

## Ionizing Radiation Induces Expression and Binding Activity of the Nuclear Factor $\kappa$ B

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### Abstract

Recent studies have demonstrated that treatment of mammalian cells with ionizing radiation is associated with activation of gene expression. Although the signal transduction pathways stimulated by ionizing radiation remain unclear, our previous findings indicate that radiation induces specific genes at the transcriptional level. The present work has examined the effects of ionizing radiation on the transcription factor NF- $\kappa$ B. The results demonstrate that ionizing radiation activates DNA binding of nuclear factor (NF) $\kappa$ B. This effect was detectable at 2 grays (Gy) and reached a maximum at 5–20 Gy. At a dose of 20 Gy, the increase in NF- $\kappa$ B binding activity was maximal at 2–4 h and then declined to pretreatment levels. The results also demonstrate that ionizing radiation transiently increases NF- $\kappa$ B mRNA levels. However, the finding that induction of NF- $\kappa$ B binding to DNA occurs in the presence of cycloheximide indicates that ionizing radiation activates preexisting NF- $\kappa$ B protein. NF- $\kappa$ B exists as a cytoplasmic protein before activation. Thus, our results suggest that ionizing radiation induces transduction pathways which include cytoplasmic signaling events. (*J. Clin. Invest.* 1991. 88:691–695.) Key words: DNA-binding proteins • gene expression • DNA damage

### Introduction

Ionizing radiation induces both neoplastic transformation and lethal events in mammalian cells. These effects were thought to arise from direct mutagenic or toxic events of x rays on DNA. However, recent studies have demonstrated that this agent induces the expression of specific genes, the products of which may contribute to the effects of x rays on cells. For example, ionizing radiation increases tumor necrosis factor (TNF)<sup>1</sup> gene expression (1). This cytokine enhances the killing of x rays by paracrine or autocrine pathways (2). Moreover, other work has shown that exposure to ionizing radiation is associated with

increased expression of interleukin-1, platelet-derived growth factor, and fibroblast growth factor (3, 4). The stimulation of cytokine gene expression may thus represent one aspect of the cellular response to the effects of this agent. While few insights are available regarding mechanisms responsible for the induction of specific gene expression by ionizing radiation, recent work has demonstrated that the increase in TNF mRNA levels is regulated by a transcriptional mechanism (5).

The modulation of transcription is a critical control point in the regulation of cellular processes and involves the activation of transcription factors that bind to specific DNA sequences (6). Transcriptional regulation has been proposed to be important in a variety of cellular responses to irradiation, including DNA repair, cell cycle arrest, and subsequent growth. Nuclear factor (NF)  $\kappa$ B recognizes and binds an 11-bp DNA sequence present in the  $\kappa$  immunoglobulin light chain gene enhancer (7). NF- $\kappa$ B also recognizes DNA sequences in several virus enhancers, including that of the human immunodeficiency virus (8–11). Moreover, NF- $\kappa$ B-like sites are associated with regulation of genes coding for the Class I major histocompatibility antigen (12, 13),  $\beta_2$ -microglobulin (14), granulocyte-macrophage colony stimulating factor (15, 16), IL-2 (17, 18), and IL-2 receptor  $\alpha$  chain (19). NF- $\kappa$ B is located in the cytoplasm bound to an inhibitor protein, I $\kappa$ B, which prevents uptake of NF- $\kappa$ B into the nucleus (20, 21). The affinity of NF- $\kappa$ B for DNA binding is activated by tumor-promoting phorbol esters (7, 22), the transactivator tax protein of human T cell lymphotropic virus type 1 (9, 23, 24), TNF (25), and IL-1 (25). Thus, NF- $\kappa$ B may regulate the expression of certain genes involved in cell growth or transformation.

In this report, we have examined the effects of ionizing radiation on activation of NF- $\kappa$ B binding to DNA. The results demonstrate that DNA binding of NF- $\kappa$ B is activated by this agent in a dose and time-dependent manner. NF- $\kappa$ B binding is induced in the absence of protein synthesis, suggesting that the effect of ionizing radiation is mediated in part by a posttranslational activation of cytoplasmic NF- $\kappa$ B.

