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Identification of NF-jun, a novel inducible transcription factor that regulates c-*jun* gene transcription

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In this study we report the identification of a novel transcription factor, termed Nuclear Factor-jun (NF-jun). This factor contributes to inducible transcription of the c-*jun* gene in human myeloid leukemia cells. NF-jun was, however, undetectable in nuclear proteins from human monocytes, granulocytes, resting T lymphocytes and lung fibroblasts. NF-jun shares several features with the well characterized NF-κB in that binding activity can be generated in cytosolic extracts by treatment with dissociating agents. In addition, binding of NF-jun to its recognition site is enhanced by treatment of cells with 12-*O*-tetradecanoylphorbol-13-acetate, tumor necrosis factor alpha or the protein synthesis inhibitor cycloheximide (CHX). However, as revealed by competition assays and electrophoretic mobility shift assays, purified NF-κB fails to bind to the c-*jun* fragment which contains the NF-jun site, and this fragment fails to compete with NF-κB for binding. UV crosslinking showed that NF-jun contains a 55 and a 125 kDa protein species. These findings demonstrate that the c-*jun* gene can be regulated by a transcription factor distinct from AP-1. Our findings also indicate that while NF-jun has several features in common with the NF-κB binding protein including its subcellular localization and its ability to translocate from the cytoplasm to the nucleus, this factor recognizes a unique DNA sequence. Moreover, the activity of this protein is differentially regulated in various cell types. NF-jun might function as a signal transducing molecule in order to mediate rapid induction of the early response gene c-*jun* in a cell type- and stimulus-specific manner.

Key words: binding specificity/c-*jun* gene/NF-jun/transcription factors

Introduction

The c-*jun* proto-oncogene belongs to a class of proto-oncogenes which code for nuclear proteins that regulate various aspects of cell growth and differentiation. As an immediate early response gene, expression of the c-*jun* gene is inducible by serum, active phorbol esters, ionizing radiation and tumor necrosis factor alpha (TNF-α) (Ryder and Nathans, 1988; Ryseck *et al.*, 1988; Lamph *et al.*, 1989; Sherman *et al.*, 1990a; Brenner *et al.*, 1989). The c-*jun* gene product is a major component of the transcription factor AP-1 which binds to the heptameric DNA consensus sequence TGAG/CTCA (TRE) (Angel *et al.*, 1989). Binding affinity of the Jun Ap-1 is determined by dimerization with other transcription factors such as c-*fos* via a leucine zipper motif (Chiu *et al.*, 1988). Binding sites for AP-1 have been identified in several growth factor and cytokine genes (Shoemaker *et al.*, 1990; Taniguchi, 1988). Recent studies have indicated that binding to the AP-1 consensus sequence within the c-*jun* promoter positively autoregulates expression of its own gene (Angel *et al.*, 1988).

Regulation of c-*jun* expression in myeloid leukemia cells treated with 12-*O*-tetradecanoylphorbol-12-acetate (TPA), 1,25-dihydroxyvitamin D3, TNF-α or the DNA damaging agent 1-β-D-arabinofuranosylcytosine (Ara-C) is controlled at the transcriptional level (Sherman *et al.*, 1990b; Kharbanda *et al.*, 1990). The molecular mechanisms which regulate transcriptional activation of c-*jun* are, however, poorly understood. We have recently shown that activation of AP-1 by Ara-C is involved in induction of the c-*jun* gene in human myeloid leukemia cells. Furthermore, these findings suggested that a site upstream of the Sp1 box contributes to basal and inducible transcriptional activity of c-*jun* in these cells (Brach *et al.*, 1992).

In this article we show that a transcription factor distinct from AP-1 and NF-κB mediates transcriptional activation of the c-*jun* gene. This factor, designated NF-jun (Nuclear factor-jun), recognizes an 11 bp sequence within the c-*jun* promoter. NF-jun has several features in common with the transcription factor NF-κB, including its subcellular localization and its inducibility by TPA, TNF-α and the protein synthesis inhibitor cycloheximide (CHX). Similar to NF-κB, NF-jun also translocates from the cytoplasm to the nucleus upon stimulation. Binding of NF-jun to its recognition site is enhanced in the presence of guanosine triphosphate (GTP). Expression of NF-jun appears, however, to be restricted to rapidly proliferating cells such as myeloid leukemia cells, and is not detectable in non-proliferating diploid lung fibroblasts, blood monocytes, granulocytes or resting T-cells.

Results

We have previously shown that the c-*jun* promoter is activated in human myeloid cells by Ara-C due to enhanced binding of the transcription factor AP-1. In addition, our data also indicated that an enhancer element distinct from AP-1 located between positions −166 and −128 of the c-*jun* promoter confers inducibility of the c-*jun* gene (Brach *et al.*, 1992). In order to determine whether this fragment contains a transcriptional enhancer element, the f(−166/−115) fragment was subcloned in front of the herpes thymidine kinase promoter (TK) and linked to the human growth hormone gene (hGH) as a reporter [designated as p(NF-jun)TKGH]. As shown in Figure 1A, growth hormone activity (hGH) in supernatants of TNF-α treated KG-1 cells transfected with p(NFjun)TKGH increased 4-fold, while peripheral blood

