signals leading to cellular proliferation. The present study, therefore, aimed at investigating the significance of *c-jun* activation in the mitogenic response of both fibroblasts and primary AML blasts to TNF-α.

MATERIALS AND METHODS

Cells. Cultures of embryonic lung fibroblasts (FH 109) were established as previously described (22). Samples of leukemic blasts were obtained from the bone marrow of five consecutive patients with newly diagnosed AML. All samples were obtained after informed consent. The diagnosis of AML was established by morphology and cytochemical staining, and cases were classified according to the French-American-British criteria. T lymphocytes and monocytes/macrophages were removed from all leukemia cells by rosetting with sheep erythrocytes and repeated adherence to plastic surfaces after initial isolation of mononuclear cells by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were cryopreserved in 10% dimethyl sulfoxide in the vapor phase of liquid nitrogen until use. Samples under study contained always >95% blast cells as determined by examination of cell morphology.

Cell culture and proliferation assay. FH 109 cells were cultured in RPMI 1640 supplemented with 5% low-endotoxin fetal calf serum (Hazleton, Vienna, Va.), 100 U of penicillin per ml, 100 U of streptomycin (GIBCO, Grand Island, N.Y.) per ml, and 2 mM L-glutamine (standard culture medium) in the presence or absence of recombinant human TNF-α

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(rhTNF-α) (1 to 100 ng/ml; kindly provided by G. Adolf, Bender KG, Vienna, Austria). Leukemic blasts were cultured under serum-free conditions as previously described (6) in the presence or absence of rhTNF-α (25 ng/ml). For proliferation assays, FH 109 cells growing at a confluency of 30% or primary AML blasts (10^5/ml) costimulated with rhIL-3 (25 ng/ml) were cultured in the presence or absence of TNF-α (25 ng/ml) with or without sense (S), antisense (AS), or nonsense (NS) *c-jun* oligodeoxynucleotide (see below) added every 18 h to the culture medium for up to 72 h, with 15 kBq of tritiated thymidine ([^{3}H]thymidine; specific activity, 2 kBq/mmol) being present during the last 6 h. After this culture period, cells were harvested, and [^{3}H]thymidine uptake was measured in a liquid scintillation counter. Concentrations of oligonucleotides used in these experiments ranged from 2 to 16 μM.

cDNA probes, isolation of total cellular RNA, Northern (RNA) blot analysis, and nuclear run-on transcription assay. The plasmids and cDNA probes used were the *c-jun* 1.2-kb *Eco*RI-*Bam*HI fragment in pBR322 (kindly provided by P. Angel, Institut für Genetik, Karlsruhe, Germany) and the 0.8-kb *Bam*HI-*Pst*I α-actin fragment in pBR322 (kindly provided by R. J. Schwarz, Baylor College of Medicine, Houston, Tex.). FH 109 fibroblasts cultured in the presence or absence of TNF-α (25 ng/ml) were harvested after the appropriate culture period as indicated above. The cells were resuspended in guanidinium isothiocyanate (Sigma, Munich, Germany) and extracted with an equal volume of acetate-EDTA-equilibrated phenol (60°C for 25 min with frequent vortexing). The aqueous phase was recovered after centrifugation and extracted once with an equal volume of phenol-chloroform and twice with chloroform. The resulting RNA was precipitated overnight at −20°C with 2.5 volumes of ethanol. The total RNA from each sample was then electrophoresed on a 1% agarose gel containing 20 mM sodium borate (pH 8.3), 0.5 mM EDTA, and 3% formaldehyde. The RNA was transferred overnight to nitrocellulose paper (Schleicher & Schuell, Dassel, Germany) in 10× SSC (10× SSC is 1.5 M sodium chloride and 150 mM sodium citrate) by capillary blotting. The blots were baked and prehybridized at 65°C in 7% sodium dodecyl sulfate (SDS)–10× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin [fraction V; Sigma], and 0.02% polyvinylpyrrolidone)–5× SSC–20 mM salmon sperm DNA (Sigma). Probes were radiolabeled by random priming with [α-^{32}-P]dCTP (>6,000 Ci/mmol; Amersham Buchler, Braunschweig, Germany). The blots were washed at 55°C in 1% SDS–1× SSC and were autoradiographed with Kodak X-Omat film (Eastman Kodak, Rochester, N.Y.) at −70°C with an intensifying screen. The autoradiographs were scanned with an LKB Ultro Scan XL laser densitometer and analyzed with an LKB Gel Scan XL software package.