### Methods

**Cell culture.** Human KG-1 myeloid leukemia cells (American Type Culture Collection, Bethesda, MD) were maintained in Iscove's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were irradiated using a Gammacell 1000 (Atomic Energy of Canada, Ottawa) with a <sup>137</sup>Cs source emitting at a fixed dose rate of 14.3 grays (Gy)/min as determined by dosimetry. Control cells were handled under the same conditions but not irradiated. Cells were also treated with 10  $\mu$ g/ml cycloheximide (Sigma Chemical Co., St. Louis, MO).

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1. Abbreviations used in this paper: EMSAs, electrophoretic mobility shift assays; NF, nuclear factor; TNF, tumor necrosis factor.

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**Electrophoretic mobility shift assays.** Nuclear proteins were prepared according to previously described methods (26). A 22-bp synthetic oligomer (5' GATCGAGGGGACTTTCCCTAGC 3') containing the 11-bp NF- $\kappa$ B binding sequence (GGGGACTTTC) was end labeled with [ $\alpha$ - $^{32}$ P]dATP using DNA polymerase I and purified in a 12% polyacrylamide gel. Radiolabeled DNA (1 ng) was incubated with 10  $\mu$ g nuclear protein for 20 min at 20°C in 25 mM Tris-HCl, pH 7.6, 250 ng/ml poly dI/dC, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM DTT, and 10% (vol/vol) glycerol. Competition studies with unlabeled NF- $\kappa$ B oligonucleotide were performed by adding up to a 25-fold excess compared to the end-labeled fragment. The reaction products were analyzed by 5% polyacrylamide gel electrophoresis and autoradiography.

**Northern blot analysis.** Total cellular RNA (30  $\mu$ g) was isolated as described (27, 28), separated in a 1% agarose/2.2 M formaldehyde gel, transferred to a nitrocellulose filter, and hybridized to the following  $^{32}$ P-labeled DNA probes: (a) the 3.9-kb EcoRI fragment isolated from the NF- $\kappa$ B cDNA (29); and (b) the 2.0-kb PstI  $\beta$ -actin cDNA insert purified from the pA1 plasmid (30).

## Results

To study the effects of ionizing radiation on NF- $\kappa$ B binding activity, we first performed electrophoretic mobility shift assays (EMSAs) with nuclear proteins isolated from KG-1 cells exposed to varying doses of ionizing radiation. Incubation of a radiolabeled 22-bp oligonucleotide containing the NF- $\kappa$ B binding site with nuclear proteins from untreated KG-1 cells resulted in the retardation of a single band (Fig. 1 A). The intensity of this band was increased when nuclear extracts from cells exposed to 2 Gy ionizing radiation were used in these assays (Fig. 1 A). Maximal increases in signal intensity were observed when using doses of 5–50 Gy.

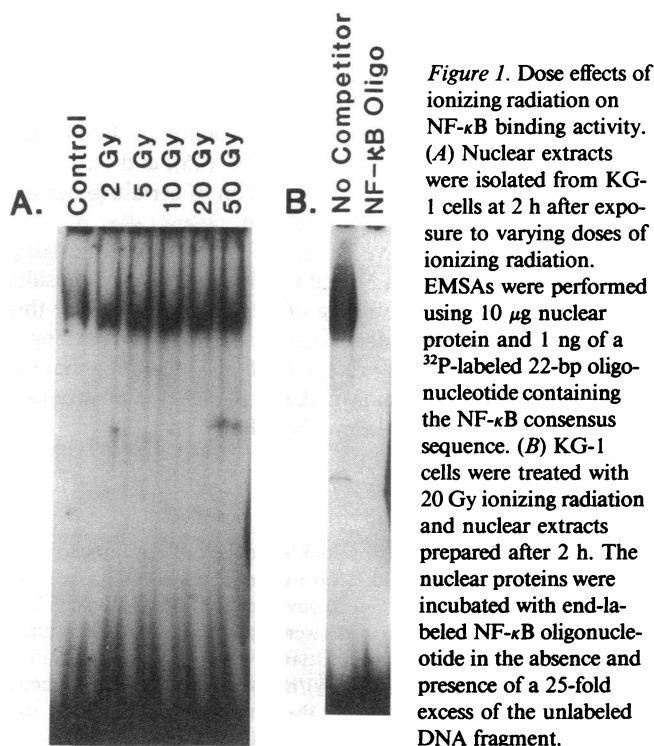
Competition studies were next performed with unlabeled NF- $\kappa$ B oligonucleotide to ensure specificity of the DNA-pro-

