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ACTIVATION OF THE jun-D GENE IN HUMAN MYELOID CELLS
BY 1- β -D-ARABINOFURANOSYLCYTOSINE

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Abbreviations used are: ara-C, 1- β -D-arabinofuranosylcytosine; TRE, phorbol ester-responsive element; CRE, cAMP-responsive element; TPA, 12-O-tetradecanoylphorbol-13-acetate; CREBP, CRE-binding proteins; CHX, cycloheximide; PKC, protein kinase C.

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INTRODUCTION

The transcription factor AP-1 binds to a DNA consensus sequence TGA^G/cTCA (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 related to the formation of homodimers or heterodimers with certain other proteins that contain a leucine zipper and DNA binding domain (11). This structure is shared by a family of transcription factors which includes 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 to Jun/Jun homodimers (12-14). Moreover, the half-lives of Jun/Fos complexes with DNA are prolonged compared to those formed between DNA and Jun/Jun dimers (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). These findings have suggested that the jun genes confer distinct regulatory patterns on different target genes.

The c-jun, jun-B and jun-D genes are differentially expressed in mouse tissues. jun-D mRNA is more abundant than c-jun and, while jun-B and jun-D expression is similar in most tissues, jun-B mRNA levels are higher than that for jun-D in testis and ovary

(17). Levels of jun-D expression are also higher compared to 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 has a distinct role in 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 and functions as a relative chain terminator (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). However, transcriptional activation of the c-jun and jun-B genes and the mechanisms responsible for these effects remain unclear. 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, we also demonstrate that this agent has little effect on expression of genes coding for proteins that bind to the related cAMP-responsive consensus sequence (CRE) (29).

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MATERIALS AND METHODS

Cell culture. Human HL-525 myeloid leukemia cells (30) were grown in RPMI 1640 medium containing 20% fetal bovine serum (FBS) with 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. 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 (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) was subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to the following ³²P-labeled DNA probes: 1) the 1.7-kb EcoRI fragment of a murine jun-D cDNA purified from the XHJ-12.4 plasmid (18); 2) the pAI plasmid containing a 2.0-kb PstI insert of the chicken β-actin gene (32); 3) the CREBP1 gene (33); 4) the CREBP2 gene (34); and 5) the gadd153 gene (35). 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 with a laser densitometer.

Nuclear run-on assays. Nuclei were isolated from 10⁸ HL-525 cells and suspended in 100 µl of glycerol buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA). An equal volume of reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 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°C for 30 min with 250 μ Ci of [α -³²P]UTP (3000 Ci/mmol; Dupont, Boston, MA). The nuclear RNA was isolated as described (26-28) and hybridized to the following digested DNAs: 1) the 1.7-kb EcoRI fragment of the mouse jun-D cDNA (18); and 2) the 2.0-kb PstI fragment of the chicken β -actin gene (31). The digested DNAs were run in 1% agarose gels and transferred to nitrocellulose filters. Hybridizations were performed with 10⁷ cpm of ³²P-labeled RNA/ml in 10 mM Tris-HCl, pH 7.4, 4x SSC (0.15 M sodium chloride, 0.15 M sodium citrate), 1 mM EDTA, 0.1% SDS, 2x Denhardt's solution (0.02% polyvinylpyrrolidone), 40% formamide, 100 μ g/ml yeast tRNA, for 72 h at 42°C. The filters were washed in 1) 2x SSC, 0.1% SDS, at 37°C for 30 min; 2) 200 ng/ml RNase A in 2x SSC at room temperature for 5 min; and 3) 0.1x SSC, 0.1% SDS, at 42°C for 30 min.

RESULTS

Previous studies have demonstrated that the phorbol ester-resistant variant of HL-60 cells, designated HL-525, is defective in protein kinase C (PKC)-mediated signal transduction (30). Treatment of these cells, as well as wild type HL-60 cells, with 32 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) had little if any effect on jun-D mRNA levels (data not shown). In contrast, exposure of HL-525 cells to ara-C was associated with increases in jun-D expression. Low levels of -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 h. However, longer exposures to this agent resulted in increases in jun-D mRNA levels which were first apparent at 3 h and maximal at 6 h (Fig. 1). This effect of ara-C was transient with decreases in jun-D expression to near baseline levels at 24 h (Fig. 1). Moreover, these changes in jun-D expression were associated with little if any change in actin mRNA levels (Fig. 1). This stimulation of jun-D expression by ara-C was concentration and time-dependent. At 6 h 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 12 and 24 h 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 which specifically bind to the CRE (29). For example, two CRE-binding proteins (CREBP), designated CREBP1 and CREBP2, contain leucine zipper motifs similar to those present in Jun and Fos (33,34). CREBP1 transcripts were detectable in HL-525 cells (Fig. 3). However, in contrast to the effects on jun-D expression, ara-C had little effect on CREBP1 mRNA levels (Fig. 3). Similar findings were obtained for the CREBP2 gene (Fig. 3). Moreover, we were unable to demonstrate changes in expression of the growth arrest and DNA damage-inducible gene, gadd 153 (35) (Fig. 3). Taken together, these findings suggested that ara-C inducibility is selective for jun-D, as well as other jun family members (26-28).

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 h 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 X-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.

