Transcriptional Regulation of c-*jun* Gene Expression by Arabinofuranosylcytosine in Human Myeloid Leukemia Cells

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Abstract

Previous studies have demonstrated that 1-β-D-arabinofuranosylcytosine (ara-C) induces terminal differentiation of human myeloid leukemia cells. Other studies have shown that the c-*jun* protooncogene is expressed during phorbol ester–induced myeloid differentiation. This work examines the effects of ara-C on c-*jun* gene expression in human KG-1 myeloid leukemia cells. The results demonstrate that c-*jun* transcripts are undetectable in uninduced KG-1 cells and that ara-C induces expression of this gene in a concentration- and time-dependent manner. Ara-C treatment was also associated with increases in c-*jun* transcripts in U-937, THP-1, and HL-60 myeloid leukemia cells. Furthermore, transcriptional run-on analysis has demonstrated that exposure to ara-C increases the rate of c-*jun* gene transcription. The results also demonstrate that while inhibition of protein synthesis superinduces c-*jun* mRNA levels in phorbol ester–treated KG-1 cells, cycloheximide had no effect on the induction of c-*jun* transcripts during ara-C treatment. Moreover, the half-life of c-*jun* transcripts in ara-C-treated KG-1 cells was 42 min. These findings suggest that the increase in c-*jun* mRNA observed during ara-C treatment is regulated by a transcriptional mechanism, and that c-*jun* may be involved in the induction of differentiation and regulation of gene expression by ara-C. (*J. Clin. Invest.* 1990. 86:1517–1523.) Key words: transcriptional activator AP-1 • differentiation • proliferation • phorbol esters • cycloheximide

Introduction

Avian sarcoma virus 17 (ASV 17)[1] is a retrovirus that induces progressively growing fibrosarcomas in chickens and transforms cultured chicken embryo fibroblasts into elongated refractile neoplastic cells (1). The normal cellular homologue of the ASV 17 transforming gene is the c-*jun* protooncogene (2–4). The protein product of c-*jun* is structurally and functionally similar to a component of the mammalian transcriptional activator AP-1 (5, 6). Subsequent studies have demonstrated that c-*jun* is a member of a multigene family of mammalian transcription factors, which also includes jun-B, jun-D, c-fos, fos-B and fra-1 (reviewed in references 7–9). Protein members of this family interact via a common structural motif identified as a leucine zipper. Dimerization of these proteins allows the DNA binding region to recognize and bind to the heptameric DNA consensus sequence $TGA^G/_CTCA$. Expression of the c-*jun* gene is an early response event during activation of fibroblasts and is rapidly activated by serum or phorbol esters (10–12). Furthermore, c-*jun* expression is induced by a number of growth factors including platelet-derived growth factor (10), fibroblast growth factor (10), epidermal growth factor (13), transforming growth factor-β (14), tumor necrosis factor (15, 16), nerve growth factor (17), and IL 1 (18). Moreover, c-*jun* protein increases transcription of the c-*jun* gene by an autoregulatory mechanism (19).

1-β-D-Arabinofuranosylcytosine (ara-C) is one of the most effective agents in the treatment of human acute myelogenous leukemia (20). The mechanism(s) of action of ara-C and the basis for selectivity against leukemic cells, however, remain unclear. Ara-C is a potent inhibitor of DNA replication (21, 22). Studies with ara-C have demonstrated that inhibition of DNA synthesis is significantly related to the extent of drug incorporation into DNA (23). Moreover, there is a highly significant relationship between the formation of (ara-C) DNA and loss of clonogenic survival (24, 25). Ara-C also induces differentiation of mouse leukemic myeloblasts (26). Similarly, exposure of the human HL-60 (27), ML-1 (28), and U-937 (29) cell lines to ara-C results in the induction of terminal differentiation along the monocytic lineage. In this regard, induction of U-937 cells by ara-C is associated with down regulation of c-*myc* transcripts as well as an increase in c-*fos* gene expression (29).

There is presently little known about the mechanisms by which ara-C regulates gene expression. In this study, we monitored the effects of ara-C on the regulation of c-*jun* gene expression in human myeloid leukemia cells. We demonstrate that ara-C increases the level of c-*jun* transcripts in these cells and that this induction is regulated at the level of transcription.