For nuclear run-on transcription assays, 10^8 cells were lysed in RSB (10 mM Tris-HCl, 5 mM KCl, 3 mM $MgCl_2$) containing 0.5% Nonidet P-40 (Sigma) and were washed once in ice-cold phosphate-buffered saline (PBS). Nuclei were incubated at 26°C in a mixture of 15% glycerol, 70 mM KCl, 2.5 mM $MgCl_2$, 10 mM EDTA, 4 mM concentrations each of ATP, CTP, and GTP, 2 mM UTP, 0.5 mM dithiothreitol, and 60 U of RNasin (Boehringer Mannheim, Mannheim, Germany) per ml in the presence of 100 μCi of [^{32}P]UTP (3,000 Ci/mmol; Amersham Buchler) for 30 min. The mixture was digested with DNase I and precipitated in 70% ethanol before hybridization in 5 × 10^5 cpm/ml of hybridization buffer (50% formamide, 2× SSC, 1% SDS, 5× Denhardt's solution) containing 5 μg of tRNA per ml. Filters contained 10 μg each of linearized plasmids immobilized on nitrocellulose (Schleicher & Schuell) after blötting with a slot-blot apparatus (Schleicher & Schuell). After hybridization at 42°C for 3 days, the filters were rinsed in 2× SSC at 55°C, 2× SSC containing 10 μg of RNase A per ml at 37°C, and finally 0.5× SSC at 55°C, for 30 min each time, and were exposed to Kodak X-Omat film for 10 days.

Immunoprecipitation of c-Jun protein. Cells were cultured in the presence or absence of AS, NS, or S oligonucleotides for 16 h in methionine-free medium, washed twice with PBS, and then labeled with 300 μCi of [^{35}S]methionine (Amersham Buchler) in the presence or absence of the respective oligonucleotides with or without TNF-α or IL-3 for another 6 h. Cells were lysed in radioimmunoprecipitation assay buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS) and then centrifuged at 4°C for 20 min at 20,000 × g to remove insoluble material. Incorporated radioactivity was measured by scintillation counting. The amount used for immunoprecipitation was 10^7 cpm. c-Jun and β2-microglobulin proteins were immunoprecipitated with monoclonal antibodies directed to the c-Jun protein (Oncogene Science, Manhasset, N.Y.) or β2-microglobulin (Sigma) and then subjected to SDS-polyacrylamide gel electrophoresis (17).

Preparation of nuclear extracts and electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed essentially as previously described (7). Briefly, nuclear extracts were prepared from untreated cells and cells exposed to TNF in the presence or absence of S, NS, or AS oligodeoxynucleotides by the method of Dignam et al. (14). Protein concentrations were determined by the Bradford assay (9). S and AS oligonucleotides corresponding to positions −78 to −56 of the *c-jun* promoter, containing the AP-1 binding site, were synthesized, annealed, and end labeled with the large fragment of DNA polymerase (Klenow fragment) in the presence of [α-^{32}P]dCTP. The end-labeled oligonucleotides (1 ng; approximately 10,000 cpm) were incubated with 10 μg of nuclear proteins in an incubation buffer containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 10% (vol/vol) glycerol (Sigma) for 20 min at room temperature. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat film at −70°C with an intensifying screen.