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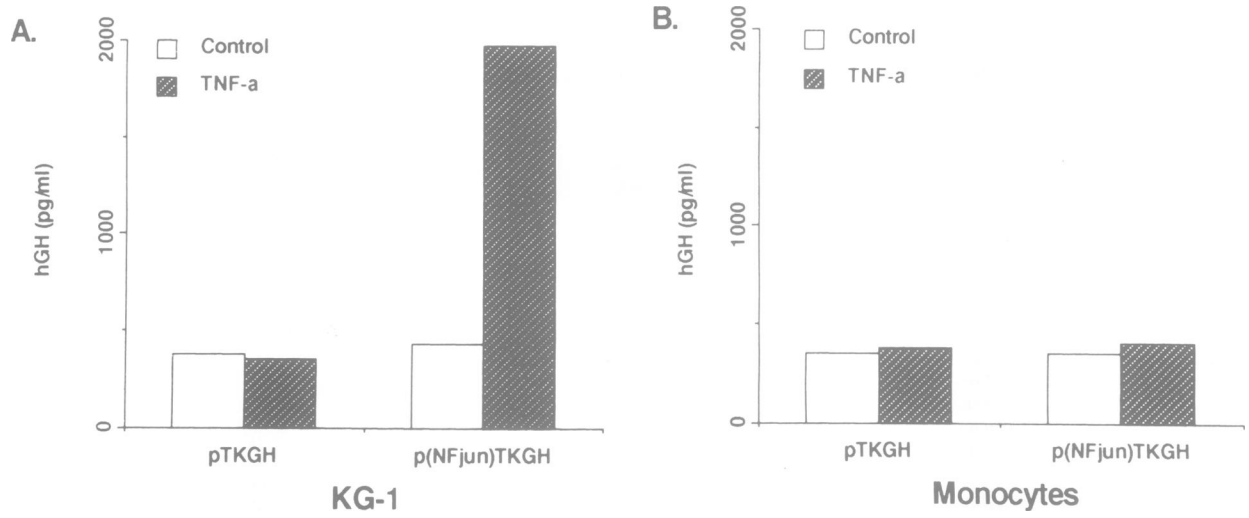

Fig. 1. TNF-α induces enhancing activity of the −166/−115 c-*jun* promoter fragment linked to the TK promoter. The −166/−115 c-*jun* promoter fragment was inserted in both orientations upstream of the TK promoter and linked to the growth hormone reporter gene. Plasmids [(pTKGH as a control plasmid and p(NF-jun)TKGH] were transfected into KG-1 cells (**A**) and monocytes (**B**) using the DEAE dextran technique. Cells were maintained in SCM for 24 h after transfection and then for an additional 24 h in the absence (open bars) or presence (hatched bars) of TNF-α (100 U/ml). Human GH activities were assessed in cell-free culture supernatants by enzyme-linked immunoassay. Shown are the results obtained with the NF-jun fragment inserted in the sense direction. Comparable results were obtained with the NF-jun fragment inserted in the antisense direction. Two separate experiments gave identical results.

monocytes (PMBO) failed to respond with any increase in hGH synthesis under identical conditions. Both KG-1 cells and PBMO transfected with the control plasmid (pTKGH) showed no increase in hGH activity upon stimulation with TNF-α. Comparable results were obtained when transfections were performed with a plasmid containing the f(−166/−115) fragment in antisense direction 5′ to the TK promoter (data not shown). These data provide support for the notion that an enhancer element that confers inducibility by TNF-α to a heterologous promoter is located between positions −166 and −115 of the c-*jun* promoter. Similar results were obtained when the leukemic cell lines THP-1, U 937, HL-60 and Mono Mac 1 were studied (data not shown). In contrast, there was no evidence for activation of this fragment in PBMO (Figure 1B).

These results prompted further analysis of the interaction between constitutively expressed and TNF-α-induced nuclear proteins and their potential *cis*-regulatory elements. While the transfection data suggested that a regulatory protein recognizes a site within this fragment, computer analysis revealed that this region does not contain a known transcription factor binding site (Ghosh, 1990). Therefore, DNA footprinting analysis was performed to determine the binding sequence of this factor. To this end, the 3′ end-labeled fragment f(−166/−103) of the c-*jun* promoter containing the Sp1 binding site as an internal control was incubated with various amounts of nuclear extracts from untreated and TNF-α treated KG-1 cells. As shown in Figure 2, the Sp1 site at the 3′ end of this fragment was equally protected when using nuclear extracts from untreated or TNF-α treated cells. Binding of this protein was competed with 25-fold molar excess of the synthetic oligonucleotide containing the Sp1 consensus sequence (data not shown) and was not enhanced upon exposure to TNF-α. In addition, an 11 bp fragment 5′ to the Sp1 site at positions −139 to −129 was protected (Figure 2). In contrast to the Sp1 footprint, however, protection was less pronounced when proteins from untreated KG-1 cells were used as compared with the footprint