Methods

Cell culture. KG-1 human myeloid leukemia cells (American Type Culture Collection, Rockville, MD) were grown in Iscove's modified Dulbecco media (Gibco Laboratories, Grand Island, NY) containing 10% FBS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at a density of $3–5 \times 10^5$/ml in 5% CO_2 humidified atmosphere at 37°C. U-937, THP-1, and HL-60 cells were grown as previously described (30). Ara-C (Sigma Chemical Co., St. Louis, MO) was diluted in media without serum and added directly to the cell cultures. Viability was determined by trypan blue exclusion. Cytocentifuge smears of cultured cells were examined for nitroblue tetrazolium (NBT) reduction and α-naphthyl acetate esterase (NSE) staining (31). Cell cycle analysis was performed after propidium iodide

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1. *Abbreviations used in this paper:* ara-C, 1-β-D-arabinofuranosylcytosine; ASV, avian sarcoma virus; NBT, nitroblue tetrazolium; NSE, α-naphthyl acetate esterase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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(Calbiochem-Behring Corp.) staining and fluorescence flow cytometry using a fluorescein-activated cell sorter equipped with the Becton Dickinson CellFIT cell cycle analysis software (Becton Dickinson & Co., Oxnard, CA).

Preparation of RNA and Northern blot hybridization. Total cellular RNA was isolated by a modification of the guanidine-isothiocyanate technique as described previously (30, 32). 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 ^{32}P-labeled DNA probes: (*a*) the 1.8-kb Bam HI/Eco RI insert of a human c-*jun* DNA probe containing a 1.0-kb cDNA and 0.8-kb 3′-untranslated sequences (5) purified from a pBluescript SK(+) plasmid (provided by Dr. R. Tjian, University of California Berkeley); and (*b*) the pA1 plasmid containing a 2.0-kb Pst I insert of the chicken β-actin gene (33). The hybridization was carried out for 16–24 h at 42°C in 50% (vol/vol) formamide, 2× SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1× Denhardt's solution, 0.1% (wt/vol) 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 a laser densitometer.

Nuclear run-on assays. KG-1 cells were treated as indicated, pelleted at 500 *g* and washed twice with ice-cold PBS. The cells (10^8) were then resuspended in 4 ml of ice-cold lysis buffer (10 mM Tris HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40), vortexed gently for 20 s and left on ice for 5 min. Nuclei were then pelleted for at 500 *g* for 5 min. The supernatant was removed and the nuclei resuspended in 100 μl glycerol buffer (50 mM Tris HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA). An equal volume of reaction buffer (10 mM Tris HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM DTT) was added to the nuclei suspension and incubated at 26°C for 30 min with 200 μCi (α-^{32}P) UTP (> 800 Ci/mmol; Amersham Corp., Arlington Heights, IL). Transcription was terminated by addition of 40 U DNase I, 10 mM Tris HCl, pH 7.4, 100 mM NaCl, 1 mM Na_2EDTA, 60 μg/ml yeast tRNA and 150 U/ml RNasin for 15 min at 26°C. Proteinase K (750 μg/ml) and 1% (vol/vol) SDS were then added for 30 min at 37°C. Nuclear RNA was isolated by phenol/chloroform extractions and then ethanol precipitated three times in 2.5 M ammonium acetate. RNA was purified through a spin column prepared with Sephadex G50 equilibrated in and eluted with column buffer (0.3 M NaCl, 0.1% SDS, 1 mM Na_2EDTA, 10 mM Tris HCl, pH 7.5).

Plasmid DNAs containing various cloned inserts were digested with restriction endonucleases as follows: (*a*) the 2.0-kb Pst I fragment of the chicken β-actin pA1 plasmid; and (*b*) the 1.8-kb Bam HI/Eco RI fragment of the human c-*jun* cDNA from the pBluescript SK(+) plasmid. The digested DNA was denatured by heating to 65°C for 15 min, separated in a 1% agarose gel and transferred to nitrocellulose filters by the method of Southern. The filters were prehybridized in 5× Denhardt's solution, 40% formamide, 4× SSC, 5 mM Na_2EDTA, 0.4% SDS, and 100 μg/ml yeast tRNA for 2 h. Hybridizations was performed with 10^7 cpm of ^{32}P-labeled RNA per ml hybridization buffer for 72 h at 42°C. The filters were then washed in 2× SSC and 0.1% SDS at 37°C for 30 min, 10 μg/ml RNase A in 2× SSC at 37°C for 20 min and 0.1× SSC and 0.1% SDS at 42°C for 30 min.