Oligonucleotide design, stability assay, and determination of intracellular duplex formation. An oligonucleotide corresponding to the translation initiation site of the *c-jun* gene, the respective AS oligonucleotide, and an NS oligonucleotide with the same overall base-pair composition as the AS oligonucleotide (AS, 5′ TCATAGAACAGTCCGTCACT TCAC 3′; S, 5′ GTGAAGTGACGGACTGTTCTATGA 3′; NS, 5′ CAAGCTTCAGTCCTAACTCCAGAT 3′) were synthesized, stabilized by addition of thiosulfate groups, purified by high-performance liquid chromatography, and then sequenced. Oligonucleotide uptake studies were performed essentially as described previously (35). Briefly, the oligomers were 5′ end labeled with [γ-^{32}P]ATP with bacteriophage T4 polynucleotide kinase and purified by denaturing polyacrylamide gel electrophoresis. A total of 5 × 10^5 cpm of 5′-labeled oligonucleotides was added to 4 × 10^6 AML blasts or 4 × 10^6 FH 109 cells. The cells were incubated at 37°C in 7% CO_2 in air for up to 24 h, and aliquots were collected every 4 h over a period of 24 h. The cells were pelleted, and the supernatant was harvested and saved. The cells were then washed twice in PBS (GIBCO) and pelleted, and the

cell pellet was lysed in 0.1 ml of Tris-buffered saline (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% SDS) and phenol extracted. Aliquots of the aqueous phase, the cell wash, and culture medium supernatants were analyzed by liquid scintillation counting. The percentage of oligonucleotides taken up by the cells was calculated by dividing the counts in the aqueous phase by the total counts (aqueous phase plus cell wash plus culture medium supernatant). To assess the stability of the oligonucleotides, aliquots of the aqueous phase were lyophilized, redissolved in 20 μl of loading buffer (80% deionized formamide, 0.01% bromphenol blue, 0.01% xylene cyanol FF), and subjected to electrophoresis through a 12% denaturing polyacrylamide gel and fluorography.

Experiments designed to analyze intracellular duplex formation were performed as previously described by Holt et al. (17). Briefly, oligonucleotides were 5′ end labeled as described above to a specific activity of 2×10^7 to 4×10^7 cpm/μg and added to FH 109 cells in culture medium at a concentration of 8 μM for 4 h. After several washing steps in prewarmed PBS, the cells were lysed in 100 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 0.5% SDS, 100 μg of proteinase K per ml). A 10,000-fold excess of unlabeled oligomer was added as a carrier. After phenol-chloroform extraction and ethanol precipitation, an S1 nuclease protection assay was performed as previously described (4). The products were analyzed on a 15% denaturing polyacrylamide gel. In order to confirm that duplexes had been formed intracellularly, a control experiment was performed; in the control experiment, an equal amount of cell-associated radioactivity together with an excess of unlabeled carrier oligomer was added to lysates of cells that had been cultured in the absence of labeled oligonucleotides.

RESULTS

In agreement with previous studies, we found that TNF-α promotes proliferation of fibroblasts. TNF-α enhanced [^{3}H]thymidine incorporation by FH 109 fibroblasts in a dose-dependent fashion in the range of 1 to 25 ng/ml. The stimulatory effect was optimal (10-fold) when cells were exposed to 25 ng of rh TNF-α for 48 hours, while higher TNF doses and prolongation of the culture period did not further increase the proliferative response (Fig. 1).

In concert with previous reports (10, 28), we also found that TNF-α treatment of fibroblasts was associated with an immediate and transient accumulation of c-*jun* mRNA. c-*jun* mRNA levels began to rise as early as 30 min after TNF-α exposure, peaked within 120 min, declined thereafter, and returned to starting levels within 6 to 8 h (data not shown).

In the next set of experiments, nuclear run-on assays were performed to determine whether TNF-α-mediated c-*jun* mRNA accumulation was due to transcriptional activation of the gene. Figure 2 shows that FH 109 cells responded to TNF-α with an at least fivefold increase of the transcription rate of the c-*jun* gene within 20 min of TNF-α exposure. In contrast, the transcription rate of the α-actin gene remained unaffected by TNF-α exposure at that time.

