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ACTIVATION OF THE *jun*-D GENE DURING TREATMENT OF HUMAN MYELOID LEUKEMIA CELLS WITH 1-β-D-ARABINOFURANOSYLCYTOSINE

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Abstract—The *jun*-D gene is a member of the c-*jun* family of early response genes that code for DNA binding proteins. The present studies demonstrate that 1-β-D-arabinofuranosylcytosine (ara-C) increases *jun*-D expression in HL-525 myeloid leukemia cells. This induction by ara-C was maximal at 6 hr and transient. In contrast, ara-C had no detectable effect on the gene coding for the cAMP-responsive element binding protein 1. Nuclear run-on assays demonstrated that ara-C treatment is associated with an increased rate of *jun*-D transcription. The results also show that *jun*-D transcripts are stabilized at a posttranscriptional level in ara-C-treated cells. Taken together, these results demonstrate that ara-C induces expression of the *jun*-D gene and that this effect is regulated by transcriptional and posttranscriptional mechanisms.

The transcription factor AP-1 binds to a DNA consensus sequence TGA$^{G}/_{C}$TCA (TRE§) that regulates the activation of genes responsive to phorbol esters [1–3]. This factor is a complex composed of proteins encoded by different members of the *jun* and *fos* gene family. The c-*jun* gene, which is induced as an immediate early event by phorbol esters, as well as serum and growth factors [4–7], codes for a major form of AP-1 [8–10]. The affinity of Jun/AP-1 binding to DNA is a function of the type of protein, each containing a leucine zipper and DNA binding domain, present in the dimer complex [11]. This structure is shared by a family of transcription factors composed of products of the *jun*-B, *jun*-D, c-*fos*, *fos*-B and *fra* genes [11]. Heterodimers formed between different Jun and Fos proteins exhibit enhanced binding activity compared with Jun/Jun homodimers [12–14]. Moreover, the half-lives of Jun/Fos–DNA complexes are prolonged compared with those formed between Jun/Jun dimers and DNA [14]. Although various dimers have the potential to bind to the AP-1 sequence, the biologic properties of these complexes appear to differ. In this context, Jun-B functions as a negative regulator of c-Jun [15, 16].

The c-*jun*, *jun*-B and *jun*-D genes are differentially expressed in mouse tissues. jun-D mRNA is more abundant than c-jun mRNA and, while *jun*-B and *jun*-D expression is similar in most tissues, jun-B mRNA levels are higher than jun-D levels in testis and ovary [17]. Levels of *jun*-D expression are also higher compared with c-*jun* and *jun*-B in quiescent 3T3 cells [17, 18]. Moreover, while c-*jun* and *jun*-B transcription is stimulated by phorbol esters and serum, similar conditions have minimal effects on *jun*-D expression [17, 18]. These findings have suggested that the *jun*-D gene may have a distinct role in the control of gene activity. However, little is known about mechanisms responsible for the regulation of *jun*-D expression. While other studies have indicated that cAMP plays a role in c-*jun*, *jun*-B and *jun*-D induction [19, 20], the absence of phorbol ester inducibility distinguishes the *jun*-D gene from other members of this family.

Recent work has demonstrated that cytotoxic agents, such as 1-β-D-arabinofuranosylcytosine (ara-C), activate the expression of certain early response genes. Ara-C is a potent inhibitor of DNA replication, which incorporates into the elongating DNA strand [21–23]. The extent of ara-C incorporation into DNA correlates with inhibition of DNA synthesis, as well as loss of clonogenic survival [21–23]. Other studies have shown that the strand terminating effects of ara-C are also related to the sequence of the DNA template [24, 25]. The treatment of human myeloid leukemia cells with ara-C is associated with transient increases in c-*jun* and *jun*-B gene expression [26–28]. The present studies extend these findings to the *jun*-D gene. The results demonstrate that ara-C increases *jun*-D expression by both transcriptional and posttranscriptional mechanisms. In contrast, this agent had little effect on expression of a gene coding for another leucine zipper containing protein that binds to the related cAMP-responsive consensus sequence (CRE) [29].