Table I. Effect of Ara-C on NBT and NSE Staining of KG-1 Cells

Time of exposure	NBT positive	NSE positive
h	%	
0	4.4±0.2	7.5±1.1
12	7.4±1.0	13.5±1.6
24	7.0±1.9	24.1±3.8
48	9.5±2.3	39.5±5.7

KG-1 cells were treated with 5×10^{-6} M ara-C for the indicated times. The percentage of NSE and NBT positive cells was determined by counting 200 cells in duplicate. Results are expressed as mean±SD of two experiments each performed in duplicate.

Results

KG-1 cells were treated with various concentrations of ara-C to determine the effects of this agent on cell growth. Although 10^{-7} M ara-C had partial growth inhibitory effects, proliferation was completely inhibited in the presence of 10^{-6} and 10^{-5} M drug (Fig. 1). In contrast, exposure to 10^{-4} M ara-C for 72 h was associated with cell lethality (Fig. 1). The effects of ara-C on phenotypic differentiation were also determined by moni-

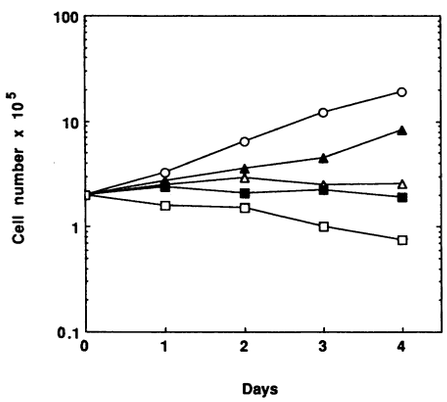

Figure 1. Effects of ara-C on KG-1 cell growth. KG-1 cells in logarithmic growth phase were seeded at 2×10^5/ml. Ara-C was added at varying concentrations and cell number monitored at the indicated times. Viability was determined by the trypan blue exclusion. Control, ○; 10^{-7} M ara-C, ▲; 10^{-6} M ara-C, △; 10^{-5} M ara-C, ■; and 10^{-4} M ara-C, □.

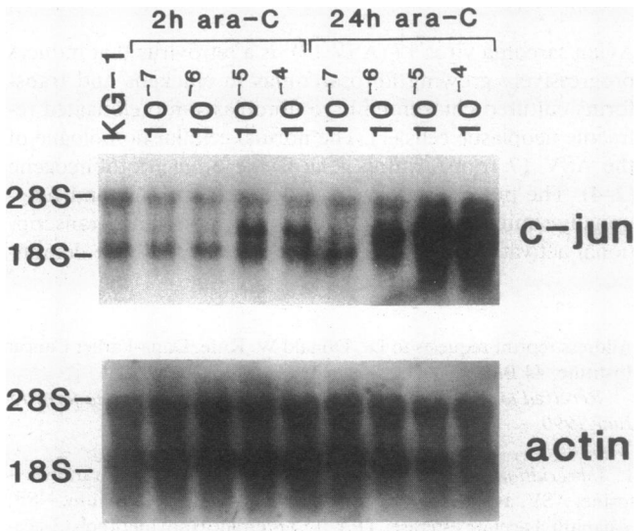

Figure 2. Effects of ara-C on c-*jun* and actin RNA levels in KG-1 cells. Northern blot analysis of RNA levels was performed in KG-1 cells after treatment with varying concentrations of ara-C for 2 and 24 h. Total cellular RNA (20 μg/lane) was hybridized to a ^{32}P-labeled c-*jun* or β-actin DNA probe. The KG-1 lane represents RNA from untreated cells.

toring changes in histochemical staining. While 7.5% of untreated KG-1 cells were NSE positive, 39.5% of these cells stained positively for NSE after 48 h of exposure to ara-C (Table I). In contrast, ara-C treatment was associated with little, if any, change in the percentage of cells that reduced NBT (Table I). These results suggest that ara-C induces KG-1 cells along the monocytic lineage.

