**Transcriptional and Post-transcriptional Regulation of c-jun Expression during Monocytic Differentiation of Human Myeloid Leukemic Cells**

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AP-1, the polypeptide product of c-jun, recognizes and binds to specific DNA sequences and stimulates transcription of genes responsive to certain growth factors and phorbol esters such as 12-0-tetradecanoylphorbol-13-acetate (TPA). We studied the effects of TPA on the regulation of c-jun gene expression in HL-60 cells during monocytic differentiation. Low levels of c-jun transcripts were detectable in untreated HL-60 leukemic cells, increased significantly by 6 h, and reached near maximal levels by 24 h of exposure to 32 nM TPA. Similar kinetics of c-jun induction by TPA were observed in human U-937 and THP-1 monocytic leukemia cells. Similar findings were obtained with bryostatin 1 (10 nM), another activator of protein kinase C and inducer of monocytic differentiation. Furthermore, 1,25-dihydroxyvitamin D$_3$ (0.5 μM), a structurally distinct agent which also induces HL-60 myeloid differentiation, increased c-jun expression. TPA treatment of HL-60 cells in the presence of cycloheximide was associated with superinduction of c-jun transcripts. Run-on analysis demonstrated detectable levels of c-jun gene transcription in untreated HL-60 cells, and that exposure to TPA increases this rate 3.3-fold. Treatment of HL-60 cells with both TPA and cycloheximide had no effect on the rates of c-jun transcription. The half-life of c-jun RNA as determined by treating HL-60 cells with TPA and actinomycin D was 30 min. In contrast, the half-life of c-jun RNA in TPA-treated HL-60 cells exposed to cycloheximide and actinomycin D was greater than 2 h. These findings suggest that the increase in c-jun mRNA observed during TPA-induced monocytic differentiation is mediated by both transcriptional and post-transcriptional mechanisms.

Avian sarcoma virus 17, isolated from a spontaneously growing chicken sarcoma, is a retrovirus which induces fibrosarcomas in chickens and neoplastic transformation of cultured chicken embryo fibroblasts (1). Avian sarcoma virus 17 contains the v-jun oncogene whose product is a 55-kDa gag-protein. The carboxyl-terminal portion of p55$^{\text{imm}}$-jun was shown to contain amino acid homology with the DNA-binding region of the yeast GCN4 regulatory protein (2). GCN4 binds to a specific DNA sequence which is also recognized by the mammalian transcriptional activator AP-1. Indeed, several investigators have demonstrated that the protooncogene c-jun-encoded product is structurally and functionally similar, if not identical, to AP-1 (3, 4).

The expression of c-jun is an immediate early event during activation of fibroblasts. For example, expression of c-jun is transcriptionally and post-transcriptionally regulated by known, platelet-derived growth factor, and fibroblast growth factor in mouse Balb/c 3T3 fibroblasts (5). c-jun transcription is rapidly induced during the G1 to G0 transition following serum stimulation of quiescent NIH 3T3 cells (6). Epidermal growth factor also stimulates transcription of c-jun in rat fibroblasts (7). Moreover, nerve growth factor transiently induces c-jun expression in rat PC12 pheochromocytoma cells (8), while stimulation of c-jun expression is an early event in several human and mouse cell lines after exposure to transforming growth factor-β (9). More recent studies have shown that tumor necrosis factor induces c-jun in both human fibroblasts and umbilical vein endothelial cells (10, 11). Other studies have demonstrated that induction of c-Ha-ras oncoprotein is associated with stimulation of c-jun expression in mouse NIH 3T3 fibroblasts using a heavy metal-inducible model (12).