The next set of experiments was designed to investigate the role of c-Jun accumulation in the proliferative response of fibroblasts to TNF-α. AS oligodeoxynucleotides have been previously shown to enter cells when added into the culture medium and to form intracellular duplexes with their specific complementary mRNAs, thereby interfering with translation of the respective mRNAs. Thus, AS technology may provide a useful tool to assess the physiological role of their target proteins (34). We therefore synthesized an AS oligonucleotide directed against the translation initiation site of the c-*jun* gene, as well as a corresponding S oligonucleotide and an unrelated NS oligonucleotide which served as controls. In pilot experiments (data not shown), the oligonucleotides in cell culture medium were determined to be stable for 18 to 24 h. To examine oligodeoxynucleotide uptake by FH 109 fibroblasts, cells were cultured in the presence of radioactively labeled AS or S oligonucleotides for up to 24 h. The cells were washed several times, and the amount of radioactivity retained by the washed cell pellet

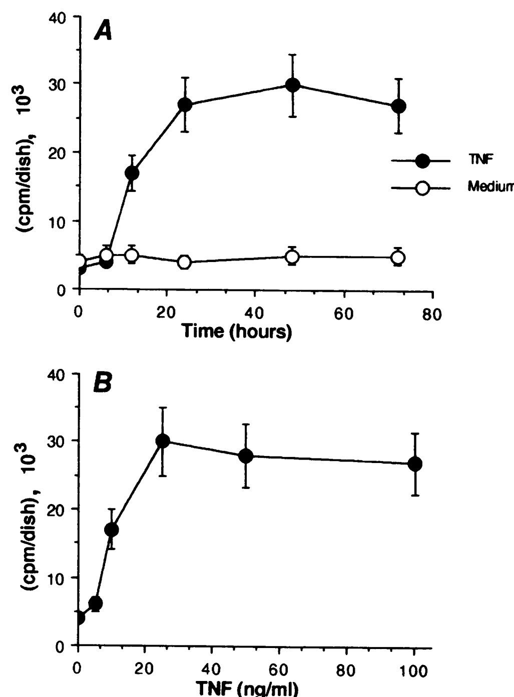

FIG. 1. TNF-α induces proliferation of human fibroblasts. (A) Time kinetics; (B) dose responses. (A) FH 109 fibroblasts grown at a confluency of 30% were cultured in the presence or absence of TNF-α (25 ng/ml) for 2 to 72 h. (B) The culture period was 48 h, and rhTNF-α was used in the dose range of 1 to 100 ng/ml. In all experiments, [^{3}H]thymidine was present during the last 6 h of culture. After the appropriate culture time, cells were harvested and [^{3}H]thymidine incorporation was quantitated by scintillation counting. Values represent means ± standard deviation of three independent experiments. Each experiment was performed in triplicate (the standard deviation never exceeded 8% of the mean), and for further calculation means of triplicates were used.

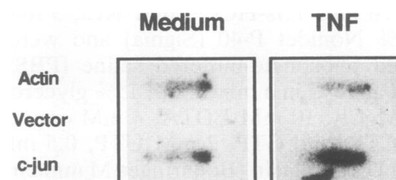

FIG. 2. Run-on assays with nuclei of TNF-stimulated fibroblasts. Cultures were treated for 20 minutes with 25 ng of TNF-α per ml (TNF) or were left untreated (Medium). RNA from a plasmid containing α-actin cDNA (Actin) and RNA from plasmid pBR322 without an insert (Vector) were used as positive and negative controls, respectively.

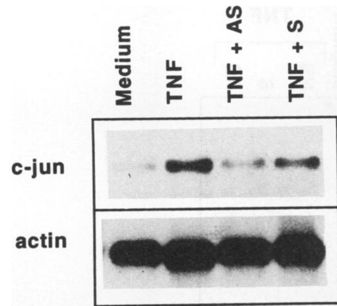

FIG. 3. Northern blot analysis probing for *c-jun* RNA accumulation in FH 109 cells. FH 109 cells were incubated for 4 h in the presence or absence of oligomers (AS or S) before TNF-α was added for a further 2-h incubation. Control cultures (Medium) received medium only. RNA was hybridized with a *c-jun*-specific cDNA. The filters were reprobed with α-actin cDNA controls for integrity of RNA and comparable loading in single lanes.