MATERIALS AND METHODS

Cell culture. Human HL-525 myeloid leukemia

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§ Abbreviations: TRE, phorbol ester-responsive element; ara-C, 1-β-D-arabinofuranosylcytosine; CRE, cAMP-responsive element; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CREBP, CRE-binding protein; CHX, cycloheximide; PKC, protein kinase C; SSC, 0.15 M sodium chloride and 0.15 M sodium citrate.

cells [30] were grown (2×10^5/mL) in RPMI 1640 medium containing 15% fetal bovine serum (FBS) with 1 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Viability as determined by Trypan blue exclusion exceeded 95%. Ara-C (Sigma Chemical Co., St. Louis, MO) was diluted in medium without serum and then added to the cultures. The cells were also exposed to 5 μg/mL actinomycin D (Sigma) and 10 μg/mL cycloheximide (CHX; Sigma).

RNA isolation and analysis. RNA was isolated by a modification of the guanidine-isothiocyanate technique [31]. Total cellular RNA (20 μg) or poly $(A)^+$-selected RNA (2 μg; Ref. 32) was subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to the following DNA probes labeled with ^{32}P by random priming: (1) the 1.7-kb *Eco*RI fragment of a murine jun-D cDNA purified from the XHJ-12.4 plasmid [18]; (2) the pA1 plasmid containing a 2.0-kb *Pst*I insert of the chicken β-actin gene [33]; and (3) the cAMP responsive element (CRE)-binding protein 1 (CREBP1) gene [34]. Hybridizations were performed as described [26–28]. The filters were washed and exposed to Kodak X-Omat XAR film using an intensifying screen. Autoradiograms were scanned using an LKB Produkter (Bromma, Sweden) Ultrascan XL laser densitometer and analyzed with the Gelscan XL software package. Signal intensity was determined in a linear range and normalized to that for the actin control.

Nuclear run-on assays. Nuclei were isolated from 10^8 HL-525 cells and suspended in 100 μL of glycerol buffer (50 mM Tris–HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA). 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, and 5 mM dithiothreitol) was added to the nuclei suspension and incubated at 26° for 30 min with 250 μCi of [α-^{32}P]UTP (3000 Ci/mmol; Dupont, Boston, MA). The nuclear RNA was isolated as described [26–28] and hybridized to the following DNAs: (1) the 1.7-kb *Eco*RI fragment of the mouse jun-D cDNA [18]; and (2) the 2.0-kb *Pst*I fragment of the chicken β-actin gene [33]. The fragments were run in 1% agarose gels and transferred to nitrocellulose filters. Hybridizations were performed with 10^7 cpm of ^{32}P-labeled RNA/mL in 10 mM Tris–HCl, pH 7.4, 4 × SSC (0.15 M sodium chloride, 0.15 M sodium citrate), 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 2 × Denhardt's solution (0.02% polyvinylpyrrolidone), 40% formamide, 100 μg/mL yeast tRNA, for 72 hr at 42°. The filters were washed in (1) 2 × SSC, 0.1% SDS, at 37° for 30 min; (2) 200 ng/mL RNase A in 2 × SSC at room temperature for 10 min; and (3) 0.1 × SSC, 0.1% SDS, at 42° for 30 min.

RESULTS

Low levels of 2.0-kb *jun*-D transcripts were present in untreated HL-525 cells (Fig. 1). Similar levels of *jun*-D expression were detectable after treatment with 10^{-5} M ara-C for 1 hr. However, longer exposures to this agent resulted in increases in jun-D mRNA levels that were first apparent at 3 hr and maximal at 6 hr (4-fold) (Fig. 1). This effect of ara-C was transient with decreases in *jun*-D expression to near baseline levels at 24 hr (Fig. 1). Moreover, these changes in *jun*-D expression were associated with little if any change in actin mRNA levels (Fig. 1). HL-525 cells were similarly exposed to 10^{-5} M ara-C for 6 hr and then analyzed by fluorescence flow cytometry. While 50% of untreated HL-525 cells were in G_1 phase, 61% of these cells were in G_1 after 6 hr of ara-C exposure. In contrast, the percentage of cells in S and G_2/M phases decreased from 29 and 21% (control) to 27 and 12% (ara-C-treated), respectively. These findings suggest that cell cycle distribution has little if any effect on induction of jun-D mRNA by ara-C.

The stimulation of *jun*-D expression by ara-C was both concentration- and time-dependent. At 6 hr of exposure, 10^{-8} and 10^{-7} M ara-C had little effect and 10^{-6} M increased jun-D mRNA levels to a lesser extent than 10^{-5} M (Fig. 2A). However, treatment of cells with lower concentrations of this agent for longer periods was associated with increased *jun*-D expression. In this regard, *jun*-D transcripts were induced at 48 and 72 hr of exposure to 10^{-7} M ara-C (Fig. 2B).