We next studied the effects of various concentrations of ara-C on c-*jun* gene expression in KG-1 cells. Northern blot analysis of KG-1 cellular RNA collected at 2 and 24 h of drug exposure is shown in Fig. 2. c-*jun* transcripts were undetectable in untreated KG-1 cells, and exposure to 10^{-7} or 10^{-6} M ara-C for 2 h had little effect on expression of this gene. In contrast, treatment with 10^{-5} M and 10^{-4} M ara-C for 2 h resulted in induction of 2.7-kb c-*jun* transcripts. Lower concentrations of ara-C (10^{-6} M) also induced c-*jun* expression after 24 h of treatment (Fig. 2). Furthermore, the changes in c-*jun* mRNA levels were associated with little if any effect of ara-C on actin gene expression. These findings suggested that ara-C treatment specifically increases c-*jun* expression in a concentration- and time-dependent manner.

The effects of ara-C on the kinetics of c-*jun* expression were next studied using a cytostatic concentration of drug. c-*jun* transcripts reached maximal levels by 6–8 h of exposure to 5×10^{-6} M ara-C and declined by 10 h (Fig. 3 *A*). Furthermore, c-*jun* mRNA levels remained elevated at 24 h of ara-C exposure before returning to that in control cells by 72 h (Fig. 3 *B*). These findings were also associated with the absence of a detectable effect on actin gene expression. Similar studies were performed using serum-starved cells. KG-1 cells were grown in 1% serum for 18 h and then exposed to ara-C. After 6 h, a significant increase in c-*jun* mRNA levels was observed in the absence of serum (Fig. 3 *C*).

To determine the effects of cell cycle on induction of c-*jun* by ara-C, KG-1 cells were treated with 5×10^{-6} M ara-C for varying times and analyzed by fluorescence flow cytometry. While 53.4% of untreated KG-1 cells were in G_1 phase, 75.2% of these cells were in G_1 after 24 h of ara-C exposure (Table II).

