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Genetics

Reactive oxygen intermediates target CC(A/T)$_6$GG sequences to mediate activation of the early growth response 1 transcription factor gene by ionizing radiation

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ABSTRACT **The cellular response to ionizing radiation includes induction of the early growth response 1 gene (*EGR1*). The present work has examined the involvement of reactive oxygen intermediates (ROIs) in this response. Exposure of human HL-525 cells, an HL-60 subclone deficient in protein kinase C-mediated signaling, to both ionizing radiation and H_2O_2 was associated with increases in EGR-1 transcripts. These increases in EGR-1 expression were inhibited by the antioxidant *N*-acetyl-L-cysteine (NAC). Nuclear run-on assays demonstrate that NAC inhibits the activation of *EGR1* transcription by these agents. Previous studies have shown that induction of *EGR1* by x-rays is conferred by serum response or CC(A/T)$_6$GG (CArG) elements. The present studies demonstrate similar findings with H_2O_2 and the finding that activation of the *EGR1* promoter region containing CArG elements is abrogated by NAC. Moreover, we show that NAC inhibits the ability of a single CArG box to confer x-ray and H_2O_2 inducibility when linked to a heterologous promoter. Taken together, these findings indicate that ROIs induce *EGR1* transcription by activation of CArG elements.**

The early growth response 1 gene (*EGR1*; also known as zif/268, TIS-8, NFGI-A, and Krox-24) encodes a 533-amino acid nuclear phosphoprotein (1–5). The EGR-1 protein contains three tandem repeat units of the Cys_2-His_2 zinc-finger type and interacts with the DNA sequence CGCCCCCGC in a zinc-dependent manner (6). EGR-1, thus, belongs to a class of transcription factors that includes Sp1 (7), TF111A (8), and SW15 (9). Members of the *EGR* gene family (*EGR2*, *EGR3*, and *EGR4*) encode finger domains strikingly similar to EGR-1 but differ outside the finger regions (10–13). *EGR1* is induced during mitogenic stimulation of a variety of cell types—including fibroblasts, epithelial cells, and B cells (13, 14). Moreover, this gene is activated by the protein tyrosine kinase activity of v-src and v-fps (15, 16). Other studies have demonstrated that *EGR1* is induced during cardiac and neuronal differentiation of the pleuripotent EC line (1) and monocytic differentiation of human myeloid leukemia cell lines (17, 18). These findings have suggested that EGR-1 is involved in both cell growth and differentiation.

Recent work has also demonstrated that ionizing radiation increases expression of *EGR1* (19). The response of cells to ionizing radiation includes cell-cycle-specific growth arrest, induction of DNA-repair mechanisms, and reinitiation of DNA synthesis in surviving cells. Although the events responsible for these effects remain unclear, ionizing-radiation treatment is associated with the formation of reactive oxygen intermediates (ROIs) and direct damage to DNA (20). One or both of these events probably contribute to the induction of specific gene expression. Indeed, in addition to *EGR1*, exposure of cells to ionizing radiation is associated with activation of the *jun/fos* gene family (21). Other work has shown that ionizing radiation induces expression and DNA-binding activity of the nuclear factor κB (NF-κB) (22). Activation of these transcription factors probably contributes to longer-term changes in gene expression that constitute the radiation response. For example, this response also includes increased expression of the tumor necrosis factor, fibroblast growth factor, platelet-derived growth factor, and interleukin-1 gene (23–26).

The present studies have addressed the signaling events that mediate induction of *EGR1* by ionizing radiation. The findings indicate that this effect is related to the formation of ROIs. In this regard, similar results have been obtained with H_2O_2. Moreover, we demonstrate that ROIs induce *EGR1* transcription through serum response or CC(A/T)$_6$GG (CArG) domains.

MATERIALS AND METHODS

Cell Culture. Human HL-525 myeloid leukemia cells (27) were grown in RPMI 1640 medium/20% fetal bovine serum/1 mM L-glutamine/penicillin at 100 units/ml/streptomycin at 100 μg/ml. Irradiation (20 Gy) was at room temperature in a Gammacell 1000 (Atomic Energy of Canada, Ottawa) with a ^{137}Cs source emitting a fixed dose of 13.3 Gy/min. Cells were also exposed to 32 nM phorbol 12-myristate 13-acetate (PMA; Sigma), 50 μM H_2O_2 (Sigma), and 30 mM *N*-acetyl-L-cysteine (NAC; Sigma).