While these findings demonstrated that ara-C stimulates *jun*-D expression, the selectivity of this response was addressed by studying other classes of genes coding for transcription factors. Several genes have been described that encode proteins that specifically bind to the CRE [29]. For example, the CRE-binding protein (CREBP), designated CREBP1, contains a leucine zipper motif similar to those present in Jun and Fos [34]. CREBP1 transcripts were detectable in HL-525 cells (Fig. 3). However, in contrast to the effects on *jun*-D expression, ara-C had no detectable effect on CREBP1 mRNA levels (Fig. 3). Taken together, these findings suggested that *jun*-D is selectively induced by ara-C as compared with that for CREBP1.

Run-on assays were performed to determine whether ara-C increases *jun*-D expression by transcriptional mechanisms. Nuclear RNA was isolated from cells treated with 10^{-5} M ara-C for 6 hr and hybridized to actin and jun-D DNAs. The actin gene was constitutively transcribed in untreated HL-525 cells (Fig. 4). Furthermore, the rate of actin gene transcription was unaffected by ara-C treatment (Fig. 4). Transcription of the *jun*-D gene was also detectable in untreated cells. However, this rate of transcription was increased 7-fold in ara-C-treated cells (Fig. 4). These findings indicated that ara-C induces *jun*-D expression, at least in part, by a transcriptional mechanism.

To determine whether ara-C also regulates *jun*-D expression by altering mRNA stability, we first treated cells with actinomycin D alone to inhibit further transcription. Using this approach, the half-life of *jun*-D transcripts was 37 min (Fig. 5). In contrast, this half-life was increased to 90 min when cells were first treated with ara-C to induce *jun*-D expression (Fig. 5). Moreover, stability of jun-D mRNA was further increased ($T_{\frac{1}{2}} > 120$ min) when the ara-C-treated cells were exposed to both actinomycin D and CHX. These findings indicated that the increase in jun-D mRNA levels during ara-C

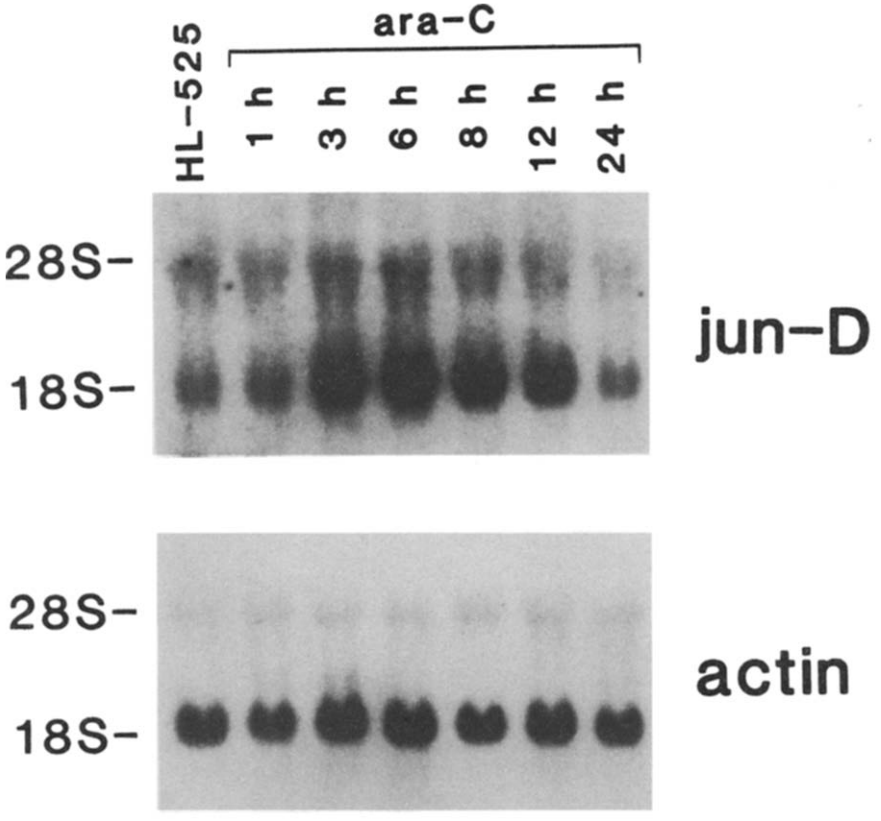

Fig. 1. Induction of *jun*-D expression by ara-C. HL-525 cells (2×10^5/mL) were treated with 10^{-5} M ara-C for the indicated times. Total cellular RNA (20 μg) was hybridized to the ^{32}P-labeled jun-D and actin DNA probes. Viability of the cells treated with ara-C for 24 hr was 92% as determined by Trypan blue exclusion.

treatment was also mediated by a posttranscriptional mechanism.