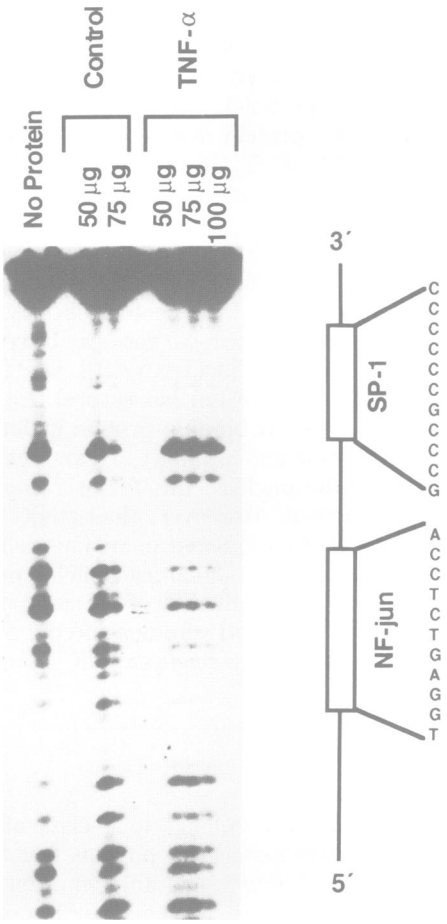

Fig. 2. DNase I analysis of protein binding to the −166/−103 c-*jun* promoter fragment. End-labeled DNA was incubated with the indicated amounts of nuclear proteins from untreated and TNF-α treated KG-1 cells. The first lane shows a control digest without addition of protein. G and G+A bases were determined for sequencing (not shown).

Table I. Expression of NF-jun in various human cell types

Origin	Cell type	Constitutive	TPA	TNF-α	CHX
Myelo/monocytic leukemia	KG-1	+[a]	++	++	++
Myelo/monocytic leukemia	HL-60	+	++	++	++
Myelo/monocytic leukemia	U 937	+	++	++	++
Myelo/monocytic leukemia	THP-1	+	++	++	++
Myelo/monocytic leukemia	Mono Mac 1	+	++	++	++
Hodgkin's disease	KM-H2	+	−	n.i.[b]	n.i.
Hodgkin's disease	HDLM-2	+	++	n.i.	n.i.
Embryonic lung fibroblasts	FH109	−	−	−	−
	monocytes	−	−	−	n.i.
	T-cells	−	(+)	(+)	(+)

[a]−, no binding detectable; (+), weak binding; +, constitutive binding; ++, enhanced binding upon stimulation with the respective agent.
[b]n.i., not investigated.

obtained with comparable amounts of nuclear proteins extracted from TNF-α treated KG-1 cells. These data suggested that TNF-α induces a DNA binding protein in human myeloid leukemia cells which recognizes an as yet unidentified binding sequence in the c-*jun* promoter. We therefore designated this element NF-jun.

Next, electrophoretic mobility shift assays (EMSAs) with the −166/−103 fragment of the c-*jun* promoter containing the Sp1 binding site were performed. As shown in Figure 3, two faint but readily detectable retarded bands were apparent, when nuclear proteins extracted from untreated KG-1 cells were incubated with this labeled fragment. The intensity of these bands was enhanced 3- to 4-fold when nuclear extracts from TNF-α treated KG-1 cells were analyzed. Comparable results were obtained with the THP-1, HL-60, U 937 and Mono Mac 1 cells (Table I). Both retarded bands were efficiently competed in the presence of 25-fold molar excess of an unlabeled synthetic oligonucleotide containing the 11 bp binding sequence determined in footprinting analysis. Moreover, both untreated and TNF-α stimulated cells displayed an additional retarded band which could be competed with 25-fold molar excess of unlabeled synthetic oligonucleotide containing the Sp1 binding site. Intensity of the Sp1 band did not change regardless of whether nuclear proteins derived from unstimulated or TNF-α exposed KG-1 cells were employed (Figure 3).

The next set of experiments was designed to study the functional role of the novel DNA binding protein NF-jun. To this end, the −286/+170 *Sma*I−*Sma*I c-*jun* promoter fragment was linked to the human growth hormone gene as a reporter yielding p(−286)GH (Figure 4A). Subsequently, the NF-jun binding site was deleted by site directed mutagenesis yielding p(−286, mt) GH (Figure 4A). Both constructs were transiently transfected into the myeloid leukemia cell line KG-1 and reporter gene activity of untreated and TNF-α treated cells was assessed in cell free supernatants. While KG-1 cells transfected with p(−286)GH responded to TNF-α with 3- to 4-fold enhanced hGH activity (Figure 4B), KG-1 cells transfected with the p(−286, mt)GH