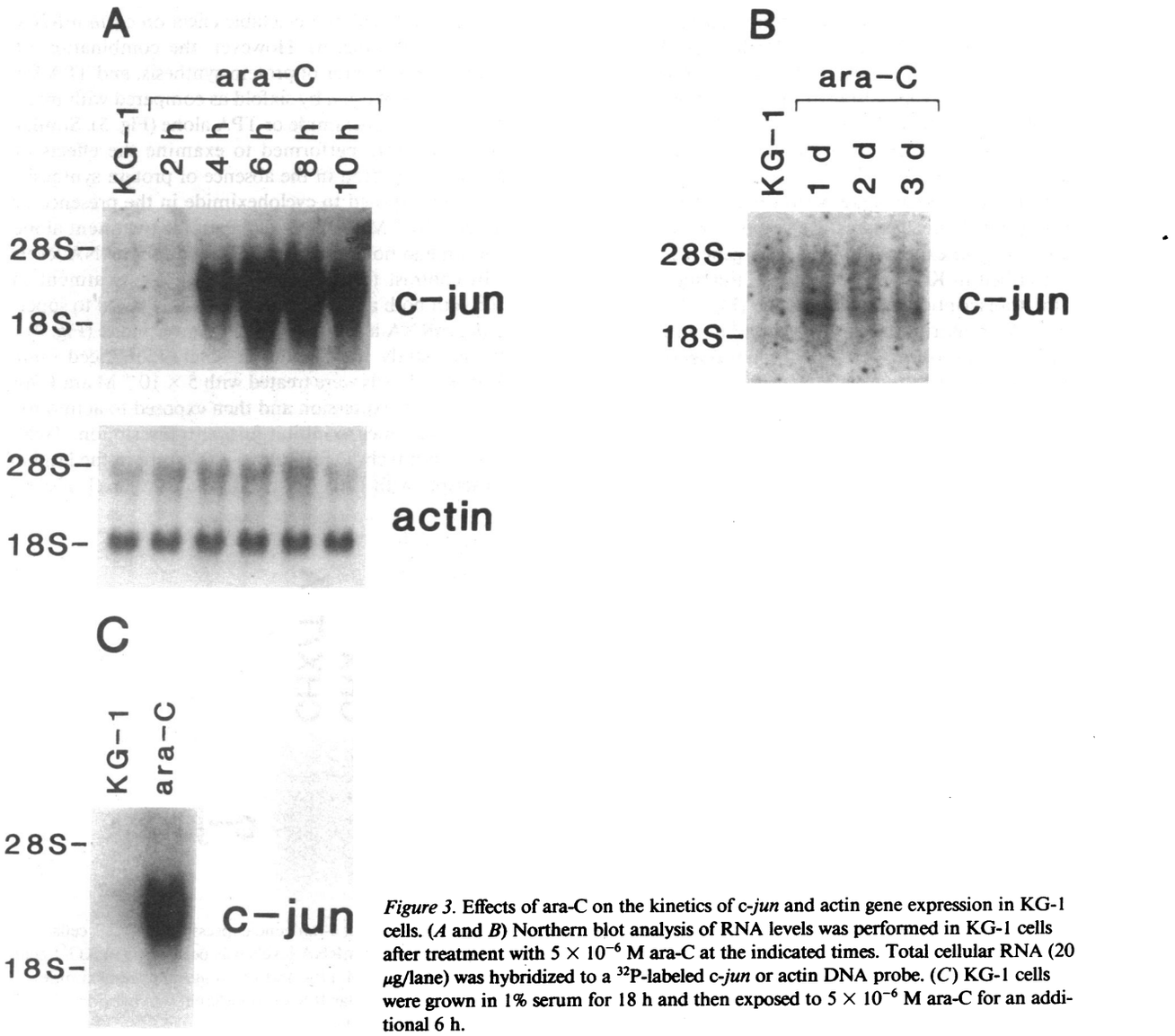

Figure 3. Effects of ara-C on the kinetics of c-*jun* and actin gene expression in KG-1 cells. (*A* and *B*) Northern blot analysis of RNA levels was performed in KG-1 cells after treatment with 5×10^{-6} M ara-C at the indicated times. Total cellular RNA (20 μg/lane) was hybridized to a ^{32}P-labeled c-*jun* or actin DNA probe. (*C*) KG-1 cells were grown in 1% serum for 18 h and then exposed to 5×10^{-6} M ara-C for an additional 6 h.

Table II. Cell Cycle Analysis of Ara-C-treated KG-1 Cells

Time of exposure	G_1	S	G_2/M
h		%	
0	53.4±2.4	36.0±0.8	10.5±1.6
6	72.7±1.1	21.8±0.7	5.5±0.3
24	75.2±8.3	20.2±6.8	4.7±1.5

KG-1 cells were exposed to 5×10^{-6} M ara-C for the indicated times. The percentage of cells in G_1, S, and G_2/M phase was determined by fluorescence flow cytometry.

In contrast, the percentage of cells in S and G_2/M phase decreased from 36 and 10.5% to 20.2 and 4.7%, respectively (Table II). These results confirm that ara-C inhibits S-phase DNA synthesis and accumulates cells in the G_1/S interphase.

Previous studies have demonstrated that exposure of human HL-60 and U-937 cells to ara-C results in the induction of terminal differentiation along the monocytic lineage (27, 29). Thus, it was also of interest to determine the effects of ara-C on c-*jun* expression in other myeloid cell lines. Ara-C treatment was associated with increases in c-*jun* transcripts in U-937, THP-1, and HL-60 cells, although the relative levels of induction varied among the cell lines (Fig. 4). These results indicate that ara-C induces c-*jun* expression in several human myeloid leukemia cell lines.

Run-on transcription assays were performed to determine whether transcriptional mechanisms are responsible for the effects of ara-C on c-*jun* expression. The actin gene was constitutively transcribed in KG-1 cells and ara-C treatment had no effect on the transcription rate of this gene (Fig. 5). A low level of c-*jun* gene transcription was detectable in untreated KG-1 cells. However, exposure to ara-C for 6 h was associated with a sixfold increase in c-*jun* gene transcription (Fig. 5). Taken together, these results suggested that the induction of c-*jun* expression by ara-C is regulated by a transcriptional mechanism.

We and others have recently shown that treatment of human myeloid leukemia cells with phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is associated with induction of c-*jun* transcripts (30, 34). Consequently, it

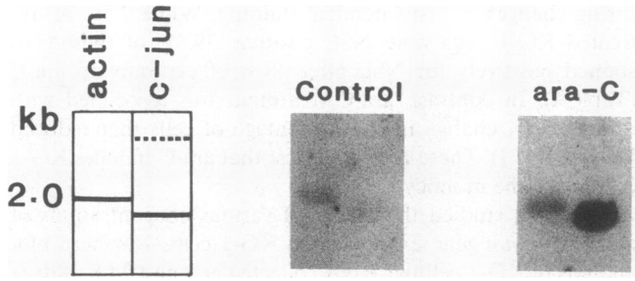

Figure 5. Effects of ara-C on rates of c-*jun* gene transcription in KG-1 cells. Digested actin and c-*jun* plasmid DNAs were run on a 1% agarose gel, transferred to nitrocellulose paper by Southern blotting, and hybridized to equal amounts of ^{32}P-labeled nuclear RNA ($5–6 \times 10^6$ cpm/ml) isolated from control and ara-C-treated KG-1 cells. In the schematic on the left, the solid lines indicate the relative positions of the cDNA inserts while the dashed line indicates the position of the plasmid vector (negative control).