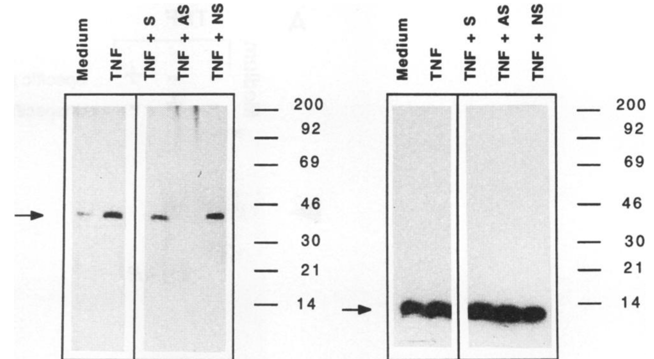

FIG. 4. Immunoprecipitation of FH 109 cell lysates with anti-c-Jun/AP-1 and anti-β2-microglobulin monoclonal antibodies. FH 109 cells either were left untreated for 16 h and then exposed to medium or TNF-α for a further 6 h or were cultured in the presence of S, AS, or NS oligonucleotides for 16 h and then exposed to TNF-α for an additional 6 h. Cell lysates were immunoprecipitated as detailed in Materials and Methods. The arrows show the specific c-Jun/AP-1 protein (left panel) and the specific β2-microglobulin complex (right panel). Molecular mass markers are indicated on the right in kilodaltons.

was compared with the amount of radioactivity left in the culture medium. Approximately 1% of the radioactivity was associated with the cell pellet after 1 h of culture (data not shown). This fraction further increased to 5.6% of the total radioactivity after 4 h of culture and remained at that level for up to 24 h. Comparable results were obtained for the S and NS oligodeoxynucleotides. In order to assess oligodeoxynucleotide stability, denaturing gel electrophoresis was performed with lyophilized aliquots of lysed cells. In the intact cell pellets, the AS oligonucleotide was stable for up to 24 h. Comparable results were obtained for the S and NS oligodeoxynucleotides (data not shown). Next, we assessed intracellular duplex formation of *c-jun* mRNA and the respective oligonucleotides as previously described by Holt et al. (17). To this end, FH 109 cells were cultured in the presence or absence of the respective S or AS oligonucleotide for 4 h, and mRNA was extracted. An S1 nuclease-resistant duplex of endogenous mRNA and the 5′-end-labeled oligomer was detectable in RNA extracted from FH 109 cells cultured for 4 h in the presence of AS oligomers but not in RNA extracted from cells cultured with S or NS oligomers (data not shown). We also failed to detect duplex formation in the S1 nuclease assay when labeled oligomers were added after the culture period and immediately before RNA extraction, indicating that oligomer-mRNA duplexes had been formed intracellularly and not during the RNA preparation (for details, see Materials and Methods).

Next, we studied whether intracellular duplex formation was associated with modulation of *c-jun* mRNA accumulation and c-Jun protein biosynthesis. To this end, Northern blot analyses of FH 109 cells treated with medium only or exposed to TNF-α with or without AS or S oligonucleotides were performed. As shown in Fig. 3, AS but not S oligomers were capable of interfering with the capacity of TNF-α to induce *c-jun* transcripts. Although the AS oligonucleotide is directed against the translation initiation site, inhibition of *c-jun* mRNA accumulation by TNF-α may be explained by positive autoregulation of the *c-jun* gene through its own product, c-Jun/AP-1. Inhibition of c-Jun/AP-1 synthesis by AS oligonucleotides may therefore contribute to the failure of TNF-α to induce *c-jun* transcripts.

