Activation of the Early Growth Response 1 Gene and Nuclear pp90^{rsk} in Human Myeloid Leukemia Cells by 1-(β-D-Arabinofuranosyl)cytosine†

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Received April 15, 1993; Revised Manuscript Received June 21, 1993⊗

ABSTRACT: The early growth response 1 (EGR-1) gene is induced by mitogens, differentiating stimuli, and certain genotoxic agents in diverse cell types. The present work has examined the effects of 1-(β-D-arabinofuranosyl)cytosine (ara-C), an antileukemia agent that misincorporates into DNA, on EGR-1 expression. Treatment of HL-525 myeloid leukemia cells with ara-C was associated with transient increases in EGR-1 mRNA levels. Nuclear run-on assays showed that this effect is related at least in part to activation of EGR-1 gene transcription. Sequences responsive to ara-C-induced signals were determined by deletion analysis of the EGR-1 promoter. The results demonstrate that ara-C inducibility of the EGR-1 gene is conferred by a region containing six serum response or CC(A/T)$_6$GG (CArG) motifs. Further analysis demonstrated that the first two distal or 5′-most CArG elements are functional in the ara-C response. An oligomer corresponding to the first CArG element also conferred ara-C inducibility of the minimal thymdine kinase gene promoter, while no inducibility was detectable using a similar oligomer containing a mutated CArG box. Other work has demonstrated that the nuclear serum response factor (SRF) interacts with the CArG box in the EGR-1 promoter and that the serine/threonine pp90^{rsk} protein kinase phosphorylates SRF in vitro at sites phosphorylated in vivo. The present studies demonstrate that ara-C has little if any effect on cytosolic pp90^{rsk} as determined by immunoblotting to assess electrophoretic mobility and by immune-complex kinase assays using S6 peptide as substrate. In contrast, both a decrease in electrophoretic mobility and an increase in S6 peptide phosphorylation confirmed activation of this kinase in the nucleus. Taken together, these findings suggest that ara-C activates a nuclear pp90^{rsk} cascade and that this event may contribute to induction of EGR-1 transcription by a CArG motif-mediated mechanism.

The stimulation of quiescent fibroblasts with serum or polypeptide growth factors is associated with the rapid and transient activation of a variety of immediate-early genes. Certain early response genes code for transcription factors involved in nuclear signaling pathways. One such family that encodes leucine-zipper-containing nuclear proteins includes the c-*jun*, *jun*-B, c-*fos*, *fos*-B, and *fra* genes (Greenberg & Ziff, 1984; Cohen & Curran, 1988; Ryder et al., 1988; Ryder & Nathans, 1988; Hirai et al., 1989; Zerial et al., 1989; Nishina et al., 1990). Another gene family encoding zinc finger transcription factors includes the early growth response (EGR) 1 gene (also known as *zif*268, NGF1-A, Krox 24, and TIS-8) (Lim et al., 1987; Milbrandt, 1987; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988). While the *jun*/*fos* and EGR-1 genes are induced by diverse mitogenic stimuli in a variety of cell types, other studies have demonstrated increased expression of these genes by differentiating signals. For example, differentiation of human myeloid leukemia cells by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is associated with induction of both *jun* and EGR-1 (Sherman et al., 1990b; Datta et al., 1991; Kharbanda et al., 1991).

Recent studies have demonstrated that exposure of human myeloid leukemia ells to 1-(β-D-arabinofuranosyl)cytosine (ara-C) also results in induction of the c-*jun*, *jun*-B, and c-*fos* genes (Datta et al., 1990; Kharbanda et al., 1990). Ara-C incorporates into leukemic cell DNA and functions as a potent inhibitor of DNA synthesis (Major et al., 1981, 1982). Although ara-C is the most effective agent in the treatment of acute myelogenous leukemia (Frei et al., 1969), little is known about the basis for this selectivity against leukemic cells. Nonetheless, the available insights suggest that ara-C induces DNA fragmentation and that this event is associated with activation of certain early response genes (Fram & Kufe, 1982; Gunji et al., 1991). Indeed, other studies have demonstrated that expression and DNA binding activity of the transcription factor κB (NF-κB) is increased in ara-C-treated leukemic cells (Brach et al., 1992b). These findings have indicated that multiple nuclear signal transduction pathways are activated during the cellular response to this agent.

The present work extends ara-C-induced signaling to the EGR-1 gene. This gene encodes a 533 amino acid nuclear phosphoprotein with a Cys_2-His_2 zinc finger motif that is partially homologous to the corresponding domain in the Wilms tumor susceptibility gene (Gessler, 1990). The EGR-1 protein binds to the DNA sequnce CGCCCCCGC in a zinc-dependent manner and thereby functions in the regulation of gene transcription (Christy & Nathans, 1989a; Cao et al., 1990; Gupta et al., 1991). Mitogenic and differentiating signals

† This investigation was supported by PHS Grant CA29431 awarded by the National Cancer Institute, DHHS.

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⊗ Abstract published in *Advance ACS Abstracts*, August 15, 1993.

[1] Abbreviations: EGR, early growth response; TPA, 12-*O*-tetradeanoylphorbol 13-acetate; ara-C, 1-(β-D-arabinofuranosyl)cytosine; PMSF, phenylmethanesulfonyl fluoride; CAT, chloramphenicol acetyltransferase; CArG, C(A/T)$_6$GG; SRF, serum response factor.