Isolation and Analysis of RNA. Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride technique (28). The RNA (20 μg) was analyzed by electrophoresis in 1% agarose-formaldehyde gels, transferred to nitrocellulose filters, and hybridized to the following ^{32}P-labeled DNA probes: (*i*) the 0.7-kb non-zinc-finger insert of a murine EGR-1 cDNA (1) and (*ii*) the 2.0-kb *Pst* I insert of a chicken β-actin gene purified from the pA1 plasmid (29). Hybridizations were done as described (17). Autoradiographic bands were scanned by using an LKB Produkter Ultrascan XL laser densitometer and analyzed with Gelscan XL software version 1.21.

Nuclear Run-on Assays. Nuclei were isolated from 10^8 cells, and newly elongated transcripts were labeled with 250 μCi of [α-^{32}P]UTP (3000 Ci/mmol; DuPont/NEN; 1 Ci = 37 GBq) at 26°C for 45 min. The nuclear RNA was isolated as described (17) and hybridized to the following single-copy

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Abbreviations: EGR, early growth response; ROI, reactive oxygen intermediate; CArG, CC(A/T)$_6$GG or CCW$_6$CG; NAC, *N*-acetyl-L-cysteine; CAT, chloramphenicol acetyltransferase. XRT, x-ray treated; PMA, phorbol 12-myristate 13-acetate.

DNAs: (*i*) a *Pst* I digest of the pA1 plasmid containing a fragment of the chicken β-actin gene (positive control) (29), (*ii*) a 1.1-kb *Bam*HI insert of a human β-globin gene (negative control) (30), and (*iii*) the 0.7-kb insert of the EGR-1 cDNA (1). The digested DNAs were run in 1% agarose gels and transferred to nitrocellulose filters. Hybridizations were done as described (17).

Reporter Assays. The pE425 construct were prepared as described (31). pSRE1TK contains the 5′-most distal or first CArG box in the *EGR1* promoter along with 7 bp of the 5′ and 3′ flanking sequences cloned into the *Sal* I–*Bam*HI site of pTK35CAT (16). The constructs were transfected into cells by using the DEAE-dextran technique. Cells (2×10^7) were incubated at 37°C for 45 min in 1 ml of Tris-buffered saline solution (25 mM Tris·HCl, pH 7.4/137 mM NaCl/5 mM KCl/0.6 mM Na_2HPO_4/0.7 mM $CaCl_2$/0.7 mM $MgCl_2$) containing 0.4 mg of DEAE-dextran and 8 μg of plasmid. The cells were washed with medium containing 10% fetal bovine serum, resuspended in complete medium, and then incubated at 37°C for 36 hr. One sample of the cells served as a control, and the other was treated with ionizing radiation or H_2O_2. The cells were harvested after 8 hr and lysed by three cycles of freezing and thawing in 0.25 M Tris·HCl, pH 7.8/1 μM phenylmethylsulfonyl fluoride. Equal amounts of the cell extracts were incubated with 0.025 μCi of [^{14}C]chloramphenicol/0.25 M Tris·HCl, pH 7.8/0.4 mM acetylCoA for 1 hr at 37°C. The enzyme assay was terminated by adding ethyl acetate. The organic layer was analyzed by TLC with chloroform/methanol, 95%/5% (vol/vol). After autoradiography, both acetylated and unacetylated forms of [^{14}C]chloramphenicol were scraped from the plates, and the conversion of chloramphenicol to the acetylated form was calculated by measuring radioactivity.

RESULTS AND DISCUSSION

Previous studies have shown that expression of *EGR1* is increased by phorbol esters in human myeloid leukemia cell lines, as well as in other cell types. For example, treatment of wild-type HL-60 cells with PMA is associated with maximal increases in EGR-1 transcripts at 1 hr (ref. 17 and Fig. 1*A*). These findings have implicated activation of protein kinase C in the induction of EGR-1 expression. The present studies were done on HL-525 cells, which are defective in protein kinase C-mediated signaling events (27). In contrast to HL-60 cells, treatment of HL-525 cells with PMA was associated with attenuated increases in EGR-1 mRNA levels (Fig. 1*A*). However, exposure of the HL-525 line to ionizing radiation increased EGR-1 transcripts, which were detectable by 15 min and maximal at 3 hr (Fig. 1*B*). This effect was transient with declines in EGR-1 expression at 8 hr (Fig. 1*B*). That such increases in EGR-1 transcripts might be related to ROI formation in irradiated cells was explored in similar studies with H_2O_2, another agent that increases intracellular levels of these intermediates. Treatment of HL-525 cells with H_2O_2 also caused increased expression of *EGR1*. This induction was detectable at 15 min, and EGR-1 mRNA levels remained above control at 8 hr (Fig. 1*C*). Although the kinetics of *EGR1* induction differed with ionizing radiation and H_2O_2, these findings suggested that ROIs might act as messengers that confer induction of *EGR1*. Moreover, the finding that both ionizing radiation and H_2O_2 induce EGR-1 expression in HL-525 cells suggests that this event is mediated by signaling pathways distinct from those activated by PMA.