DISCUSSION

Recent studies have demonstrated that ara-C transiently induces expression of the c-*jun* gene in myeloid leukemia cells [26, 28]. This effect appears to involve the stimulation of a protein kinase activity similar to that of protein kinase C (PKC) [28]. In this context, other investigators have demonstrated that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activates Jun/AP-1 through PKC, and that this event is sufficient for induction of c-*jun* gene transcription [35, 36]. While c-*jun*, as well as *jun*-B, are induced in myeloid leukemia cells by TPA [37, 38], similar exposures to this agent had little effect on *jun*-D gene expression (data not shown). This finding is in concert with a previous demonstration that phorbol esters and serum have minimal effects on jun-D mRNA levels in other cell types [17, 18]. Nonetheless, since ara-C has been shown to induce PKC-like activity [28], we have used HL-525 cells, which are deficient in TPA-induced PKC-mediated signaling [30] including increases in *jun/fos* expression [39], to distinguish events that are regulated by that transduction pathway. The present results demonstrate that ara-C induces *jun*-D expression in HL-525 cells and that this effect is controlled by both transcriptional and posttranscriptional mechanisms. Previous work has demonstrated that treatment of these cells with ara-C is also associated with induction of c-*jun* and *jun*-B gene expression ([39] and unpublished data). Although the precise role of Jun-D in *trans*-activation is unclear, this factor, in contrast to Jun-B, appears to function more closely to c-Jun [40]. However, at high concentrations, human Jun-D displays decreased DNA binding activity [40].

Characterization of the mouse *jun*-D promoter has demonstrated the presence of an AP-1-like sequence [41]. While this element contributes to *jun*-D promoter activity, a more significant effect is conferred by an octamer motif (TATGCAAATC) [41]. Moreover, this octamer motif appears to block TPA-inducibility of the *jun*-D promoter [41]. Regulation of the human *jun*-D promoter is presently unknown. However, the finding that ara-C activates *jun*-D transcription in the TPA-unresponsive HL-525 cells supports the hypothesis that ara-C induces *jun*-D transcription by a mechanism other than through PKC and stimulation of AP-1 binding. The present results also demonstrate that ara-C-induced *jun*-D expression is controlled by a posttranscriptional