was of interest to determine if the effects of TPA on c-*jun* mRNA could also be demonstrated in KG-1 cells. In contrast to HL-60 cells, TPA had no detectable effect on c-*jun* mRNA levels in KG-1 cells (Fig. 6). However, the combination of cycloheximide, an inhibitor of protein synthesis, and TPA for 4 h increased levels of c-*jun* by sixfold as compared with treatment with either cycloheximide or TPA alone (Fig. 5). Similar studies were therefore performed to examine the effects of ara-C on c-*jun* expression in the absence of protein synthesis. KG-1 cells were exposed to cycloheximide in the presence or absence of 5×10^{-6} M ara-C. Cycloheximide treatment alone for 1, 3, or 6 h had no detectable effect on c-*jun* mRNA levels (Fig. 7). In contrast to the findings with TPA, treatment of KG-1 cells with both ara-C and cycloheximide failed to superinduce c-*jun* mRNA levels compared to ara-C alone (Fig. 7).

To further study the regulation of ara-C-induced c-*jun* RNA levels, KG-1 cells were treated with 5×10^{-6} M ara-C for 6 h to induce c-*jun* expression and then exposed to actinomycin D for varying times to inhibit further transcription. Treatment with actinomycin D resulted in a decrease in the level of c-*jun* transcripts with little if any effect on the level of actin

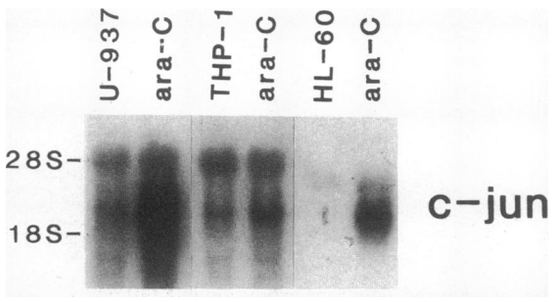

Figure 4. Effect of ara-C on c-*jun* RNA levels in other myeloid leukemia cell lines. Northern blot analysis of mRNA levels were performed in U-937, THP-1 and HL-60 cells after treatment with 5×10^{-6} M ara-C for 6 h. Total cellular RNA (20 μg/lane) was hybridized to a ^{32}P-labeled c-*jun* probe.

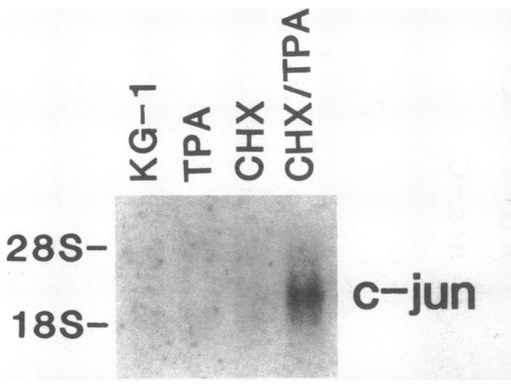

Figure 6. Effects of TPA on c-*jun* gene expression in KG-1 cells. Northern blot analysis of mRNA levels was performed in KG-1 cells after treatment with 32 nM TPA and/or 10 μg/ml cycloheximide (CHX) for 4 h. Total cellular RNA (20 μg/lane) was hybridized to a ^{32}P-labeled c-*jun* DNA probe.