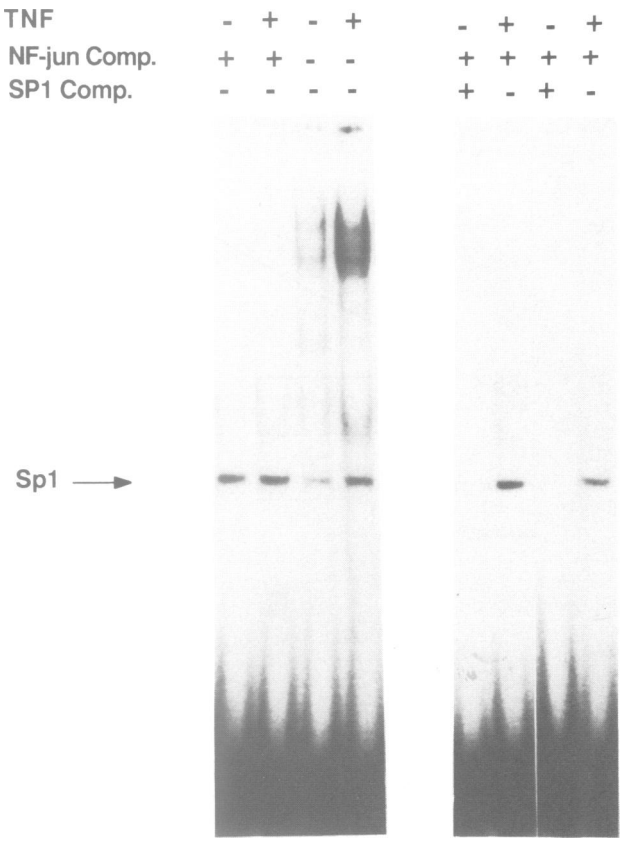

Fig. 3. Binding of constitutive and TNF-α induced nuclear proteins to the −166/−103 c-*jun* promoter fragment. The fragment was end-labeled and purified by polyacrylamide gel electrophoresis. Binding assays were performed with ~1 ng of labeled DNA (10 000 c.p.m.) and 10 μg nuclear proteins extracted from untreated (−) or TNF-α treated (+) KG-1 cells. Incubations were performed in the presence (+) or absence (−) of specific oligonucleotides containing the Sp1 or the NF-jun recognition sequence. The arrow indicates the Sp1 complex.

failed to respond to TNF-α. These data further underline the functional character of NF-jun.

The binding sequence of NF-jun (TGGAGCTCCA) shares considerable similarities with the recognition site of the well characterized transcription factor NF-κB (GGGGG GA TTTCC) of the human κB immunoglobulin gene (Shakhov *et al.*, 1990). The NF-κB consensus sequence was previously shown to undergo several alterations while still being recognizable by NF-κB proteins (Shakhov *et al.*, 1990). We therefore aimed at determining whether characteristics ascribed to NF-κB applied for NF-jun as well. NF-κB is known to be released from its inhibitor IκB in the cytoplasm upon phosphorylation of IκB (Bäuerle and Baltimore, 1988a; Ghosh and Baltimore, 1990). Furthermore, NF-κB binding activity can be generated from cytoplasmic extracts by treatment with dissociating agents such as sodium deoxycholic acid (DOC) or formamide (Bäuerle and Baltimore, 1988b). As shown in Figure 5, cytoplasmic extracts obtained from untreated KG-1 cells disclosed binding activity with the −166/−115 fragment of the c-*jun* promoter in the presence, but not the absence of DOC and formamide. Cytoplasmic extracts from TNF-α treated cells, however, failed to display binding activity in the presence or absence of DOC/formamide, suggesting that the factor NF-jun was already translocated to the nucleus