In order to further study the control of jun-D expression by ara-C, cells were pretreated with cycloheximide (CHX) to inhibit protein synthesis. CHX alone had no detectable effect on jun-D

mRNA levels (Fig. 5). However, the combination of CHX and ara-C demonstrated superinduction of jun-D transcripts at 1, 3 and 6 h compared to that obtained with ara-C alone (Fig. 5). These findings indicated that ara-C induces jun-D expression in the absence of protein synthesis and that ara-C-induced jun-D transcripts may be regulated posttranscriptionally by a labile protein. In order to determine whether ara-C 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 approximately 15 min (Fig. 6A). In contrast, this half-life was increased to 60 min when cells were first treated with ara-C to induced jun-D expression (Fig. 6B). Moreover, stability of jun-D mRNA was further increased ($t^{1/2}$ =120 min) when the ara-C-treated cells were exposed to both actinomycin D and CHX (Fig. 6C). These findings indicated that the increase in jun-D mRNA levels during ara-C treatment is also mediated by a posttranscriptional mechanism involving the synthesis of a labile protein.

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 PKC (28). In this context, other investigators have demonstrated that TPA activates Jun/AP-1 through PKC, and that this event is sufficient for induction of c-jun gene transcription (36,37). While c-jun, as well as jun-B, are induced in myeloid leukemia cells by TPA (38,39), similar exposures to this agent had little effect on jun-D gene expression. This finding is in concert with the 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), to distinguish from 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. Although the precise role of Jun-D in transactivation 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).

Little is known about the regulation of jun-D gene expression. Indeed, other than the demonstration that cAMP increases jun-D mRNA levels (20), we are unaware of other studies

which have addressed the signaling events that control this gene. 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: 1) ara-C activates jun-D transcription in the TPA-unresponsive HL-525 cells; and 2) TPA is ineffective in inducing jun-D in HL-60 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 mechanism. The half-life of ara-C-induced jun-D transcripts was prolonged compared to that in untreated cells. Moreover, the results indicate that levels of ara-C-induced jun-D transcripts are regulated at the posttranscriptional level by a labile protein. The finding that inhibition of protein synthesis is associated with superinduction of jun-D transcripts is in concert with the demonstration that early response genes have AT-rich regions in their 3'-untranslated regions which may serve as 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).

The present studies further demonstrate that ara-C-induced jun-D expression occurs in the absence of changes in other genes

that code for leucine zipper DNA binding proteins. Another major signal transduction pathway involves the CREB 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 which 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 (33). 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 limited to induction of jun, but not CREBP, gene expression. In this regard, ara-C had little effect on CREBP1 and CREBP2 mRNA levels. Other work has demonstrated that the gadd153 gene, which similarly encodes a leucine zipper containing protein, is transcriptionally activated by certain DNA-damaging agents, but not by growth arrest signals (35,43). In the present studies, ara-C had no detectable effect on gadd153 mRNA levels. Taken together, these findings indicate that the cellular response to ara-C is selective for induction of the jun gene family and that distinct leucine zipper encoding genes are activated by other classes of DNA-damaging agents.

Finally, few insights are available regarding the signaling pathways by which ara-C regulates early response gene expression. Previous work has demonstrated that cells respond to ara-C

treatment with activation of a protein kinase which may contribute to inducing members of the jun gene family. However, the initial event that stimulates this protein kinase activity or jun-D gene expression remains unclear. The effects of ara-C appear to be limited to inhibition of DNA replication and are associated with DNA fragmentation (44). This fragmentation includes internucleosomal DNA cleavage (45). Other studies have shown that c-jun transcripts are increased in cells treated with DNA-damaging agents, such as ionizing radiation, UV light and etoposide (46-48). Taken together, these findings have suggested that activation of jun gene transcription may represent a physiologic response to damage of genomic DNA.

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FIGURE LEGENDS

Figure 1. Induction of jun-D expression by ara-C. HL-525 cells were treated with 10^{-5} M ara-C for the indicated times. Total cellular RNA (20 μ g) was hybridized to the 32 P-labeled jun-D and actin DNA probes.

Figure 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 h. B. Cells were treated with 10^{-7} M ara-C for the indicated times. Total cellular RNA (20 μ g) was hybridized to the jun-D and actin probes.

Figure 3. Effects of ara-C on CREBP and gadd153 gene expression. HL-525 cells were exposed to 10^{-5} M ara-C for the indicated times. Poly A⁺-selected RNA (2 μ g) was hybridized to 32 P-labeled CREBP1, CREBP2 or gadd153 DNA probes.

Figure 4. Transcription of the jun-D gene is activated by ara-C. HL-525 cells were treated with 10^{-5} M ara-C for 6 h. 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.

Figure 5. Effects of CHX on jun-D mRNA levels in ara-C-treated cells. HL-525 cells were treated with 10^{-5} M ara-C, 10 μ g/ml CHX

or both agents for the indicated times. Total cellular RNA was hybridized to the jun-D and actin probes.

Figure 6. Stability of jun-D mRNA. A. HL-525 cells were treated with 5 µg/ml actinomycin D (act D) for the indicated times. B. Cells were treated with 10^{-5} M ara-C for 6 h followed by the addition of act D. C. Cells were treated with 10^{-5} M ara-C for 6 h followed by the addition of both act D and CHX. Total cellular RNA (20 µg) was hybridized to the jun-D and actin probes.