In the next set of experiments, we studied the effects of AS, NS, and S oligonucleotides on c-Jun synthesis. FH 109 cells were exposed to TNF-α in the presence or absence of AS, S, or NS oligomers, and c-Jun/AP-1 protein expression was assessed by immunoprecipitation. TNF-α present in the culture over a period of 6 h enhanced synthesis of c-Jun/AP-1, while synthesis of the β2-microglobulin protein remained unaffected. When FH 109 cells were pretreated with AS, NS, or S oligonucleotides and then stimulated with TNF-α, c-Jun/AP-1 synthesis was impaired in cells exposed to AS but not to S or NS oligonucleotides (Fig. 4). Again, the protein biosynthesis of β2-microglobulin remained unaffected. Taken together, these findings indicate that intracellular duplex formation of the AS oligonucleotide and the *c-jun* mRNA is associated with complete inhibition of TNF-α-induced c-Jun protein synthesis. These findings could be further substantiated by gel mobility shift assays. As shown in Fig. 5, c-Jun/AP-1 binding was elevated approximately fourfold after TNF exposure for 2 h in S- or NS-oligomer-pretreated cultures. However, when cells were pretreated with the AS oligomer, c-Jun/AP-1 binding was less than that in nuclear extracts from untreated FH 109 cells, suggesting that the AS oligomer had efficiently blocked TNF-α-induced and constitutive c-Jun/AP-1 binding. In contrast, binding of an unrelated transcription factor, NF-κB, which is also known to be inducible by TNF-α (26), remained unaffected by AS, S, or NS oligomers in the culture medium (data not shown).

Taken together, these findings indicate that c-Jun/AP-1 synthesis is efficiently inhibited in fibroblasts by specific AS oligomers but not by S or NS oligomers. Therefore, the following experiments were designed to assess the role of c-Jun/AP-1 in TNF-α-induced proliferation. FH 109 cells were seeded in standard culture medium in the presence or absence of TNF-α with either AS, S, or NS oligomers present in the culture medium, and [^{3}H]thymidine incorporation was quantified by liquid scintillation counting. The AS oligomer directed against *c-jun* efficiently abolished the TNF-induced proliferative response; the optimal AS oligomer concentration was 8 μM. Both S and NS oligomers, however, had no significant effect, except at a concentration

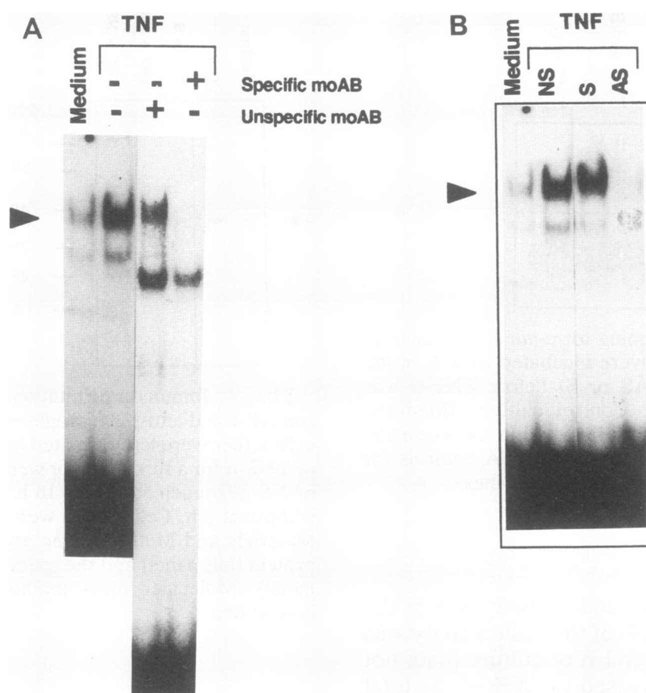

FIG. 5. Electrophoretic mobility shift assay of nuclear extracts obtained from FH 109 cells. (A) Cells were cultured in standard culture medium (Medium) or were exposed to TNF-α for 2 h (TNF). The specificity of complexes was demonstrated by a monoclonal antibody (moAB) (50 ng/ml; Oncogene Science) directed against the binding epitope of c-Jun/AP-1. The unspecific monoclonal antibody used in this system was directed against the c-Rel–NF-κB transcription factor (Neosystem Laboratoire, Strasbourg, France) and was used at the same concentration as the specific monoclonal antibody. (B) FH 109 cells were incubated in the presence or absence of oligomers (NS, S, or AS) for 4 h before TNF was added and incubation was continued 2 h. Control cultures received medium only.