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have been found to induce the rapid and transient expression of EGR-1 in diverse cell types (Lau & Nathans, 1987; Sukhatme et al., 1987, 1988; Kharbanda et al., 1991). EGR-1 transcription is activated by the protein tyrosine kinase activity of v-*src* and v-*fps* (Qureshi et al., 1991a,b; Alexandropoulos et al., 1992). Other work has shown that the serine/threonine kinase activity of c-*raf* is also involved in mediating inducibility of this gene (Qureshi et al., 1991c). Moreover, additional studies have demonstrated that induction of EGR-1 expression is coordinate with c-*fos* in many situations and that such regulation is mediated by the presence of serum response elements in both promoters (Sukhatme et al., 1988; Christy & Nathans, 1989b; Guis et al., 1990).

The present results demonstrate that ara-C increases EGR-1 expression and that this effect is related to regulation at the transcriptional and posttranscriptional levels. We also demonstrate that ara-C inducibility of the EGR-1 gene is conferred through serum response elements [CC(A+T-rich)$_6$GG domains] and that this induction is associated with nuclear activation of the protein phosphorylation cascade regulating the protein serine/threonine kinase pp90^{rsk}.

MATERIALS AND METHODS

Cell Culture. Human HL-525 myeloid leukemia cells (Homma et al., 1988) were maintained in RPMI 1640 medium containing 15% fetal bovine serum (FBS) with 1 mM L-glutamine, 100 units mL penicillin, and 100 μg/mL streptomycin. The cells were treated with varying concentrations of ara-C (Sigma Chemical Co., St. Louis, MO), 10 μg/mL cycloheximide (Sigma), and 10 μg/mL actinomycin D (Sigma). Cell viability was determined by trypan blue exclusion.

Isolation and Analysis of RNA. Total cellular RNA was isolated by the guanidine isothiocyanate/cesium chloride method (Chirgwin et al., 1979), analyzed by electrophoresis in 1% agarose/formaldehyde gels, transferred to nitrocellulose filters, and hybridized to the following ^{32}P-labeled DNA probes: (1) the 0.7-kb non-zinc-finger insert of the murine Egr-1 cDNA (Sukatme et al., 1988) and (2) the 2.0-kb *Pst*I fragment of a chicken β-actin DNA purified from the pA1 plasmid (Cleveland et al., 1980). Hybridizations were performed as described (Kharbanda et al., 1991). Autoradiograms were scanned by laser densitometry.

Nuclear Run-On Assays. Nuclei were isolated and newly elongated transcripts were labeled with [α-^{32}P]UTP (800 Ci/mmol; Dupont-New England Nuclear, Boston, MA) at 26 °C for 30 min (Kharbanda et al., 1991). The labeled RNA was hybridized to the following DNAs: (1) the 2.0-kb *Pst*I fragment of the chicken β-actin pA1 plasmid (Cleveland et al., 1980) and (2) the 0.7-kb *Hin*dIII/*Eco*RI fragment of the murine Egr-1 cDNA (Sukhatme et al., 1988). The digested DNAs were denatured by heating to 65 °C for 15 min, separated in 1% agrose gels, and transferred to nitrocellulose filters. The filters were prehybridized in 5× Denhardt's solution, 40% formamide, 4× SSC, 5 nM Na_2EDTA, 0.4% SDS, and 100 μg/mL yeast tRNA for 2 h. Hybridizations were performed with 10^7 cpm of ^{32}P-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, in 200 ng/mL RNase A in 2× SSC at 37 °C for 20 min, and finally in 0.1× SSC/0.1% SDS at 42 °C for 30 min.

Reporter Assays. Construction of pE425, pE395, pE359, pE342, pE70, ptkSRE1, and ptkSRE1m was as described (Gius et al., 1990). The vectors were transfected into cells using the DEAE-dextran technique (Grosschedl & Baltimore, 1985). Cells (2×10^7) were harvested, washed with phosphate-buffered saline (PBS) and resuspended in 1 mL of 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 mg/mL DEAE-dextran, 8 μg of plasmid, 140 mM NaCl, 5 mM KCl, 375 μM $Na_2HPO_4 \cdot 7H_2O$, 1 mM $MgCl_2$, and 675 μM $CaCl_2$. The cells were incubated at 37 °C for 45 min, washed with medium containing 10% FBS, resuspended in complete medium, and then incubated at 37 °C. Thirty-six hours after transfection, the cells were distributed equally into two aliquots; one aliquot served as a control, and the other was treated with ara-C. The cells were harvested after 9 h and lysed by three cycles of freezing and thawing in 0.25 M Tris-HCl (pH 7.8) and 1 mM phenylmethanesulfonyl fluoride (PMSF). Equal amounts of cell extract were incubated with 0.025 μCi of [^{14}C]chloramphenicol, 0.15 M Tris-HCl (pH 7.8), and 0.4 mM acetyl-CoA for 1 h at 37 °C. The enzyme assay was terminated by addition of ethyl acetate. The organic layer containing the acetylated [^{14}C]chloramphenicol was separated by thin-layer chromatography using chloroform/methanol (95%/5%, v/v). Following autoradiography, both acetylated and unacetylated forms of [^{14}C]chloramphenicol were cut from the plates, and conversion of chloramphenicol to the acetylated form was calculated by measurement of radioactivity.