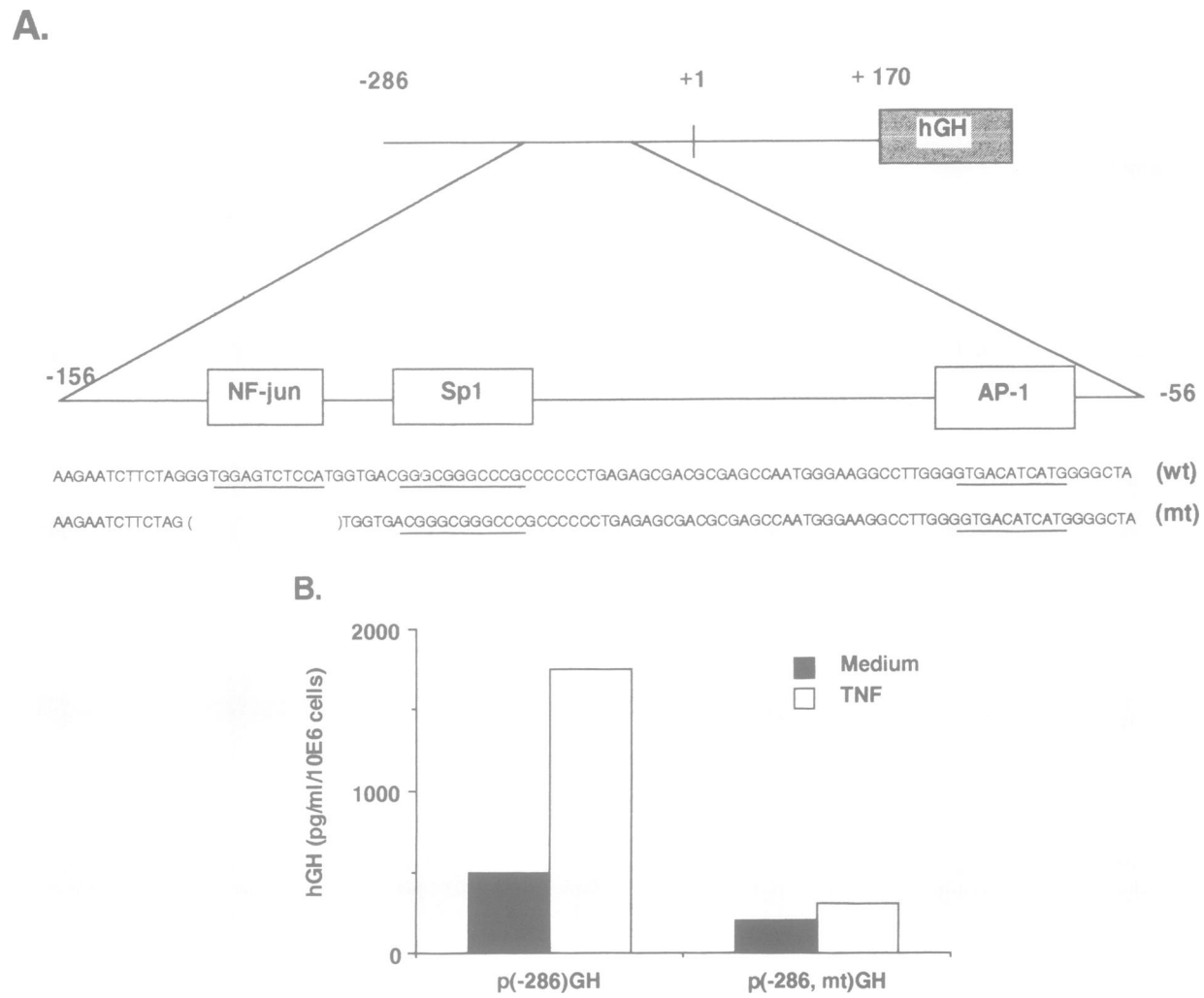

Fig. 4. TNF-α induces transcriptional activity of the c-*jun* wt promoter, but not the c-*jun* promoter with deleted NF-jun binding site. (**A**) Schematic demonstration of the c-*jun* promoter constructs containing either the $-286/+170$ wt c-*jun* promoter linked to the human growth hormone gene [hGH; p(−286)GH] or the $-286/+170$ c-*jun* promoter with a deleted NF-jun binding site [p(−286, mt) GH]. (**B**) KG-1 cells (107) were transiently transfected with either construct and then maintained in medium (closed bars) only or exposed to TNF-α (open bars). hGH activity was assessed in cell-free supernatants.

in response to TNF-α. Control experiments with an unrelated oligonucleotide containing the AP-1 consensus sequence failed to bring about binding activity in the presence or absence of DOC/formamide of cytoplasmic extracts from untreated or TNF-α treated cells (not shown). Complex formation was inhibited in the presence of 25-fold molar excess of a synthetic oligonucleotide containing the NF-jun recognition sequence. In line with these data is our previous report demonstrating that the transcriptional activation of the c-*jun* gene occurs in the presence of the protein synthesis inhibitor CHX (Sherman *et al.*, 1990b). In addition, as previously shown for NF-$\varkappa$B (Sen and Baltimore, 1987), CHX treatment was associated with enhanced binding of NF-jun to its recognition site. Finally, guanosine triphosphate (GTP) is known to specifically enhance binding of the NF-$\varkappa$B protein to its consensus sequence as shown in EMSAs (Leonardo *et al.*, 1988). The present findings demonstrate that binding of NF-jun isolated from TNF-α treated KG-1 cells is also enhanced when 3 mM GTP is added to the incubation mixture (Figure 6).

Taken together, these data suggested that NF-jun and NF-$\varkappa$B are highly related in their functional characteristics and share similarity in their respective recognition sites. We therefore tested whether NF-jun and NF-$\varkappa$B can interact with their respective binding sites. Competition assays with the labeled $-166/-115$ fragment using an unlabeled oligonucleotide containing the NF-$\varkappa$B consensus sequence (GGGG GA TTTCC) revealed that a 50- to 100-fold molar excess of unlabeled oligonucleotide was necessary to show some inhibition in binding, but not sufficient to completely inhibit binding of the NF-jun factor to its recognition site within the *jun* promoter (not shown). These data indicate that the binding site for NF-$\varkappa$B is only to a limited extent recognized by NF-jun. We next determined whether the NF-$\varkappa$B protein would recognize the 11 bp site of the c-*jun* promoter. To this end, NF-$\varkappa$B protein, purified from human placenta (Zabel *et al.*, 1991) was incubated with the f($-166/-115$) fragment under conditions previously described (Bäuerle and Baltimore, 1988b). As shown in Figure 7, 50 pg of purified NF-$\varkappa$B protein did not recognize the $-166/-115$ fragment, but showed strong binding to an oligonucleotide containing the NF-$\varkappa$B consensus sequence. These data indicate that NF-jun has several features in common with the NF-$\varkappa$B transcription factors, but that both

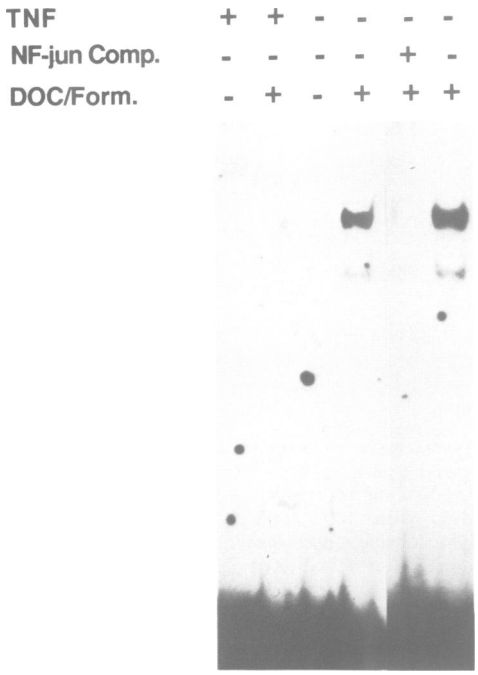

Fig. 5. Binding of constitutive or TNF-α induced cytoplasmic proteins to the −166/−115 c-*jun* promoter fragment. Cytoplasmic proteins from untreated (−) or TNF-α treated (+) KG-1 cells were incubated with the end-labeled −166/−115 c-*jun* fragment in the absence (−) or presence (+) of the dissociating agents sodium DOC (0.2%) and formamide (7.5%). Competition assays were performed in the presence (+) or absence (−) of excess unlabeled oligonucleotide containing the NF-jun recognition sequence.