tein interaction. Addition of the unlabeled fragment to nuclear proteins from 20 Gy irradiated cells and end-labeled oligonucleotide decreased appearance of the retarded band. Complete inhibition was obtained when using a 25-fold excess of unlabeled NF- $\kappa$ B fragment (Fig. 1 B). Taken together, these findings indicated that ionizing radiation activates DNA binding of NF- $\kappa$ B.

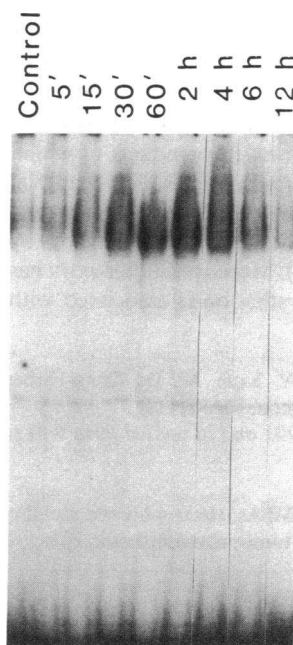
EMSAs were also used to study the kinetics of NF- $\kappa$ B binding. Nuclear proteins were isolated at varying intervals after treatment with 20 Gy. Ionizing radiation treatment was associated with an increase in intensity of the retarded fragment by 15 min (Fig. 2). This effect was maximal at 2–4 h and was associated with a decline in DNA binding to nearly pretreatment levels by 12 h (Fig. 2).

The stimulation of NF- $\kappa$ B binding to DNA by ionizing radiation could be related to increases in expression of this gene or to activation of preexisting protein. To examine these possibilities, we initially performed Northern analyses of RNA from irradiated cells. There was a low but detectable level of 4.0-kb NF- $\kappa$ B transcripts in control cells (Fig. 3). Moreover, there was little if any effect of 20 Gy on NF- $\kappa$ B mRNA levels at 1 h (Fig. 3). In contrast, there was a detectable increase in NF- $\kappa$ B expression at 3 and 6 h after irradiation. This effect was transient and levels of NF- $\kappa$ B transcripts returned to that in control cells by 15 h. These findings and the lack of an effect on actin gene expression indicated that ionizing radiation regulates NF- $\kappa$ B expression at the mRNA level.

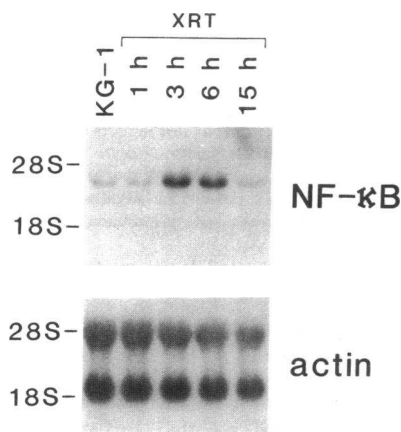
The absence of a detectable increase in NF- $\kappa$ B transcripts at 1 h suggested that the rapid stimulation of NF- $\kappa$ B binding to DNA might be related at least in part to activation of preexisting NF- $\kappa$ B protein. This possibility was addressed by studying DNA binding of nuclear proteins from cells treated with cycloheximide. Nuclear proteins isolated 2 h after treating cells with 20 Gy resulted in an increase in retardation of the end-labeled NF- $\kappa$ B oligonucleotide (Fig. 4). Similar results were obtained with nuclear proteins from cells that were pretreated with 10