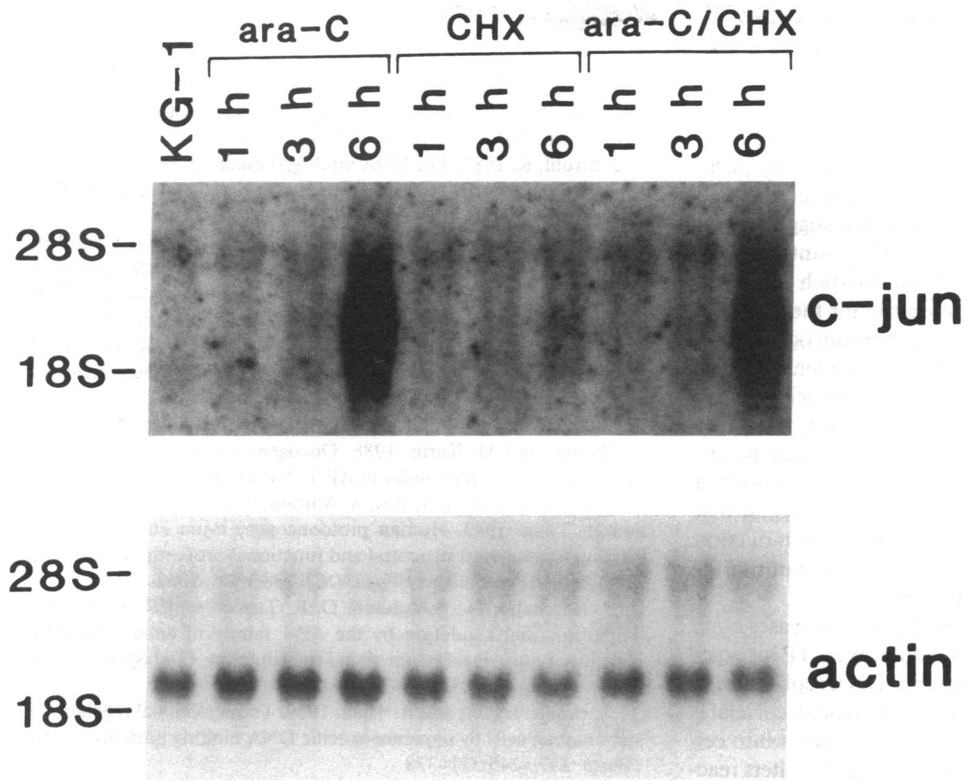

Figure 7. Effect of ara-C and cycloheximide on c-*jun* and actin gene expression. Northern blot analysis of mRNA levels was performed in KG-1 cells after treatment with 5×10^{-6} M ara-C and/or 10 μg/ml cycloheximide (CHX) at the indicated times. Total cellular RNA (20 μg/lane) was hybridized to ^{32}P-labeled c-*jun* or actin DNA probe.

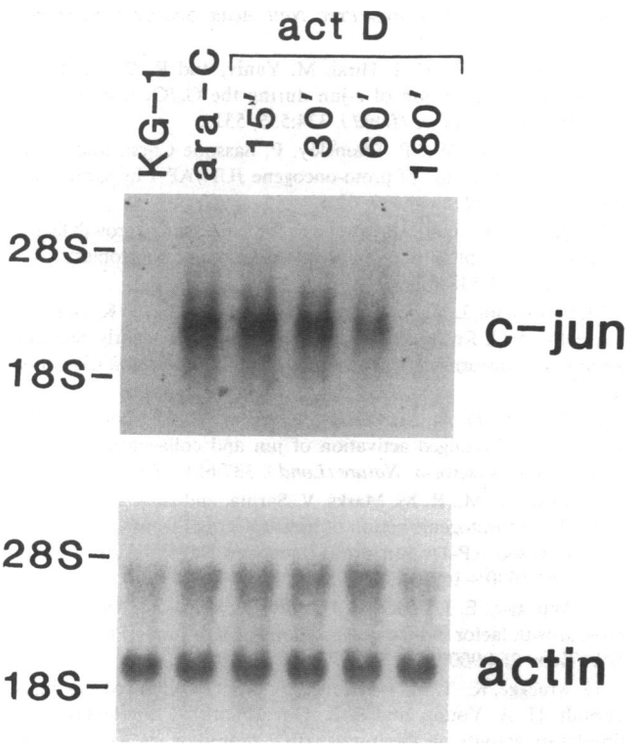

Figure 8. Effect of ara-C and actinomycin D on c-*jun* and actin gene expression. Northern blot analysis of RNA levels was performed in KG-1 cells after treatment with 5×10^{-6} M ara-C alone or in combination with 5 μg/ml actinomycin D (act D) at the indicated times. Total cellular RNA (20 μg/lane) was hybridized to ^{32}P-labeled c-*jun* or actin DNA probe.