of 16 μM, at which toxicity was observed (Fig. 6). The last set of experiments aimed at extending this observation to another cell type, IL-3-costimulated primary AML blasts, which also respond proliferatively to TNF (6). AML blasts respond to TNF-α with enhanced synthesis of c-Jun/AP-1; in contrast, IL-3 fails to mediate c-Jun/AP-1 formation by these cells (Fig. 7). These findings prompted us to investigate the role of c-Jun/AP-1 in TNF-α or IL-3 costimulation. AML blasts from five different patients were seeded in serum-free culture medium containing rhIL-3 (25 ng/ml) in the presence or absence of TNF-α with oligomers directed against *c-jun* and [^{3}H]thymidine uptake was assessed after 48 h by liquid scintillation counting. While the AS oligomers relieved TNF-stimulated growth in the presence or absence of IL-3 (Fig. 8), both S and NS oligomers had almost no effect (not shown). Also, cultures performed in the presence of IL-3 only were unaffected by the anti-*c-jun* AS oligomer (Fig. 8), confirming previous studies in which we demonstrated that IL-3 does not affect transcription of *c-jun* (15).

DISCUSSION

Upon binding to its receptor, TNF-α initiates a complex signaling cascade which includes the activation of phospholipase A_2 and subsequent release of arachidonic acid and the activation of protein kinase C, phospholipase C, and serine/threonine and tyrosine-specific protein kinases (30). Several cellular proteins, such as the heat shock protein hsp27 (18) and the transcription factor NF-κB (26), have been identified as substrates of TNF-α-activated protein kinases. It is well established that transcription factors couple extracellular signals to the intracellular response. These factors represent key regulators of gene expression and thereby direct cellular proliferation and differentiation, depending on both the nature of the incoming signal and the cell type being targeted. In contrast to the NF-κB transcription factor is the AP-1 transcription factor, which is not considered to be an immediate TNF messenger molecule but is believed to function as an effector molecule at the end of the TNF signaling pathway by regulating expression of a secondary set of genes (30). In the present study, we addressed the role of the c-Jun/AP-1 transcription factor in the mitogenic response of human fibroblasts and primary AML blasts to TNF-α. The *c-jun* proto-oncogene encodes c-Jun, a major component of the AP-1 transcription factor complex (2). c-Jun/AP-1 is capable of forming homodimers and heterodimers with various other members of the Jun-Fos family (32). These complexes bind with different affinities to a heptameric consensus sequence, also referred to as the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element, which has been identified in numerous genes, including *c-jun*, where it is located in the 5′ regulatory region (2, 3). Accumulation of *c-jun* transcripts has been linked to the induction of proliferation and to the initiation of differentiation (28, 31). Recently, it has been shown that *c-jun* also participates in the cellular response to DNA-damaging agents (13). However, no experimental evidence has been provided to suggest that c-Jun/AP-1 is a prerequisite for these cellular responses to occur. In the present study, we demonstrated not only that TNF-induced proliferation of various target cells is accompanied by increases in *c-jun*

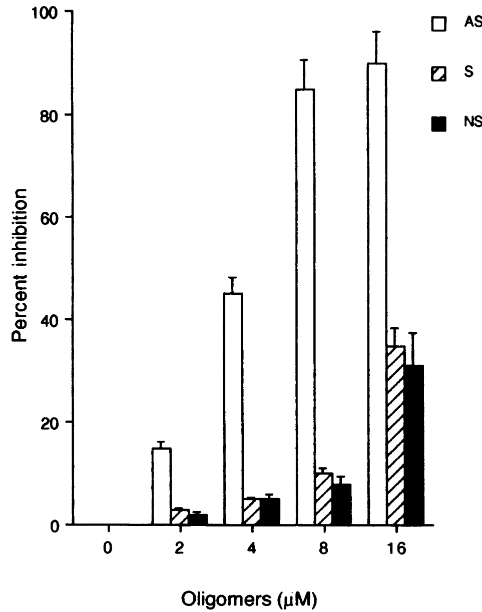

FIG. 6. Dose kinetics of the effect of c-*jun* AS, S, and NS oligodeoxynucleotides on TNF-induced proliferation of FH 109 fibroblasts. FH 109 cells grown at a confluency of 30% were cultured in culture medium supplemented with TNF-α (25 ng/ml) in the presence or absence of AS, S, or NS oligodeoxynucleotides (2 to 16 μM) for 48 h (oligodeoxynucleotides were added every 18 h), with [^{3}H]thymidine being present during the last 6 h. [^{3}H]thymidine incorporation was quantitated by scintillation counting. Values are expressed as percent inhibition in comparison with TNF-stimulated cultures in the absence of oligomers and represent means ± standard deviations of three independent experiments. Each experiment was performed in triplicate (the standard deviation never exceeded 10% of the mean), and for further calculation means of triplicates were used.