Subcellular Fractionation. Cells were washed twice with ice-cold PBS and lysed in 1 mL of hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 40 μg/mL PMSF, and 10 μg/mL each lupeptin and aprotinin). The cell suspension was incubated on ice for 30 min and then subjected to Dounce homogenization (20–25 strokes, tight pestle A). The lysate was loaded onto 1 mL of 1 M sucrose in lysis buffer and centrifuged at 1600*g* for 15 min to pellet nuclei. The nuclear pellet was solubilized in hypotonic lysis buffer containing 0.5% Nonidet P-40, 0.1% deoxycholate, and 0.1% Brij-35 and then centrifuged at 10 000 rpm for 10 min at 4 °C to remove undissolved material. The supernatant above the sucrose gradient was centrifuged at 150000*g* for 30 min. The resulting supernatant was adjusted to 0.5% NP-40, 0.1% deoxycholate, and 0.1% Brij-35 and used as the cytosolic fraction.

Immunoblot Analysis. Immunoblotting of pp90^{rsk} was performed on cytosolic and nuclear fractions as described (Chen & Blenis, 1990). Proteins were separated in SDS–7.5% polyacrylamide gels and transferred to nitrocellulose paper. The residual binding sites were first blocked by incubating the filter in 5% dry milk in PBST (PBS/0.5% Tween 20) for 1 h at room temperature. The filters were then incubated with a rabbit anti-pp90^{rsk} antiserum for 1 h with shaking. After three washes with PBST, the blots were incubated with anti-rabbit IgG peroxidase conjugate (Sigma). The antigen–antibody complexes were visualized by chemiluminescence (ECL detection system, Amersham, Arlington Heights, IL).

Immune-Complex Protein Kinase Assays for pp90^{rsk}. Cytosolic and nuclear proteins as prepared above were incubated with anti-pp90^{rsk} and immune-complex S6 protein kinase assays were performed on the immunoprecipitate (Chen & Blenis, 1990; Chen et al., 1992). The immune complexes were washed twice with buffer A (10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, and 40 μg/mL PMSF), twice with buffer B (10 mM Tris-HCl, pH 7.2, 1 M NaCl, 0.1% NP-40, 1 mM sodium orthovanadate, 40 μg/mL PMSF, and 2 mM dithiothreitol), and once with buffer C (50 mM Tris-HCl, pH 7.2, and 150

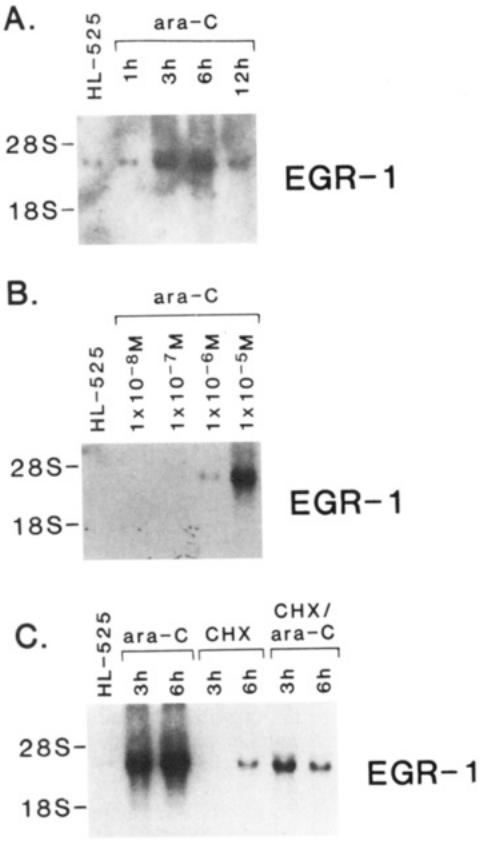

FIGURE 1: Ara-C induces EGR-1 expression in HL-525 cells. HL-525 cells were treated with 10^{-5} M ara-C for the indicated times (A) or with the indicated concentrations of ara-C for 6 h (B). Cells were also treated with 10^{-5} M ara-C, 10 μg/mL cycloheximide (CHX), or both agents for the indicated times (C). Total cellular RNA (20 μg) was hybridized to the ^{32}P-labeled EGR-1 probe. Hybridization to the actin probe demonstrated equal loading of the lanes. Viability as determined by trypan blue exclusion was over 95% for cells treated with 10^{-5} M ara-C for 12 h.