Fig. 6. Binding of TNF-α induced nuclear proteins to the −166/−115 c-*jun* promoter fragment. Nuclear proteins from TNF-α treated KG-1 cells were incubated with end-labeled DNA fragment in the absence (−) or presence (+) of 3 mM GTP and a specific competitor oligonucleotide containing the NF-jun recognition sequence.

Fig. 7. Binding of purified NF-κB protein to the −166/−115 c-*jun* fragment and to an oligonucleotide containing the NF-κB consensus sequence. 50 pg of NF-κB protein purified from human placenta was incubated with the end-labeled −166/−115 c-*jun* promoter fragment (NF-jun) or an end-labeled oligonucleotide containing the NF-κB consensus sequence (NF-κB).

proteins recognize distinct binding sites and are not interchangeable.

We then analyzed the binding specificity of NF-jun. To this end a series of synthetic oligonucleotides containing various mutations of the NF-jun recognition site were made and binding affinity was tested by EMSA. As shown in Figure 8A replacement of the purines at positions 2 and 3 by pyrimidines as well as the two pyrimidines at positions 9 and 10 by purines resulted in complete loss of binding of NF-jun to this site. In contrast, single base mutations on either side of the pallindrome did not abrogate NF-jun binding completely, but still preserved some affinity. Specificity of complex formation was confirmed by competition assays with a 25-fold molar excess of unlabeled NF-jun wild-type oligonucleotide (not shown). Functional consequences of mutations were analyzed in additional experiments. Oligomers of mutant oligonucleotides (m1 and m5) as well as the wild-type oligonucleotide (wt) were subcloned as enhancer elements in front of the heterologous herpes thymidine kinase promoter and were transiently transfected into KG-1 human myeloid leukemia cells. hGH activity was then assessed in supernatants. As shown in Figure 8B, KG-1 cells transfected with the wt enhancer construct responded to TNF-α with an ~5-fold increase in hGH release into the supernatant. In contrast, cells transfected with the m1 enhancer construct did not respond to TNF-α treatment, and also cells transfected with the m5 enhancer construct showed no significant increase in hGH synthesis.

In order to determine the molecular size of NF-jun, UV crosslinking experiments were performed. As shown in Figure 9, the NF-jun protein gives rise to protein−DNA adducts of 55 and 125 kDa in size. Homo- and heterodimer formation of these proteins might account for the appearance of two complexes in EMSAs.

We next studied whether NF-jun is also expressed by PBMO and by cells other than those of the myeloid/monocytic lineage. As already suggested by data obtained from the transfection experiments (Figure 1B), PBMO failed to

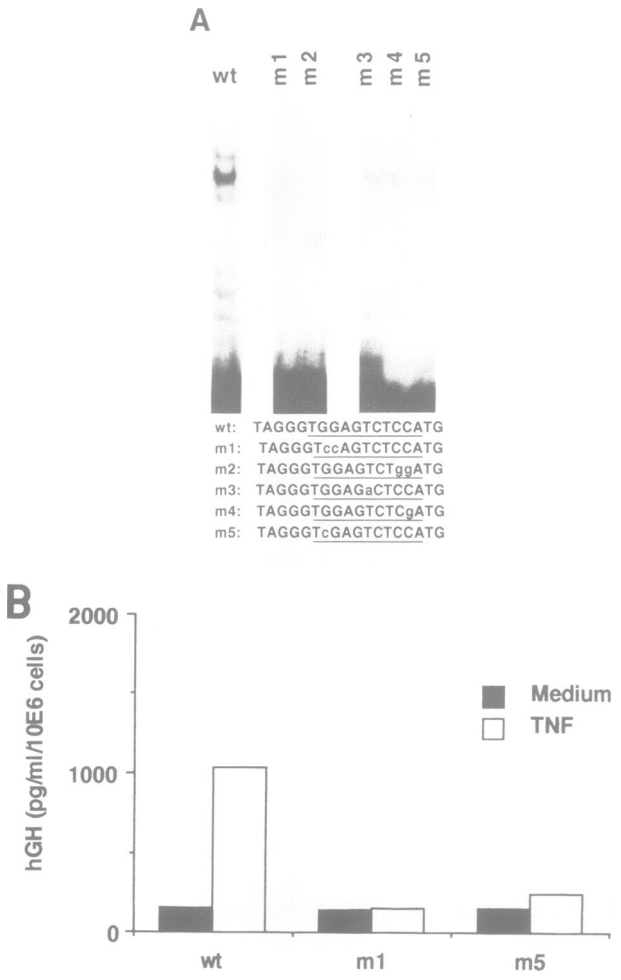

Fig. 8. (**A**) Electrophoretic mobility shift assay with nuclear proteins from TNF-α (400 U/ml; 4 h) treated KG-1 cells. Nuclear proteins (10 μg) were incubated with end-labeled synthetic oligonucleotides (10 000 c.p.m.) containing the wild-type NF-jun recognition sequence or various mutants as indicated. (**B**) Transfection assays with heterologous promoter constructs containing trimers of the m1, m5 or wt oligonucleotide (for details see A). hGH activity was assessed in supernatants of medium (closed bars) or TNF-α treated (open bars) KG-1 transfectants.