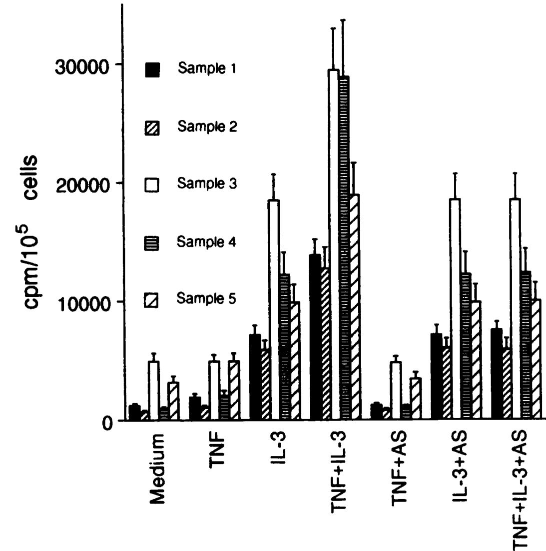

FIG. 8. Effect of AS oligodeoxynucleotides directed against c-*jun* on [^{3}H]thymidine uptake by AML blasts. Blasts from five different patients with AML (samples 1 to 5) were seeded into IL-3-containing serum-free culture medium (Medium) or with or without TNF-α (25 ng/ml) in the presence or absence of the AS oligodeoxynucleotide (added every 18 h at a concentration of 8 μM) and incubated for 48 h. During the last 6 h, the cultures were incubated in the presence of [^{3}H]thymidine. [^{3}H]thymidine incorporation was quantitated by scintillation counting. Values represent means ± standard deviations of two independent experiments. Each experiment was performed in triplicate (the standard deviation never exceeded 7% of the mean), and for further calculation means of triplicates were used.

mRNA synthesis but that this proliferative response is also abolished when c-Jun/AP-1 is eliminated with specific AS oligomers. Our data indicate for the first time that activation of c-Jun/AP-1 plays a pivotal role in the signaling cascade initiated by TNF-α, leading to a proliferative response of its target cells. These results are further substantiated by recent studies demonstrating that c-*jun* is essential for cell cycle progression (20) and DNA replication (24).

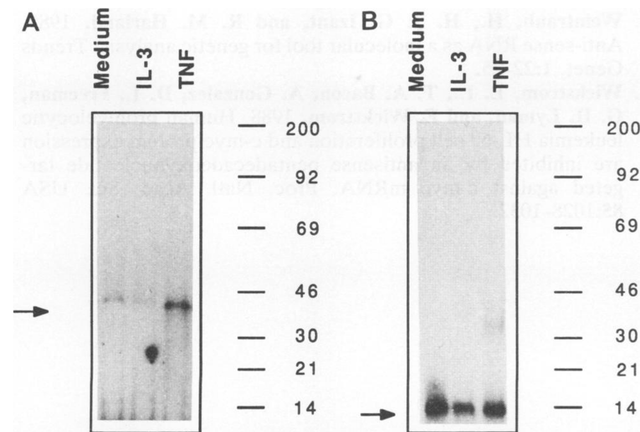

FIG. 7. Immunoprecipitation of AML cell lysates with anti-c-Jun/AP-1 (A) or anti-β2-microglobulin (B) monoclonal antibodies. AML blasts were cultured in medium or in the presence of IL-3 or TNF-α for 6 h. Cell lysates were immunoprecipitated as detailed in Materials and Methods. The arrows show the specific c-Jun/AP-1 protein (A) and the specific β2-microglobulin protein (B). Molecular mass markers are indicated on the right in kilodaltons.

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