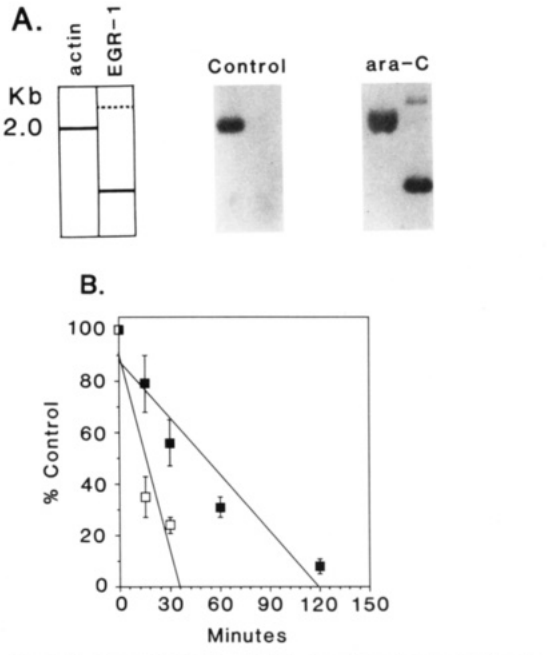

FIGURE 2: Ara-C induces EGR-1 mRNA levels by transcriptional and posttranscriptional mechanisms. HL-525 cells were treated with 10^{-5} M ara-C for 3 h. (A) Nuclei were isolated, and newly elongated ^{32}P-labeled transcripts were hybridized to 2 μg of actin and EGR-1 DNA inserts after restriction enzyme digestion and Southern blotting. Solid lines in the schematic indicate the relative positions of the inserts. The dotted line represents vector sequences. (B) Control and ara-C-treated cells were exposed to actinomycin D to inhibit further transcription. At the indicated times after the addition of actinomycin D, total cellular RNA was isolated and hybridized to the ^{32}P-labeled ERG-1 and β-actin DNA probes. Signal intensity as determined by densitometric scanning was determined for the EGR-1 hybrids and normalized to that for actin. The results represent the mean ± average deviation of two separate experiments. The half-life calculated by the method of least squares was 15 min (R = 0.86) for control cells (□) and 45 min (R = 0.91) for cells treated with ara-C (■).

mM NaCl). After washing, the immune complexes were suspended in pp90^{rsk} reaction buffer (20 mM HEPES and 10 mM $MgCl_2$) without ATP and substrate. The reaction (50 μL) was started by the addition of 50 μM ATP (20 μCi of [γ-^{32}P]ATP) and 100 μM S6 peptide (RRRLSSLRA). After incubation for 15 min at 30 °C, 25 μL was spotted onto phosphocellulose paper (P81, Whatman) followed by washing with 1% phosphoric acid and then distilled water. The incorporated [^{32}P]phosphate was determined by scintillation counting.

RESULTS

A low level of EGR-1 transcripts was detectable in untreated HL-525 cells (Figure 1A). In contrast, treatment with ara-C was associated with an increase in EGR-1 expression that was detectable at 3 and 6 h (Figure 1). Longer exposures of 12 h resulted in downregulation of these transcripts to nearly the level in control cells. This transient increase in EGR-1 mRNA levels occurred in the absence of changes in expression of the actin gene (data not shown). The effects of ara-C on EGR-1 expression were concentration-dependent. Little if any induction was detectable at 3 h with 10^{-8} and 10^{-7} M ara-C, while EGR-1 transcripts were increased at the higher drug concentrations (Figure 1B). In order to determine whether ara-C-induced increases in EGR-1 expression require protein synthesis, we performed similar studies in the presence of cycloheximide. Treatment with cycloheximide alone was associated with inreases in EGR-1 mRNA levels at 6 h (Figure 1C). However, exposure of cells to both cycloheximide and ara-C resulted in levels of EGR-1 transcripts which were lower than those obtained with ara-C alone (Figure 1C). These findings indicated that de novo protein synthesis is at least in part required for induction of EGR-1 expression by ara-C.

Nuclear run-on assays were performed to determine whether ara-C-induced EGR-1 expression is regulated at the transcriptional level. The actin gene (positive control) was constitutively transcribed in untreated cells and there was little effect on this rate at 3 h of ara-C treatment (Figure 2A). EGR-1 gene transcription was at low to undetectable levels in untreated cells. However, in contrast to the actin gene, EGR-1 transcription was increased 8-fold by ara-C treatment (Figure 2A). Similar findings were obtained in two separate experiments. Stability of EGR-1 transcripts were also studied by treating HL-525 cells with actinomycin D to inhibit further transcription. By this approach, the half-life of EGR-1 mRNA as determined by densitometric scanning was 15 min (Figure 2B). Similar stability studies were performed by adding actinomycin D at 3 h of ara-C exposure. The half-life of EGR-1 mRNA under these experimental conditions was 45 min (Figure 2B). Taken together, these results indicated that ara-C-induced increases in EGR-1 mRNA levels are regulated by transcriptional and posttranscriptional mechanisms.

In order to identify the cis-acting elements responsible for ara-C induced EGR-1 transcription, we used a fragment of the EGR-1 gene (position –425 to +65) linked to the CAT reporter gene (pE425) in transient expression assays. This