**Figure 1.** Dose effects of ionizing radiation on NF- $\kappa$ B binding activity. (A) Nuclear extracts were isolated from KG-1 cells at 2 h after exposure to varying doses of ionizing radiation. EMSAs were performed using 10  $\mu$ g nuclear protein and 1 ng of a  $^{32}$ P-labeled 22-bp oligonucleotide containing the NF- $\kappa$ B consensus sequence. (B) KG-1 cells were treated with 20 Gy ionizing radiation and nuclear extracts prepared after 2 h. The nuclear proteins were incubated with end-labeled NF- $\kappa$ B oligonucleotide in the absence and presence of a 25-fold excess of the unlabeled DNA fragment.



**Figure 2.** Kinetics of NF- $\kappa$ B binding activity. Nuclear extracts were isolated at varying times after exposure of KG-1 cells to 20 Gy ionizing radiation. EMSAs were performed as described in the legend to Fig. 1.

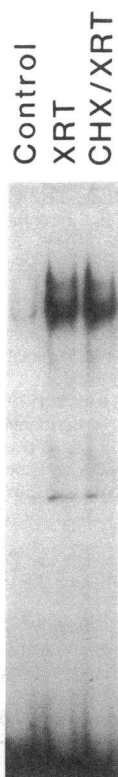


**Figure 3.** Induction of NF- $\kappa$ B mRNA levels by ionizing radiation. KG-1 cells were treated with 20 Gy ionizing radiation (XRT). Total cellular RNA (30  $\mu$ g/lane) was isolated after 1, 3, 6, and 15 h, and hybridized to  $^{32}$ P-labeled NF- $\kappa$ B or actin DNA probes. The KG-1 lane represents RNA from untreated cells.

$\mu$ g/ml cycloheximide for 2 h before and for 2 h after irradiation (Fig. 4). Although previous studies have demonstrated that treatment with cycloheximide alone modestly activates NF- $\kappa$ B (22), exposure of KG-1 cells to this agent for 4 h had no detectable effect on retardation of the NF- $\kappa$ B fragment (data not shown). These findings indicated that ionizing radiation activates NF- $\kappa$ B in the absence of de novo protein synthesis.

## Discussion

Although previous studies have demonstrated that ionizing radiation activates the transcription of specific genes such as TNF, the signaling events that contribute to gene induction by this agent remain undefined. These signaling events may be



**Figure 4.** Activation of NF- $\kappa$ B in the presence of cycloheximide. KG-1 cells were treated with 20 Gy ionizing radiation (XRT) after 2 h pretreatment with 10  $\mu$ g/ml cycloheximide (CHX). Nuclear proteins were isolated after an additional 2 h in the presence of CHX. Binding was performed with 10  $\mu$ g nuclear protein and 1 ng  $^{32}$ P-labeled 22-bp NF- $\kappa$ B oligonucleotide.

important not only for cytokine induction, but also for a wide variety of cellular events following radiation exposure. In this context, we have recently studied the effects of ionizing radiation on activation of immediate early response genes that code for transcription factors. These studies have shown that exposure to ionizing radiation is associated with activation of the *c-jun* gene (31). The *c-jun* gene codes for the major form of the 40–44-kD AP-1 leucine zipper transcription factor and is a member of a family of other related genes, such as *jun-B* and *c-fos*, which are also induced by ionizing radiation (31). Other studies have shown that a 43-kD DNA binding protein is activated by gamma irradiation in Epstein-Barr virus-transformed human lymphoblastoid cells (32). The DNA binding motif for this novel protein has been identified in the 72-bp tandem repeat of the SV40 enhancer region and thus binds to sequences distinct from the heptameric AP-1 consensus (TGA<sup>G</sup>/C/TCA) (32). More recent work has demonstrated that ionizing radiation induces expression of the *EGR-1* gene which codes for a zinc finger transcription factor that binds to the GCGGGGGCG sequence (33). Taken together, these results indicate that ionizing radiation induces distinct classes of transcription factors. These findings may contribute to our understanding of molecular events involved in cellular responses to radiation, such as DNA repair, cell cycle arrest, and cytokine induction.