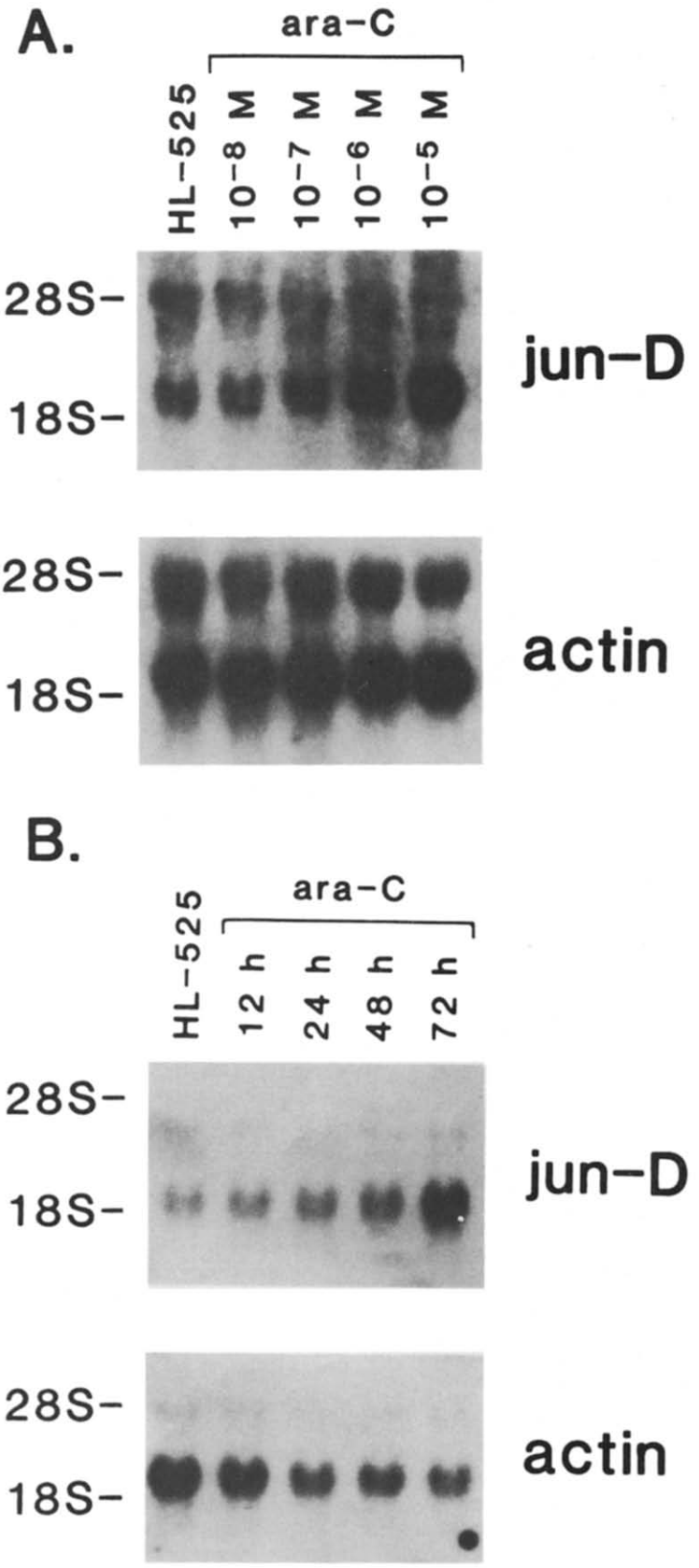

Fig. 2. Concentration- and time-dependent effects of ara-C on *jun*-D expression. (A) HL-525 cells were treated with the indicated concentrations of ara-C for 6 hr. (B) Cells were treated with 10^{-7} M ara-C for the indicated times. Total cellular RNA (20 μg) was hybridized to the jun-D and actin probes.

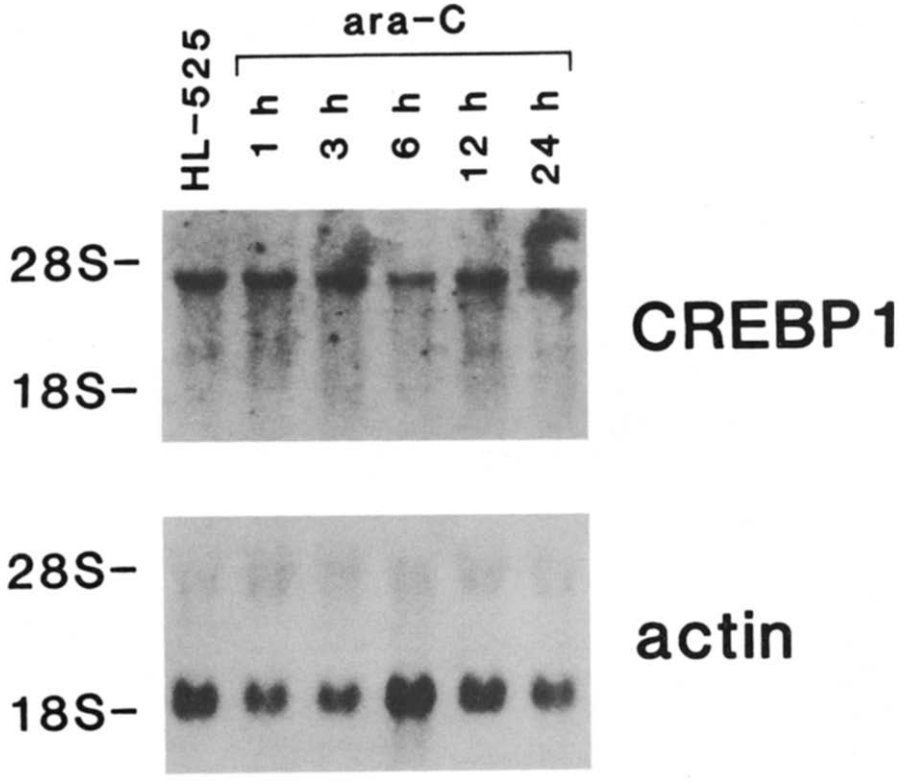

Fig. 3. Effects of ara-C on CREBP gene expression. HL-525 cells were exposed to 10^{-5} M ara-C for the indicated times. Poly $(A)^+$-selected RNA (2 μg) was hybridized to the ^{32}P-labeled CREBP1 DNA probe.