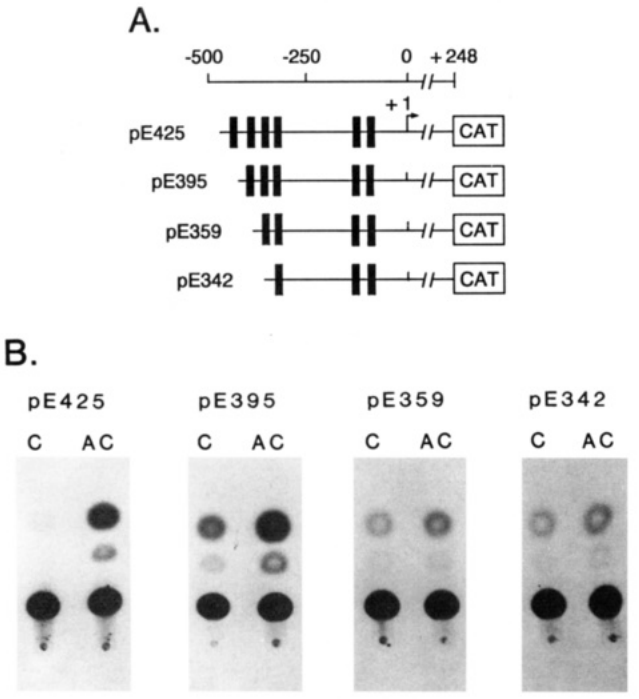

FIGURE 3: Ara-C induced EGR-1 mRNA transcription is regulated by CArG elements. (A) HL-525 cells were transfected with the indicated constructs and maintained in medium for 36 h. Ara-C (10^{-5} M) was then added and the cells were harvested after 9 h for analysis of CAT activity. CArG boxes are shown as solid bars. (B) Conversion of chloramphenicol to the acetylated forms is shown for control (C) and ara-C-treated (AC) cells transfected with the indicated constructs. Percent conversion (mean ± SD) was determined by scintillation counting. pE425 (n = 8): C, 2.1% ± 0.6%; ara-C, 10.2% ± 3.2%. pE395 (n = 4): C, 4.6% ± 1.6%, ara-C, 12.9% ± 4.7%. pE359 (n = 4): C, 2.7% ± 0.9%; ara-C, 3.3% ± 1.4%. pE342 (n = 5): C, 2.5% ± 0.6%; ara-C, 2.7% ± 0.9%.

fragment contains several potential cis elements that include six serum response elements (CArG boxes) (Figure 3A). Cells were transfected with pE425 and then treated with ara-C. Using this approach, ara-C treatment was associated with a 3.4-fold increase in CAT activity (Figure 3B). Comparable findings were obtained with pE395 (five serum response elements), although activation was somewhat lower than that obtained with pE425. However, similar experiments performed with other deleted constructs (pE359 and pE342) demonstrated little if any ara-C inducibility (Figure 3B). These findings indicated that the region encompassing the first two CArG elements is involved in ara-C-induced expression of the EGR-1 gene.

Other studies were performed with the first or 5′-most distal serum response element linked to a heterologous herpes simplex virus thymidine kinase (HSV-TK) viral promoter (Gius et al., 1990; Figure 4A). The presence of a single CArG box was able to confer ara-C responsiveness with a 3.2-fold increase in CAT activity (Figure 4B). In contrast, there was no detectable inducibility when a similar heterologous promoter construct with a mutated element was used (Figure 4B). Taken together, these results indicated that ara-C inducibility is conferred through the serum response element.

Previous studies have demonstrated that serum response factor (SRF) binds to the CArG box (Treisman, 1986; Prywes et al., 1988). Other work has shown that pp90^{rsk} phosphorylates SRF in vitro at sites phosphorylated in vivo (Rivera, Blenis, and Greenberg, unpublished data). Consequently, we asked whether ara-C treatment is associated with activation of pp90^{rsk}. These assays were performed by immunoblotting, as well as by immunoprecipitating pp90^{rsk} and then assaying

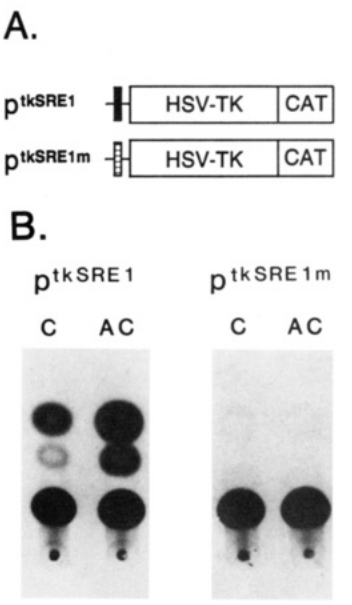

FIGURE 4: The first CArG element confers ara-C inducibility to a heterologous promoter. (A) HL-525 cells were transfected with ptkSRE1 and ptkSRE1m constructs. ptkSRE1 contains the oligomer (CTAGAGGATCCGAAACGCCATATAAGGAGCAGGAGATCTT) that includes the first CArG box cloned into the *Xba*I site of pBLCAT (minimal thymidine kinase gene promoter placed upstream to CAT). ptkSREm has the oligomer (CTAGAGGATCCGAAACGGGATATAACCAGCAGGAGATCTT) with a mutant first CArG box. The transfected cells were maintained in medium for 36 h and then exposed to 10^{-5} M ara-C. (B) Conversion of chloramphemphenicol to the acetylated forms is shown for control (C) and ara-C-treated (AC) cells transfected with the indicated constructs. Percent conversion (mean ± SD) was determined by scintillation counting. ptkSRE1 (n = 3): C, 6.1% ± 2.7%; ara-C, 19.6% ± 3.9%. ptkSREm (n = 3): C, 0.6% ± 0.22%; ara-C, 0.79% ± 0.36%.