Recent findings suggest that NF- $\kappa$ B motifs play a role in lipopolysaccharide-mediated transcriptional activation of the TNF gene (34, 35). Since ionizing radiation also induces TNF gene expression by a transcriptional mechanism (5), our studies were performed to determine if NF- $\kappa$ B is activated in irradiated cells. The results demonstrate that ionizing radiation regulates NF- $\kappa$ B expression at both the mRNA and protein levels. NF- $\kappa$ B transcripts were transiently increased after irradiation, although this effect occurred after evidence for enhanced DNA binding activity. Moreover, the finding that increased protein binding to the NF- $\kappa$ B oligonucleotide was detectable in the presence of cycloheximide indicated that preexisting NF- $\kappa$ B is also activated by ionizing radiation. These results suggest that the effects of ionizing radiation on NF- $\kappa$ B expression may occur by independent mechanisms at two levels. Alternatively, activation of NF- $\kappa$ B protein by this agent could be associated with autoinduction of the NF- $\kappa$ B gene. Previous studies have demonstrated that 4.0-kb NF- $\kappa$ B transcripts are detectable in a variety of cell types (29, 36). Furthermore, recent work has demonstrated that diverse cytoplasmic NF- $\kappa$ B complexes may exist that include the product of the *c-rel* protooncogene (29, 36–38). Although this study has not addressed the subunit structure of the NF- $\kappa$ B complexes activated by ionizing radiation, other members of the rel-related family may be regulated by this agent at the pre- or posttranslational level.

The available evidence suggests that protein kinase C may be involved in intracellular signaling pathways activated by ionizing radiation. In one study, transcriptional activation of a construct containing the long terminal repeat of Moloney murine sarcoma virus linked to the chloramphenicol acetyltransferase gene was examined after gamma irradiation (39). Irradiation stimulated expression of the construct twofold, while treatment with H7, a nonspecific isoquinolinesulfonamide inhibitor of protein kinases, or preincubation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to downregulate protein ki-

nase C, abolished the response of x rays (39). Similar inhibitory effects have been obtained with H7 and prolonged TPA stimulation during induction of TNF gene expression by ionizing radiation (unpublished data). Protein kinase C has been implicated in the activation of NF- $\kappa$ B binding to DNA. In this context, treatment of cells by certain agents, notably phorbol esters that activate protein kinase C, results in dissociation of the cytoplasmic NF- $\kappa$ B/I $\kappa$ B complex and movement of NF- $\kappa$ B to the nucleus (40). The activation of NF- $\kappa$ B by ionizing radiation could therefore be mediated by a protein kinase C-dependent mechanism.

Finally, the demonstration that ionizing radiation activates NF- $\kappa$ B implicates the activation of cytoplasmic signaling events. Previous work has clearly demonstrated that NF- $\kappa$ B is present in the cytoplasm in an inactive form (20, 21, 40). Our studies demonstrate that maximal DNA binding of the NF- $\kappa$ B complex in KG-1 cells is activated by a dose of ionizing radiation between 2 and 5 Gy. While the NF- $\kappa$ B response in other cell types may differ, doses of 2–20 Gy induce free radical formation, DNA damage, and neoplastic transformation, as well as cell killing (41). Free radical formation could directly activate a cytoplasmic signaling pathway. Alternatively, DNA strand breakage or distortion of DNA nucleoprotein conformation may transduce signals from the nucleus to the cytoplasm. Indeed, activation of NF- $\kappa$ B has recently been described during cellular exposure to UV irradiation, another DNA-damaging agent (42). While signal transduction generally proceeds from the cell membrane to the nucleus, reverse signaling mechanisms from the nucleus to cytoplasm could represent part of the cellular response to DNA damage. These findings with NF- $\kappa$ B would support this mechanism.

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