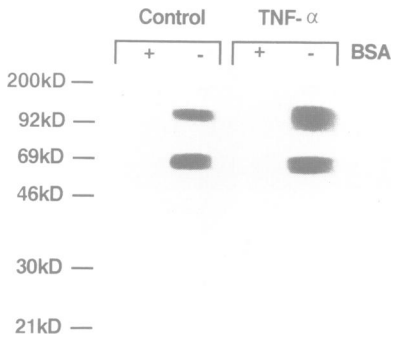

Fig. 9. Determination of the molecular size of the NF-jun protein by UV crosslinking analysis. The −166/−115 c-*jun* promoter fragment was body-labeled in the presence of [α-^{32}P]dCTP and bromodeoxyuridine triphosphate. 100 000 c.p.m. labeled DNA was incubated with either 100 μg bovine serum albumin or 100 μg nuclear extract from untreated or TNF-α treated KG-1 cells in the absence (−) or presence (+) of 25-fold molar excess unlabeled competitor DNA for 20 min and then subjected to UV irradiation (240 nm) for an additional 20 min. Samples were analyzed in a 10% SDS gel. Molecular sizes are indicated as determined with a standard molecular size marker.

display constitutive NF-jun binding (Table I) which was also not inducible by TNF-α or TPA. Two Hodgkin's disease derived cell lines, KM-H2 and HDLM-2 constitutively showed binding which was further enhanced upon stimulation with TPA in the HDLM-2 line, but down-regulated in KM-H2 cells (Table I). This finding is in concert with our previous observations showing that the transcription factor AP-1 is also differentially regulated by TPA and TNF-α in both cell lines (Gruβ *et al.*, 1992). Similar to PBMO, growth arrested human embryonic lung fibroblasts, granulocytes and resting human T cells failed to express NF-jun constitutively and also did not disclose binding activity with TPA stimulation.

Discussion

TNF-α is a potent inducer of the c-*jun* gene in human myeloid leukemia cells. Transcriptional activation of c-*jun* is enhanced 3- to 4-fold by TNF-α. This induction is independent of *de novo* protein synthesis (Sherman *et al.*, 1990b). We show that a 51 bp fragment of the c-*jun* promoter upstream of the Jun AP-1 site is able to confer a similar degree of transcription induction to of a reporter gene. This fragment contains a 11 bp sequence which is protected by a nuclear factor designated NF-jun in footprint analysis. Binding of NF-jun is enhanced by TPA, TNF-α and the protein synthesis inhibitor CHX.

Previous reports have indicated that rapid induction of c-*jun* is due to a post-translational modification of AP-1 and thereby modulation of this gene (Angel *et al.*, 1988). However, work by Angel *et al.* (1988) and Devary *et al.* (1991) has also suggested that sites further upstream of the AP-1 site may be involved in the transcriptional regulation of c-*jun*. The transcriptional regulation of the transcription factor gene c-*jun* is likely to be complex, differing from one cell type to another and possibly involves the interaction of a number of regulatory nuclear proteins. We have identified one of these factors, termed NF-jun, whose recognition site is located immediately 5′ of the Sp1 binding site. Our data further indicate that activation of NF-jun involves its translocation from the cytoplasm where it is present in an inactive form. In particular, the subcellular translocation of NF-jun suggests that this molecule functions in signal transduction in a manner previously shown for NF-κB (Bäuerle and Baltimore, 1988a). The finding that activation of NF-jun is independent of protein synthesis suggests that modification of a pre-existing molecule is sufficient for activation as it has also been demonstrated for NF-κB, the nuclear factors IL-6 DBP and AP-1 (Poli *et al.*, 1990; Boyle *et al.*, 1990). Activation of NF-jun can be induced by TNF-α or TPA, both potent activators of protein kinase C, and is followed by transcriptional induction of c-*jun*. Translocation of NF-jun into the nucleus might thus represent a step leading to signal dependent regulation of c-*jun* transcription. In this context, our preliminary data may be of interest showing that induction of differentiation of HL-60 leukemic cells along the granulocytic lineage with dimethylsulfoxide (DMSO) failed to enhance NF-jun binding, and that normal human granulocytes did not display NF-jun (M.A.Brach, unpublished results).

Since dissociating agents such as DOC or formamide are

capable of generating NF-jun activity in the cytoplasm of untreated cells, it would be of interest to determine whether the interaction between NF-jun and an inhibitory molecule in the cytoplasm is involved in the control of NF-jun activity. A similar interplay has been described for NF-κB and its cytoplasmic receptor molecule IκB (Bäuerle and Baltimore, 1988a). Further experiments are required to explore the exact mechanism controlling the release of NF-jun. It will also be of interest to identify relevant stimuli for NF-jun activation and particularly to clarify whether decreased expression in the diploid cells investigated is associated with the proliferation status of these cells. In this context, previous reports demonstrating that overexpression of c-*jun* participates in malignant transformation of normal mammalian cells (Schütte *et al.*, 1989) are of note.