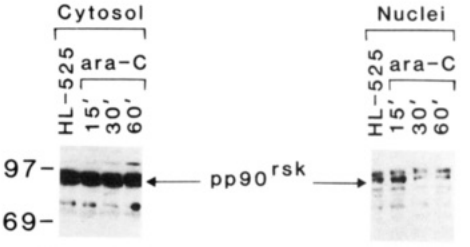

FIGURE 5: Effects of ara-C on electrophoretic mobility of pp90^{rsk}. HL-525 cells were treated with 10^{-5} M ara-C for the indicated times. Cytoplasmic (50 μg) and nuclear (100 μg) proteins were subjected to electrophoresis in SDS–7.5% polyacrylamide gels and transferred to nitrocellulose filters. The filters were incubated with an anti-pp90^{rsk} antiserum and reactivity was visualized by a peroxidase detction system.

for phosphorylation of S6 peptide. Immunoblot analysis of cytosolic fractions from HL-525 cells with the anti-pp90^{rsk} antiserum demonstrated reactivity with two major species at approximately 90 kD (Figure 5). Treatment with ara-C for up to 60 min had little effect on electrophoretic mobility of these proteins (Figure 5). Similar findings were obtained during 3 and 6 h of drug exposure (data not shown). pp90^{rsk} was also detectable as two major species in the nuclear fraction (Figure 5). While treatment with ara-C for 15 min had little effect, longer periods of drug exposure (30 and 60 min) were associated with a decrease in electrophoretic mobility of both pp90^{rsk} isoforms (Figure 5). Such decreases in electrophoretic mobility are associated with activation of pp90^{rsk} (Chen & Blenis, 1990; Chen et al., 1992).

Assays of pp90^{rsk} activity by immunoprecipitation and phosphorylation of S6 peptide demonstrated a pattern con-

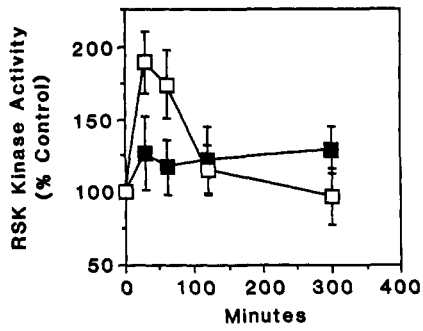

FIGURE 6: Immune-complex assays of pp90^{rsk} activity. HL-525 cells were treated with 10^{-5} M ara-C for the indicated times. Cytoplasmic (closed symbols) and nuclear (open symbols) proteins were immunoprecipitated with anti-pp90^{rsk} antiserum and the immune complexes were assayed for in vitro phosphorylation of S6 peptide (RRRLSSLRA). The results are expressed as the percent of control kinase activity (mean ± SD of two experiments, each performed in duplicate).

sistent with the immunoblotting results. Treatment of HL-525 cells with ara-C had no effect on pp90^{rsk} phosphotransferase activity in the cytosolic fraction (Figure 6). In contrast, nuclear pp90^{rsk} was stimulated by ara-C treatment. Increases in nuclear pp90^{rsk} activity were detectable at 30 min after adding ara-C and then declined by 180 min (Figure 6). Taken together with the immunoblotting studies, these results indicated that ara-C treatment is associated with stimulation of nuclear, but not cytoplasmic, pp90^{rsk} activity.

DISCUSSION

Previous studies have demonstrated that treatment of human myeloid leukemia cells with TPA is associated with induction of the EGR-1 gene (Kharbanda et al., 1991). Since TPA activates protein kinase C (Nishizuka, 1992), these findings indicated that activation of this enzyme confers inducibility of EGR-1 expression. Indeed, treatment of other cell types with phorbol esters results in increased EGR-1 mRNA levels (Christy & Nathans, 1989b). The present studies were performed on HL-525 cells which are deficient in protein kinase C-mediated signal transduction (Homma et al., 1988). These cells thus provide an opportunity to distinguish between protein kinase C-dependent and -independent signaling events. A low level of EGR-1 transcripts was detectable in HL-525 cells, and treatment with ara-C resulted in transient increases in expression of this gene. Nuclear run-on assays demonstrated that ara-C-induced increases in EGR-1 mRNA levels are related at least in part to activation of EGR-1 transcription. The finding that ara-C treatment is also associated with increases in stability of EGR-1 transcripts indicated that both transcriptional and posttranscriptional mechanisms contribute to ara-C-induced expression of this gene. In contrast to these results, treatment of HL-525 cells with TPA had no detectable effect on EGR-1 mRNA levels (data not shown). These findings further indicated that ara-C induces EGR-1 expression by a protein kinase C-independent pathway.

The mechanism of signal transduction elicited by DNA-damaging agents is presently unknown. While ara-C, ionizing radiation, topoisomerase inhibitors, alkylating agents, and UV light have all been shown to induce transcription of the c-*jun* gene (Sherman et al., 1990a; Devary et al., 1991; Rubin et al., 1991, 1992), less is known about their effects on expression of the EGR-1 gene. Since some of these agents may activate pathways unrelated to DNA damage, we have used ara-C because of the selective incorporation of this nucleoside analog into DNA (Kufe et al., 1984; Townsend & Cheng, 1987; Ohno et al., 1988). Conformational and hydrogen-bonding differences of the incorporated arabinose sugar moiety alter chromatin structure and slow chain elongation (Cozzarelli, 1977). The present results demonstrate that ara-C increases EGR-1 mRNA levels at least in part by transcriptional activation of this gene. Induction of EGR-1 promoter–CAT constructs in ara-C-treated cells further supported the stimulation of a signaling pathway that controls transcription of the EGR-1 gene. The kinetics of this activation were similar to those observed for the c-*jun* gene by ara-C (Kharbanda et al., 1990). Moreover, the finding that cycloheximide partially blocks ara-C-induced c-*jun* and EGR-1 expression reflects the involvement, at least in part, of de novo protein synthesis in the activation of both genes.