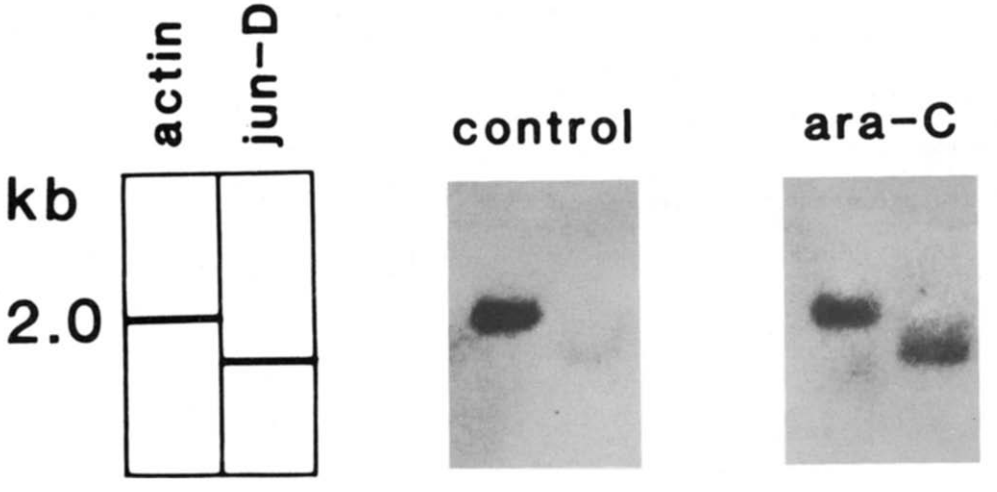

Fig. 4. Activation by ara-C of the transcription of the *jun*-D gene. HL-525 cells were treated with 10^{-5} M ara-C for 4 hr. Nuclei were isolated, and newly synthesized ^{32}P-labeled RNA was hybridized to actin and jun-D inserts subjected to restriction enzyme digestion and Southern blotting. The solid lines in the schematic represent positioning of the DNA inserts.

mechanism. The half-life of ara-C-induced *jun*-D transcripts was prolonged compared with that in untreated cells. Moreover, the results indicated that levels of ara-C-induced *jun*-D transcripts are regulated at the posttranscriptional level by CHX. The finding that inhibition of protein synthesis is associated with stabilization of ara-C-induced *jun*-D transcripts is in concert with the demonstration that certain early response genes have AT-rich regions in their 3′-untranslated regions which may serve as

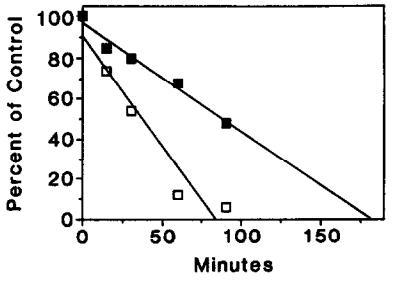

Fig. 5. Stability of jun-D mRNA. HL-525 cells were treated with 5 μg/mL actinomycin D (act D) for the indicated times (□) or cells were treated with 10^{-5} M ara-C for 6 hr followed by the addition of act D (■). Total cellular RNA (20 μg) was hybridized to the jun-D and actin probes. Signal intensity as determined by densitometric scanning (control arbitrary value: 1.2 units) was determined for the jun-D hybrids and normalized to that for actin. Control cells: $R = 0.93$; ara-C-treated cells: $R = 0.98$.

recognition sites for degradation by labile RNases [42]. Consistent with these findings is the presence of a single ATTTA element in the 3′-noncoding region of the jun-D cDNA [18].

Another major signal transduction pathway involves the CREBP family of transcription factors. The cAMP-responsive promoter element (TGACGTCA) recognized by these proteins is similar to the AP-1 binding site. Several genes have been described that encode proteins that bind to the CRE [29]. These CRE-binding proteins contain the leucine zipper motif and, like Jun and Fos, interact with DNA as both homodimers and heterodimers [29]. Moreover, certain CREB proteins form dimers with Jun that bind to the CRE and not the AP-1 site [34]. Although the similarity between CRE and AP-1 sequences may provide an opportunity for cross-talk between the TPA- and cAMP-responsive pathways, the present results indicate that the cellular response to ara-C is selective for induction of *jun*, and not CREBP1, gene expression.

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