Materials and methods

Cells and cell culture

The human myeloid leukemia cell lines KG-1, HL-60, U 937, THP-1 cells (American Type Culture Collection, Bethesda, MD) and Mono Mac 1 (Ziegler-Heitbrock *et al.*, 1988), the Hodgkin's Disease (HD) derived cell lines HDLM-2 and KM-H2 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and the embryonic lung fibroblast cells FH 109 (Mantovani *et al.*, 1990) were maintained in RPMI 1640 medium (Gibco Laboratories, Heidelberg, Germany) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 400 μg/ml streptomycin (Gibco)(= Standard Culture Medium, SCM) in 37°C at 5% CO_2 in humidified air. Human monocytes and T-cells were obtained from buffy coats of consenting healthy volunteer blood donors as previously described (Herrmann *et al.*, 1989). Cells were treated with 32 nM TPA (Sigma Chemicals Co., St Louis, MO), 100 U/ml TNF-α (Asahi Chemical Co., Tokyo, Japan) or 10 μg/ml cycloheximide (CHX; Sigma).

Promoter constructs

The *Asu*I−*Asu*I fragment of the c-*jun* promoter (position −166 to −115) was blunt ended and then subcloned into the blunt ended *Hin*dIII site of pTKGH (Selden *et al.*, 1986) yielding p(NFjun) TKGH. The *Sma*I−*Sma*I c-*jun* fragment (position −286 to +170) isolated from a genomic c-*jun* probe (kindly provided by P.Angel, San Diego, CA) was subcloned into the *Hin*cII site of p0GH (Selden *et al.*, 1986) referred to as p(−286)GH. In addition, the *Sma*I−*Sma*I fragment of the c-*jun* promoter (position −286 to +170) was subcloned into the *Sma*I site of pGEM 3 (Promega, Heidelberg, Germany) yielding pGEM3-jun. pGEM3-jun was then digested with *Nco*I and *Xba*I, the 160 bp *Xba*I−*Nco*I fragment was digested with *Mae*I, blunt ended and religated into the blunt ended *Nco*I−*Xba*I sites of pGEM3-jun yielding now pGEM3-jun, mt. Deletion of the binding site and orientation of religated fragments was confirmed by DNA sequencing using the Sanger dideoxy sequencing method (Sanger *et al.*, 1977), and is shown in part in Figure 4A. The *Sma*I−*Sma*I c-*jun* promoter fragment of pGEM3-jun, mt with the NF-jun binding site deleted was subsequently cloned into the *Hin*cII site of p0GH yielding p(−286, mt)GH. In addition, synthetic oligonucleotides containing the NF-jun binding site (wt), TAGGGTGGAGTCTCCATG or mutations of the NF-jun binding site (m1, TAGGGTccAGTCTCCATG; m5, TAGGGTcCAGTCTCCATG; binding sites are underlined), were phosphorylated, annealed, ligated and blunt ended and then inserted into the blunt ended *Hin*dIII site of pTKGH. Clones were sequenced and those containing trimers of the respective oligonucleotides were used for transfection assays.

Transient transfection and reporter gene assays

Leukemia cell lines and human blood monocytes were transfected by electroporation or the DEAE dextran technique as previously described (Toneguzzo *et al.*, 1986; Groschedl and Baltimore, 1985). Twenty-four hours after transfection, cells were split and cultured for an additional 24 h in the presence or absence of TNF-α (100 U/ml). hGH activity was assessed in cell-free supernatants using a commercially available ELISA assay (hGH EIA, Eurogenetics, Tessenderio, Belgium).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed essentially as previously described (Brach *et al.*, 1991). Briefly, nuclear extracts were prepared from untreated cells and cells exposed to TNF-α, TPA or CHX according to the method described by Dignam *et al.* (1983). Protein concentrations were determined by the Bradford assay (Bradford, 1976). The c-*jun* fragment from position −166 to −115 was end-labeled with [α-^{32}P]dCTP using DNA polymerase I (Stratagene, La Jolla, CA). This fragment was purified on a 12% polyacrylamide gel. End-labeled DNA (1 ng; ~10 000 c.p.m.) was incubated with 10 μg nuclear proteins in an incubation buffer containing 25 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA and 10% (v/v) glycerol for 20 min at room temperature. Competition assays were performed by adding up to 500-fold molar excess of unlabeled oligonucleotide containing the NF-κB, the AP-1- or Sp1 consensus sequence. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel. The gel was dried and exposed to a Kodak XAR film at −70°C using an intensifying screen.

DNase I footprinting analysis

DNase I footprinting analysis was performed as described (Angel *et al.*, 1987). Briefly, the end-labeled c-*jun* fragment from positions −166 to −103, containing the Sp1 site, (10 000 c.p.m.) was incubated with increasing amounts of nuclear proteins in a binding buffer as described (Angel *et al.*, 1987) for 30 min, digested with DNase I (Stratagene) for 1−2 min. DNA was purified by phenol−chloroform extraction, ethanol precipitation and analyzed in a 10% sequencing gel. The gel was dried and autoradiographed at −70°C using an intensifying screen.

UV crosslinking

UV crosslinking experiments were performed as previously described (Chodosh *et al.*, 1986). Briefly, the −166/−128 *jun* fragment was body-labeled in the presence of [α-^{32}P]dCTP, dATP, dGTP and bromodeoxyuridine triphosphate (Sigma), 100 000 c.p.m. of labeled fragment were incubated with 100 μg nuclear proteins as described above for 20 min at room temperature and were then exposed to UV light (240 nm) for 20 min. Samples were analyzed in a Laemmli buffer in a 10% SDS gel. The gel was dried and autoradiographed using an intensifying screen.

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