The effects of ROIs are inhibited by the antioxidant NAC (32, 33). This agent abrogates oxidative stress by scavenging ROIs and increasing intracellular glutathione levels (34, 35). Exposure of HL-525 cells to NAC had little, if any, effect on basal expression of *EGR1* (Figs. 2 *A* and *B*). However, this

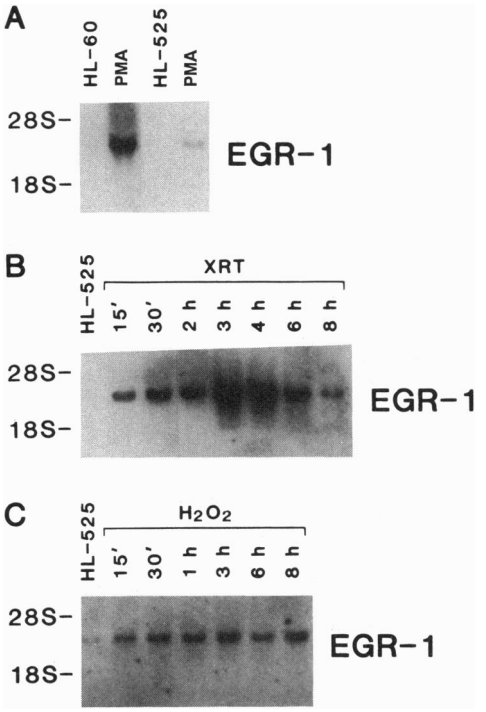

FIG. 1. Ionizing radiation and H_2O_2 induce EGR-1 expression in PMA-resistant HL-525 cells. (*A*) Wild-type HL-60 and variant HL-525 cells were treated with 32 nM PMA for 1 hr. HL-525 cells were also exposed to 20 Gy of ionizing radiation [x-ray treated (XRT)] (*B*) or 50 μM H_2O_2 for the indicated times (*C*). Total cellular RNA (20 μg) was hybridized to ^{32}P-labeled EGR-1 probe. Hybridization to the actin probe demonstrated equal loading of lanes (data not shown). ′, Min; h, hr.

antioxidant completely inhibited x-ray-induced increases in EGR-1 mRNA levels (Fig. 2*A*). Similar findings were obtained in cells exposed to H_2O_2 (Fig. 2*B*). Taken together, these results suggested that ROIs contribute to induction of *EGR1*. Other studies have demonstrated that NAC inhibits activation of NF-κB by H_2O_2 and phorbol esters (33, 36). These findings have indicated that ROIs activate nuclear translocation of NF-κB through disassociation from the inhibitory subunit IκB (36). NAC is also a potent inhibitor of phorbol ester-induced activation of the human immunodeficiency virus type 1 long terminal repeat (32). Moreover, recent work has shown that NAC blocks induction of the c-*jun* gene by ionizing radiation and H_2O_2 (37). Although these findings might suggest that NAC nonspecifically blocks activation of early response genes coding for transcription factors, this agent had no detectable effect on induction of the c-*jun* gene in HL-525 cells by 1-β-D-arabinofuranosylcytosine (ara-C) (37). In this context, ara-C incorporates into DNA and is not known to induce its cytotoxic effects through the formation of ROIs (38, 39).