Although AP-1 binds to DNA sequences that regulate transcription of genes responsive to phorbol esters (13-15), less is known about the effects of these agents on the transcriptional and post-transcriptional regulation of c-jun gene expression. For example, treatment of epithelial cells with 12-O-tetradecanoylphorbol-13-acetate (TPA)$^1$ is associated with stimulation of a c-jun promoter/reporter gene by an autoregulatory mechanism involving the AP-1 protein (16). Other studies have demonstrated that exposure of human fibroblasts to TPA results in enhanced c-jun expression (10). In human myeloid leukemia cells, treatment with TPA is associated with induction of monocytic differentiation (17). Similar findings have been obtained with other agents, such as bryostatin 1, which also activate protein kinase C (18). The effects of these agents on the regulation of c-jun gene expression during induction of monocytic differentiation, however, are unknown.

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* The abbreviations used are: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; SDS, sodium dodecyl sulfate; TRE, TPA-responsive element; kb, kilobase(s).

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tional and post-transcriptional mechanisms. We also demonstrate that bryostatin 1, and 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), another inducer of monocytic differentiation, also increase expression of the c-jun gene.

MATERIALS AND METHODS

Cell Culture—The HL-60 and U-937 cells were grown as previously described (19, 20). THP-1 monocytic cells were grown in RPMI 1640 medium containing 10% fetal bovine serum supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cell cultures were maintained in a humidified 5% CO₂ in air atmosphere at 37°C. Cells were treated with 32 nM TPA (Sigma), 0.5 μM 1,25-(OH)₂D₃ (Hoffmann-La Roche), or 10 nM bryostatin 1 (provided by Dr. George Pettit, Arizona State University). Actinomycin D and cycloheximide were obtained from Sigma.

Preparation of RNA and Northern Blot Hybridization—Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride technique as described (19). Total cellular RNA (20 μg) was subjected to electrophoresis in a 1% agarose, 2.2 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to one of the following 32P-labeled DNA probes: 1) the 1.8-kb BamHI/EcoRI insert of a human c-jun DNA probe containing 1.0-kb CDNA and 0.8-kb 3'-untranslated sequences (3) purified from a pBluescript SK(+) plasmid (provided by Dr. R. Tjian, University of California); and 2) the 2.0-kb PstI insert of a chicken β-actin CDNA gene purified from the pA1 plasmid (21). Hybridization reactions were carried out for 16–24 h at 42°C in 50% (v/v) formamide, 2 × SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1 × Denhardt's solution, 0.1% (w/v) sodium dodecyl sulfate (SDS), and 200 μg/ml salmon sperm DNA. Filters were washed and exposed to Kodak X-Omat XAR film using an intensifying screen. The autoradiograms were scanned using an LKB (Bromma, Sweden) Ultrosan XL laser densitometer and analyzed using the Gelscan XL software package. The intensity of c-jun hybridization was normalized against β-actin expression.

Run-on Transcriptional Analyses—HL-60 cells (10⁶/assay) were washed with ice-cold phosphate-buffered saline, and the nuclei were isolated by lysis in 0.5% Nonidet P-40 buffer as described (19). Nuclei were resuspended in glycerol buffer and incubated in an equal volume of reaction buffer containing 100 mM KCl, 0.5 mM each of ATP, GTP, and CTP, and 200 μCi of [α-32P]UTP (800 Ci/mmol, Du Pont-New England Nuclear) at 26°C for 30 min. The reaction was terminated by the addition of 100 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 20 mM MgCl₂, 150 units/ml RNasin, and 40 μg/ml DNase and incubated at 25°C for 15 min. After proteinase K digestion, the RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated in ethanol and 2.5 M ammonium acetate. The RNA was further purified by Sephadex G-50 column separation.

Plasmid DNAs containing various cloned inserts were digested with restriction endonucleases as follows: 1) the 2.0-kb PstI fragment of the chicken β-actin pA1 plasmid (positive control); 2) the 1.1-kb BamHI insert of the human β-globin gene (negative control, Ref. 22); and 3) the 1.8-kb BamHI/EcoRI fragment of the human c-jun cDNA from the pBluescript SK(+) plasmid. The digested DNA was run in a 1% agarose gel and transferred to nitrocellulose filters by the method of Southern. The filters were prehydrated in 5 × Denhardt's solution, 40% formamide, 4 × SSC, 5 μM Na*EDTA, 0.4% SDS, and 100 μg/ml yeast tRNA for 2 h. Hybridization was performed with 10⁶ cpm of 32P-labeled RNA/ml of hybridization buffer for 72 h at 42°C. The filters were then washed in 2 × SSC, 0.1% SDS at 37°C for 30 min, 10 μg/ml RNAse A in 2 × SSC at 37°C for 30 min, and 0.1 × SSC, 0.1% SDS at 42°C for 30 min. Autoradiography was performed for 3–10 days.