Recent work has shown that binding of Jun/AP-1 to the AP-1 site in the c-*jun* promoter contributes to ara-C inducibility of this gene (Brach et al., 1992b). The present studies indicate that ara-C induces EGR-1 by activation of serum response elements. The EGR-1 promoter includes six of these CArG-containing elements (Sukhatme, 1988; Christy & Nathans, 1989b). Our findings indicate that the two 5′-most distal CArG boxes are involved in ara-C inducibility of the EGR-1 gene. Other studies have demonstrated that the three most 5′ CArG elements are responsible for induction of the EGR-1 gene in HL-525 cells by ionizing radiation (Datta et al., 1992). Only the first CArG box is functional during okadaic acid-induced EGR-1 expression in myeloid leukemia cells (Kharbanda et al., 1993), while multiple CArG elements confer greater inducibility than that obtained with a single element in 3T3 cells exposed to serum, platelet-derived growth factor and TPA (Christy & Nathans, 1989b). These findings suggest that functionality of the six CArG boxes is inducer-, as well as cell line-, dependent and that protein interaction with these elements may be influenced by surrounding sequences. Indeed, since other sequences between these boxes could be functional cis-elements, we used the first CArG element linked to a heterologous promoter. The finding that this element was sufficient for ara-C inducibility, while mutations in the CArG box abrogated this effect, strongly support the serum response element as the target for ara-C-induced EGR-1 expression.

Previous studies with both the EGR-1 and c-*fos* promoters have demonstrated that the CArG box functions as a binding site for SRF (Treisman, 1986; Prywes et al., 1988; Christy & Nathans, 1989b). Phosphorylation of SRF on serine appears to be required for this DNA binding activity (Prywes et al., 1988). While the protein kinase (or kinases) responsible for this phosphorylation remains unclear, recent studies have shown that pp90^{rsk} phosphorylates SRF in vitro at the same sites phosphorylated in vivo (Rivera, Blenis, and Greenberg, unpublished data). pp90^{rsk} also exhibits phosphotransferase activity toward a variety of substrates including S6 protein, MAP2, c-Fos, and histone H3 (Chen & Blenis, 1990l Chen et al., 1992). Previous studies have demonstrated that the pp90^{rsk} signaling cascade transduces growth regulatory signals to the nucleus (Chen & Blenis, 1990; Chen et al., 1992). The present results show that pp90^{rsk} is also activated during ara-C treatment. In this context, ara-C exposure was associated with a rapid activation of pp90^{rsk} as determined by a decrease in electrophoretic mobility and an increase in phosphorylation of S6 peptide. During mitogenesis, cytoplasmic pp90^{rsk} is activated prior to its nuclear counterpart (Chen et al., 1992). In the present studies with ara-C, activation was restricted to nuclear pp90^{rsk}. While the basis for this selective activation is unclear, ara-C-induced signaling mechanisms involving pp90^{rsk} may be confined to the nucleus following incorporation of this analog into DNA. Whatever the mechanism, the

temporal and spatial relationships between activation of pp90^{rsk} and EGR-1 transcription suggest that these two events may involve coordinate regulation, presumably through SRF phosphorylation. The activation of this nuclear protein kinase and the EGR-1 gene by ara-C also provides the basis for further defining the signaling events that constitute the cellular response to this agent.

REFERENCES

Alexandropoulos, K., Qureshi, S. A., Rim, M., Sukhatme, V. P., & Foster, D. A. (1992) *Nucleic Acids Res. 20*, 2355–2359.

Brach, M., Herrmann, F., & Kufe, D. (1992a) *Blood 79*, 728–734.

Brach, M., Kharbanda, S., Herrmann, F., & Kufe, D. (1992b) *Mol. Pharmacol. 41*, 60–63.

Cao, X., Koski, R. A., Gashler, A., McKiernan, M., Morris, C. F., Gaffney, R., Hay, R. V., & Sukhatme, V. P. (1990) *Mol. Cell. Biol. 10*, 1931–1939.

Chen, R. H., & Blenis, J. (1990) *Mol. Cell. Biol. 10*, 3204–3215.

Chen, R. H., Sarnecki, C., & Blenis, J. (1992) *Mol. Cell. Biol. 12*, 915–927.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Christy, B. A., & Nathans, D. (1989a) *Proc. Natl. Acad. Sic. U.S.A. 86*, 8737–8741.

Christy, B. A., & Nathans, D. (1989b) *Mol. Cell. Biol. 9*, 4889–4895.

Christy, B. A., Lau, L. F., & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7857–7861.

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., & Kirschner, M. W. (1980) *Cell 20*, 95–105.

Cohen, D. R., & Curran, T. (1988) *Mol. Cell. Biol. 8*, 2063–2069.

Cozzarelli, N. (1977) *Annu. Rev. Biochem. 46*, 641–668.

Datta, R., Kharbanda, S., & Kufe, D. (1990) *Mol. Pharmacol. 38*, 435–439.