To further define the effects of ROIs on the regulation of EGR-1 expression, we asked whether formation of these intermediates is associated with activation of this gene at the transcriptional level. Nuclear run-on assays showed that NAC inhibits induction of *EGR1* transcription by ionizing radiation (Fig. 3). In this regard, there was little effect of x-rays, NAC, or the combination of both agents on constitutive transcription of the actin gene (positive control) (Fig. 3). Moreover, these agents had no effect on rates of globin gene transcription (negative control) (Fig. 3). In contrast, although ionizing radiation activated transcription of *EGR1*, this effect was abrogated by >70% when done in the presence of NAC (Fig. 3). Similar results were obtained with H_2O_2 (data not shown). Although opposite-strand transcription

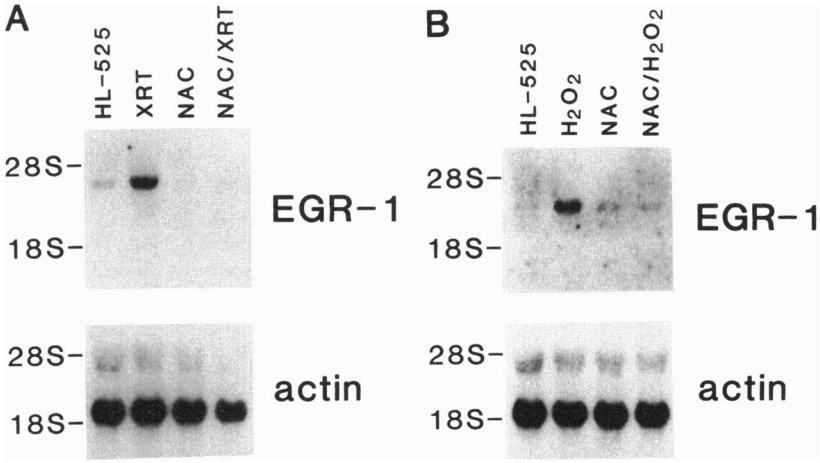

FIG. 2. NAC inhibits induction of EGR-1 expression. (*A*) HL-525 cells were exposed to ionizing radiation and/or 30 mM NAC. (*B*) Cells were exposed to H_2O_2 and/or NAC. NAC was added 30 min before irradiation or H_2O_2 and was included in the medium for an additional 3 hr. Cells treated with NAC alone were harvested after 3.5 hr. Total cellular RNA (20 μg) was hybridized to EGR-1 and actin probes.

could contribute to these findings, the results indicated that ROIs increase EGR-1 expression by transcriptional activation of this gene.

These results were extended in transient expression assays using *EGR1* promoter constructs linked to the chloramphenicol acetyltransferase (CAT) reporter gene. We have previously demonstrated that serum response or CArG domains in the *EGR1* promoter confer induction of this gene by ionizing radiation (40). Consequently, we asked whether x-ray-induced activation of the *EGR1* promoter, which contains six CArG domains, is inhibited by NAC. The results showed that although ionizing radiation induces CAT activity of the *EGR1* promoter–CAT construct, this effect of x-rays was inhibited with NAC (Fig. 4). Comparable results were obtained when cells were treated with H_2O_2 and NAC (Fig. 4). Other studies were done with the 5′-most distal or first CArG domain of the *EGR1* promoter ligated to the herpes simplex virus minimal thymidine kinase promoter proximal to the CAT gene (Fig. 5). This construct was activated after exposure of cells to both ionizing radiation and H_2O_2 (Fig. 5). Moreover, the effects of these agents were inhibited by NAC (Fig. 5). These findings indicated that the first CArG box is sufficient to confer the x-ray response and that this inducibility is mediated by ROIs.

Previous studies with the c-*fos* promoter have shown that the serum response element or CArG domain confers inducibility of this gene after treatment with serum (41). The finding that the kinetics of c-*fos* induction, as well as repression, are similar to those of *EGR1* (1, 31) have suggested that the CArG domain is functional in the regulation of both genes. Moreover, studies with both the c-*fos* and *EGR1* promoters have shown that the CArG box functions as a binding site for the serum response factor (42–44). Thus, agents such as ionizing radiation and H_2O_2, which increase ROI formation, may induce posttranslational modification(s) of serum response factor and activate transcription through the CArG domain. In this context, recent studies have demonstrated that redox changes regulate the affinity of c-Jun binding to DNA (45, 46). Oxidation of cysteine residues in the leucine zipper and adjacent basic region inhibits DNA binding and thereby autoregulation of the c-*jun* gene by Jun/AP1 (45, 46). Regulation of cysteine residues in serum response factor may also influence interaction of this protein with the CArG domain. Whatever the mechanism, the demonstration that ROIs modulate DNA binding of NF-κB and