mRNA (Fig. 8). The half-life of c-*jun* mRNA in KG-1 cells as determined by densitometeric scanning was 42 min. Moreover, cycloheximide had little if any effect on the half-life of c-*jun* transcripts in these cells (data not shown). The stability of c-*jun* mRNA was also determined in both untreated and ara-C-treated U-937 cells. Untreated U-937 exposed to actinomycin D for varying times resulted in a decrease in the level of constitutively expressed c-*jun* transcripts. The half-life of c-*jun* mRNA was determined by densitometric scanning was 30 min. U-937 cells were also treated with 5×10^{-6} M ara-C for 6 h to induce c-*jun* expression and then exposed to actinomycin D to inhibit further transcription. Under these conditions, the half-life of c-*jun* mRNA was determined to be 30 min. Taken together, these results suggest that ara-C has little if any effect on the stability of c-*jun* mRNA.

Discussion

In the present study, we have demonstrated that ara-C increases c-*jun* RNA levels in KG-1 cells in a concentration- and time-dependent manner. Analysis of relative rates of gene transcription showed that ara-C treatment of KG-1 cells is associated with significant increases in c-*jun* gene transcription. These results indicate that the induction of c-*jun* by ara-C is mediated, at least in part, by a transcriptional mechanism. Regulation of c-*jun* expression by transcriptional mechanisms has also been described for serum, phorbol esters, and certain growth factors. A rapid increase in c-*jun* transcription occurs during the G_0/G_1 transition in mouse fibroblasts (11). Because ara-C inhibits S phase DNA synthesis and accumulates cells at the G_1/S interphase, the present findings might be explained

by cell cycle–related events. However, similar exposures to cytostatic concentrations of aphidicolin, an inhibitor of DNA polymerase α (35), had no detectable effect on c-*jun* expression (data not shown).

The induction of c-*jun* RNA after treatment with TPA or growth factors in certain cell lines is also regulated by post-transcriptional mechanisms. The stabilization of c-*jun* transcripts following protein synthesis inhibition has suggested the presence of a labile ribonuclease. Indeed, the 3′-untranslated region of the c-*jun* transcript contains an AU-rich sequence that has been implicated as a recognition site for the degradation of mRNAs coding for a variety of protooncogenes and growth factors (36, 37). We have found that treatment of KG-1 cells with both TPA and cycloheximide is also associated with superinduction of c-*jun* mRNA levels. In contrast, treatment of KG-1 cells with ara-C and cycloheximide showed no increase in levels of c-*jun* mRNA compared with that obtained during treatment with ara-C alone. These results suggest that induction of c-*jun* expression by ara-C differs from induction by TPA and that there are at least two separate mechanisms for regulating c-*jun* mRNA levels in KG-1 cells.

The molecular mechanism by which ara-C increases c-*jun* transcripts is unclear. The active metabolite, ara-CTP incorporates into DNA and is a potent inhibitor of DNA replication in eukaryotic cells (23–25). Ara-C-induced cytotoxicity correlates significantly with the amount of ara-C incorporation into cellular DNA (24, 25). The incorporated ara-C residue alters reactivity of the 3′ terminus, slows chain elongation and results in DNA fragmentation (23). Moreover, the relative chain-terminating effects of ara-C are dependent on the concentration of drug and sequence of the elongating DNA strand (38, 39). Although ara-C might incorporate into the promoter region of the c-*jun* gene and thereby possibly alter rates of transcription, this mechanism would appear to be an unlikely explanation for the marked induction of this gene. Another possibility might include ara-C-induced decreases in the expression of a *trans*-acting factor involved in the negative regulation of c-*jun* transcription. The *cis*-acting elements in the c-*jun* promoter have been defined and thus additional studies are now needed to define more precisely the mechanism whereby ara-C induces transcription of this gene.

Finally, the increase in c-*jun* gene expression by ara-C may in turn induce other genes with a c-*jun*/AP-1 binding site. Treatment of myeloid leukemia cells with ara-C is associated with decreases in c-*myc* mRNA levels, as well as an increase in c-*fos* gene expression (29). In this regard, c-*jun*/AP-1-like sequences in the 5′ regulatory region of c-*myc* have been implicated in the down regulation of this gene (40, 41). Thus, the transient increase in c-*jun* expression by ara-C may initiate specific transcriptional events necessary for the regulation of certain other genes associated with ara-C-induced myeloid differentiation.

Acknowledgments

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