Datta, R., Sherman, M. L., Stone, R. M., & Kufe, D. (1991) *Cell Growth Differ. 2*, 43–49.

Datta, R., Rubin, E., Sukhatme, V., Qureshi, S., Weichselbaum, R., & Kufe, D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10149–10153.

Devary, Y., Gottlieb, R. A., Lau, L. F., & Karin, M. (1991) *Mol. Cell Biol. 11*, 2804–2811.

Fram, R. J., & Kufe, D. (1982) *Cancer Res. 42*, 4050–4053.

Frei, E., Bickers, J. N., Hewlett, J. S., Lane, M., Leary, W. V., & Tally, R. W. (1969) *Cancer Res. 29*, 1325–1332.

Gessler, M. (1990) *Nature 34*, 774–778.

Gius, D., Cao, X., Rauscher, F. J., III, Cohen, D. R., Curran, T., & Sukhatme, V. P. (1990) *Mol. Cell. Biol. 10*, 4243–4255.

Greenberg, M. E., & Ziff, E. B. (1984) *Nature 311*, 433–438.

Grosschedl, R., & Baltimore D. (1985) *Cell 41*, 885–897.

Gunji, H., Kharbanda, S., & Kufe, D. (1991) *Cancer Res. 51*, 741–743.

Gupta, M. P., Gupta, M., Zak, R., & Sukhatme, V. P. (1991) *J. Biol. Chem. 266*, 12813–12816.

Hirai, S.-I., Ryseck, R. P., Mechta, F., Bravo, R., & Yanis, M. (1989) *EMBO J. 8*, 1433–1439.

Homma, Y., Gemmell, M. A., & Huberman, E. (1988) *Cancer Res. 48*, 2744–2748.

Kharbanda, S., Sherman, M. L., & Kufe, D. (1990) *J. Clin. Invest. 86*, 1517–1523.

Kharbanda, S., Nakamura, T., Stone, R., Hass, R., Bernstein, S., Sukhatme, V., & Kufe, D. (1991) *J. Clin. Invest. 88*, 571–577.

Kharbanda, S., Rubin, E., Datta, R., Hass, R., Sukhatme, V., & Kufe, D. (1993) *Cell Growth Differ. 4*, 17–23.

Kufe, D., Munroe, D., Herrick, D., & Spriggs, D. (1984) *Mol. Pharmacol. 26*, 128–134.

Lau, L. F., & Nathans, D. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1182–1186.

Lemaire, P., Revelant, O., Bravo, R., & Charney P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4691–4695.

Lim, R. W., Varnum, B. C., & Herschman, H. R. (1987) *Oncogene 1*, 263–270.

Major, P., Egan, E., Minden, M., Beardsley, G., & Kufe, D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3235–3239.

Major, P., Egan, E., Herrick, D., & Kufe, D. (1982) *Biochem. Pharmacol. 31*, 2937–2940.

Milbrandt, J. (1987) *Science 238*, 797–799.

Nishina, H., Sato, H., Suzuki, T., Sato, M., & Iba, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3619–3623.

Nishizuka, Y. (1992) *Science 258*, 607–614.

Ohno, Y., Spriggs, D., Matsukage, A., Ohno, T., & Kufe, D. (1988) *Cancer Res. 48*, 1494–1498.

Prywes, R., Dutta, A., Cromlish, J., & Roeder, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7206–7210.

Qureshi, S. A., Cao, X.; Sukhatme, V. P., & Foster, D. A. (1991a) *J. Biol. Chem. 266*, 10802–10806.

Qureshi, S. A., Joseph, C. K., Rim, M., Maroney, A., & Foster, D. A. (1991b) *Oncogene 6*, 995–999.

Qureshi, S. A., Rim, M., Bruder, J., Kolch, W., Rapp, U., Sukhatme, V. P., & Foster, D. A. (1991c) *J. Biol. Chem. 266*, 20594–20597.

Rubin, E., Kharbanda, S., Gunji, H., & Kufe, D. (1991) *Mol. Pharmacol. 39*, 697–701.

Rubin, E., Kharbanda, S., Gunji, H., Weichselbaum, R., & Kufe, D. (1992) *Cancer Res. 52*, 878–882.

Ryder, K., & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8454–8457.

Ryder, K., Lau, L. F., & Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1487–1491.

Sherman, M., Datta, R., Hallahan, D., Weichselbaum, R., & Kufe, D. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5663–5666.

Sherman, M., Stone, R., Datta, R., Bernstein, S., & Kufe, D. (1990b) *J. Biol. Chem. 265*, 3320–3323.

Sukhatme, V. P., Kartha, S., Toback, F. G., Taub, R., Hoover, R. G., & Tsai-Morris, C.-H. (1987) *Oncogene 1*, 343–355.

Sukhatme, V. P., Cao, X.; Chang, L. C., Tsai-Morris, C.-H., Stamenkovich, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., LeBeau, M. M., & Adamson, E. D. (1988) *Cell 53*, 37–43.

Townsend, A., & Cheng, Y.-C. (1987) *Mol. Pharmacol. 32*, 330–339.

Treisman, R. (1986) *Cell 46*, 567–574.

Zerial, M., Toschi, L., Ryseck, R.-P.; Schuermann, M., Muller, R., & Bravo, R. (1989) *EMBO J. 8*, 805–813.