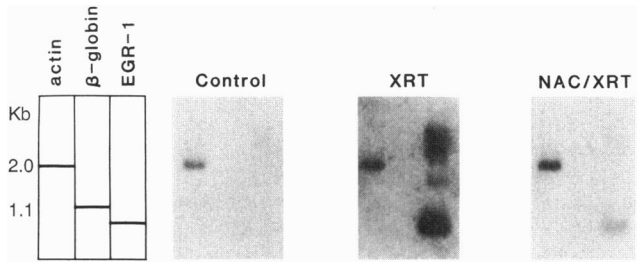

FIG. 3. Transcriptional activation of *EGR1* by ionizing radiation is inhibited by NAC. HL-525 cells were exposed to 20 Gy of ionizing radiation. NAC was added 30 min before irradiation and included in medium for an additional 2.5 hr. Cells treated with NAC alone were harvested after 3 hr. After 3 hr, nuclei were isolated, and newly elongated ^{32}P transcripts were isolated and hybridized to 2 μg of actin, β-globin, and EGR-1 DNA inserts after restriction-enzyme digestion and Southern blotting. Solid lines in schematic represent positions of DNA inserts. Hybridization above EGR-1 DNA insert represents partially digested plasmid.

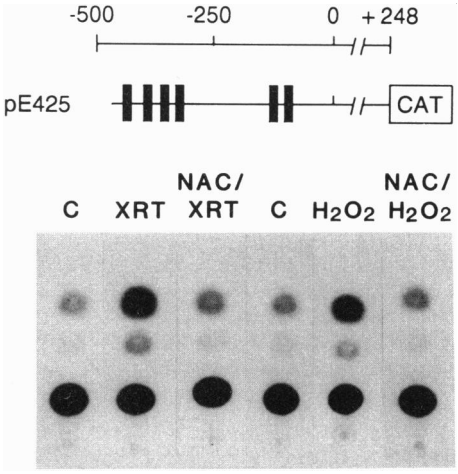

FIG. 4. NAC inhibits transcriptional activation of *EGR1* promoter–reporter constructs. HL-525 cells were transfected with pE425 and maintained in medium for 36 hr. The cells were then exposed to ionizing radiation or H_2O_2 and harvested after 8 hr for analysis of CAT activity. Studies were also done by adding NAC 30 min before irradiation or H_2O_2 treatment. Conversion of chloramphenicol to the acetylated forms is shown for control (C), XRT, and H_2O_2-treated cells transfected with the indicated constructs. Percent conversion (mean ± SD; n = 4) was determined by scintillation counting as follows: C, 3.8 ± 0.1%; XRT, 15.3 ± 1.1%; NAC/XRT, 5.2 ± 0.1%, H_2O_2, 11.3 ± 0.2%; NAC/H_2O_2, 4.1 ± 0.2%.

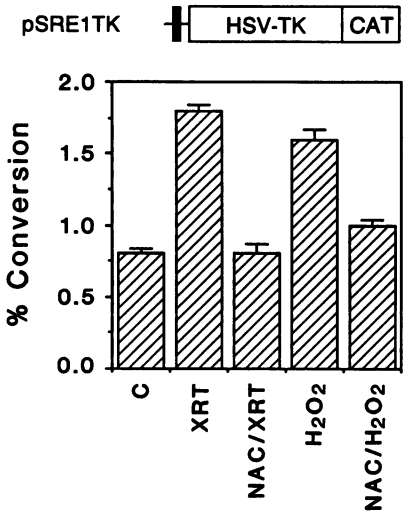

FIG. 5. Activation of CArG element by ionizing radiation and H_2O_2 is inhibited by NAC. pSRE1TK contains the 5'-most distal CArG box in the *EGR1* promoter ligated to the herpes simplex virus minimal thymidine kinase (HSV-TK) promoter upstream to CAT. HL-525 cells were transfected with pSRE1TK and maintained in medium for 36 hr. Cells were then exposed to ionizing radiation or H_2O_2 (± NAC) and harvested after 8 hr for analysis of CAT activity. Studies were also done by adding NAC 30 min before irradiation or H_2O_2 treatment. Percent conversion of chloramphenicol represents the results (mean ± SD) of four independent determinations. There was no detectable induction of pTK35CAT by ionizing radiation or H_2O_2 (ref. 40 and data not shown).

Jun/AP1, as well as induction of *EGR1*, indicates that such intermediates regulate the activity of multiple transcription factors. These nuclear signaling events are believed to confer longer-term changes in gene transcription associated with both mitogenic and differentiating signals. Thus, the cellular response to ROIs appears to involve pathways that usually confer signals from the cell membrane to the nucleus. Finally, because ROIs also damage DNA, whether these intermediates directly activate EGR-1 and other transcription factors or whether DNA damage *per se* is the initial signal that induces these cascades is unclear.

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