RESULTS

c-jun Transcripts Are Induced by TPA during Human Monocytic Cell Differentiation—The effects of TPA on c-jun expression were first studied during monocytic differentiation of HL-60 cells. Northern blot analysis of HL-60 cellular RNA using a c-jun cDNA probe is shown in Fig. 1. Low levels of a 2.7-kb c-jun transcript were detectable in untreated HL-60 cells, increased by 6 h, and reached near maximal levels by 24 h of exposure to 32 nM TPA. TPA treatment had no effect on levels of actin transcripts. Similar kinetics of c-jun induction were observed in TPA-treated human U-937 monocytic leukemia cells (Fig. 2A). Treatment of U-937 cells with TPA was also associated with the appearance of a minor 3.2-kb c-jun transcript (Fig. 2A). TPA-1 monocytic leukemia cells also have constitutive c-jun expression, and the level of c-jun transcripts was increased 2.3-fold after 24 h of exposure to TPA (Fig. 2B).

Other Inducers of Monocytic Differentiation Increase c-jun Expression—In contrast to TPA, treatment of HL-60 cells with bryostatin 1 was associated with a less rapid increase in c-jun transcripts. For example, low levels of c-jun RNA were present after 24 h, and maximal induction was observed after 48 h of exposure to bryostatin 1 (data not shown). Similar effects were observed following treatment of these cells with 0.5 μM 1,25-(OH)₂D₃ (Fig. 3). These findings suggested that...
multiple inducers of monocytic differentiation increase c-jun expression in myeloid leukemia cell lines.

c-jun Expression Is Regulated at Both the Transcriptional and Post-transcriptional Levels by TPA—In order to determine whether increased levels of c-jun RNA are associated with transcriptional activation and/or enhanced stabilization of the c-jun transcript, HL-60 cells were first treated with TPA in the absence and presence of cycloheximide. Little, if any, c-jun RNA was detectable after 2 or 4 h of TPA treatment. In contrast, treatment with cycloheximide alone was associated with an increase in the accumulation of c-jun RNA (Fig. 4). Moreover, the combination of cycloheximide and TPA for 2, 4, or 24 h increased levels of c-jun transcripts as compared with treatment with either TPA or cycloheximide alone (Fig. 4). These results indicated that cycloheximide superinduces c-jun expression, and thus de novo protein synthesis is not required for the induction of c-jun RNA by TPA.

Run-on assays were also performed to determine whether transcriptional mechanisms are responsible for the regulation of c-jun gene expression. c-jun gene transcription was detectable in untreated HL-60 cells (Fig. 5). Furthermore, exposure to TPA for 24 h was associated with a 3.3-fold increase in c-jun gene transcription. In contrast, cycloheximide had no effect on the rates of c-jun transcription (Fig. 5). These results suggested that cycloheximide affects c-jun expression by a post-transcriptional mechanism, perhaps by stabilizing the c-jun transcript.

In order to study the post-transcriptional regulation of TPA-induced c-jun RNA levels, HL-60 cells were treated with TPA for 18 h to induce c-jun expression and then exposed to actinomycin D for various times to inhibit further transcription. The half-life of c-jun RNA as determined by densitometric scanning was 30 min (Fig. 6). In contrast, inhibition of protein synthesis with cycloheximide in the absence of transcription increased the half-life of c-jun RNA in TPA-treated HL-60 cells to greater than 2 h (Fig. 6 and data not shown). Taken together, these findings suggested that the increase in c-jun RNA observed during TPA treatment is also mediated by post-transcriptional mechanisms involving the synthesis of a labile protein which affects the turnover of c-jun RNA.

DISCUSSION

The present results indicate that TPA induces c-jun expression in several myeloid leukemia cell lines. Bryostatin 1 treatment was also associated with increases in c-jun RNA levels, although the kinetics were somewhat less rapid than that observed with TPA. These findings suggested that, as in epithelial cells and fibroblasts (10, 16), activation of protein kinase C appears to play a role in the regulation of c-jun expression in hematopoietic cells. However, the present results also demonstrate that c-jun expression is increased in these cells following treatment with 1,25-(OH)2D3. This agent binds to a cytosolic protein and has not been reported to activate protein kinase C (23). Thus, other signaling mechanisms may regulate c-jun expression during monocytic differentiation. In this regard, cAMP may be involved in the regulation of c-jun expression in rat pheochromocytoma cells (8).

The present findings also suggest that post-transcriptional mechanisms requiring de novo protein synthesis regulate c-jun gene expression in myeloid cells. In this regard, inhibition of protein synthesis by cycloheximide superinduced levels of c-jun transcripts during TPA treatment. Furthermore, while cycloheximide had no effect on c-jun gene transcription, this agent did prolong the half-life of the c-jun transcript. These findings suggest that, as in the regulation of RNA levels for certain cytokines and other protooncogenes, c-jun transcripts may be selectively processed and degraded by labile ribonucleases in myeloid cells (24, 25). Similar mechanisms of post-transcriptional regulation by serum have been reported for the c-jun gene in mouse fibroblasts (5, 6) and are probably related to the presence of an AU-rich region in the untranslated 3' region of this transcript (3).

The product of the c-jun protooncogene contains a highly conserved DNA binding domain with high affinity for the TPA-responsive element (TRE). This domain, also shared by a family of mammalian transcription factors including junB, junD, c-fos, fosB, fia-1, as well as the yeast GCN4 protein, is composed of two regions, a leucine zipper and a region rich in basic amino acids (26). The leucine zipper allows for dimerization between certain members of this family while the basic motif is necessary for binding to the TRE (27-30). Specific protein complexes contribute to the DNA binding affinity (30-34). For example, c-jun/fos heterodimers bind specifically to the TRE 25 times more efficiently than c-jun homo-
dimers, while c-fos homodimers have little affinity for the TRE (27). Previous studies have demonstrated that the induction of c-fos expression is a rapid event during induction of monocytic differentiation of myeloid cells by TPA, bryostatin 1, and 1,25-(OH)₂D₃ (18, 35). The appearance of c-fos transcripts in TPA-treated HL-60 cells precedes that for the c-jun gene. Consequently, the formation of c-fos/c-jun heterodimers may play a role in the transcriptional induction of c-jun expression by TPA and other inducers of monocytic differentiation.

HL-60 cells treated with TPA express histochemical, morphological, and functional characteristics of the monocytic lineage. In addition to induction of c-fos transcripts, monocytic differentiation is associated with changes in the expression of certain other protooncogenes and growth regulatory genes. For example, TPA-induced HL-60 cell differentiation is associated with a decrease in c-myc transcripts (35). Of potential relevance is the finding that formation of c-jun/c-fos heterodimers may be involved in the down-regulation of the c-myc gene (36). Monocytic differentiation is also associated with the appearance of c-fms, platelet-derived growth factor, macrophage-specific colony stimulating factor, and tumor necrosis factor transcripts (35, 37–40). While it is not known whether induction of AP-1 is required for the changes observed in the expression of these genes, macrophage-specific colony stimulating factor and tumor necrosis factor are known to contain AP-1-like sites in their 5’ upstream promoter regions (41, 42). Thus, AP-1 may be involved in the regulation of certain genes required for the proliferation, differentiation, and activation of monocytes. Further studies are needed to determine whether the induction of AP-1 by TPA and other inducers of monocytic differentiation regulates expression of these genes by functioning as a transcriptional activator.

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