

# Chemotherapeutic DNA-damaging Drugs Activate Interferon Regulatory Factor-7 by the Mitogen-activated Protein Kinase Kinase-4-c-Jun NH<sub>2</sub>-Terminal Kinase Pathway<sup>1</sup>

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## Abstract

Chemotherapeutic drugs and energy-rich radiation cause DNA damage, inducing signaling pathways for apoptotic cell death or cell growth arrest. The tumor suppressor gene *p53* plays the critical role in the regulation of these DNA damage responses. Human tumor cells can become resistant to chemotherapy through functional inactivation of *p53*. Thus, it is important to identify *p53*-independent DNA damage signaling pathways. Here, treatment of cells with chemotherapeutic drugs or UV irradiation potentiated the transcriptional activity of IFN regulatory factor-7 (IRF7), inducing its phosphorylation and its nuclear translocation. Furthermore, IRF7 was activated by the c-Jun NH<sub>2</sub>-terminal kinase (JNK) in response to DNA-damaging agents. Activation of JNK by mitogen-activated protein kinase kinase-4 stimulated the transcriptional activity of IRF7 and induced its translocation into the nucleus. Thus, activation of IRF7 through the JNK signaling pathway may play a role in the transcriptional regulation of genes in response to DNA-damaging agents.

## Introduction

Apoptotic cell death and cell growth arrest can provide a natural defense against tumor development and underlie the effectiveness of current chemotherapeutic DNA-damaging drugs (1, 2). DNA damage induces signal transduction pathways that lead to damage repair coupled with cell cycle arrest and/or apoptotic cell death. The initiation of such DNA damage responses requires activation of the transcription factor *p53* (3–6). After DNA damage, the amount of *p53* in cells increases through attenuated proteolysis, and its transcriptional activity is enhanced to up-regulate its target genes. Consistent with its critical role in cellular responses to DNA damage, mutation of the *p53* gene can promote oncogenic transformation, tumor progression, and resistance to chemotherapeutic agents by reducing the potential of cells to undergo apoptosis and cell growth arrest.

We are interested in the identification of small molecules that affect these *p53* control pathways. Such chemicals can be used to modulate chemosensitivity and to study the molecular mechanisms of *p53*-dependent and *p53*-independent forms of apoptosis and cell growth arrest. Interestingly, most of our identified chemicals that activate *p53* are also capable of activating IRF7.<sup>3,4</sup> IRF7 is a member of the IRF

family of transcription factors, which may be involved in defensive responses to environmental stress, including viral infection (7–10). This raises the possibility that IRF7, like *p53*, could be activated by genotoxic stresses that are caused by DNA-damaging chemicals. In the present study, we demonstrate the activation of IRF7 by the JNK pathway in response to UV and chemotherapeutic agents that are known to induce DNA damage.

## Materials and Methods

**Transfection and CAT Reporter Gene Assay.** HeLa cells were transfected with plasmids using FuGene reagents (Boehringer Mannheim). At 24–36 h after transfection, cells were irradiated with 50 J/m<sup>2</sup> UV or incubated with 200 hemagglutinin unit/ml Sendai virus, 500 units/ml recombinant IFN- $\gamma$ , 1  $\mu$ g/ml Adriamycin, 1  $\mu$ g/ml mitomycin C, 0.5  $\mu$ g/ml cisplatin, or 1  $\mu$ g/ml etoposide, as indicated in the figures. Expression of the CAT reporter gene was analyzed 12–15 h after treatment, as described (11). Transfection efficiencies were monitored by transfection of a CMV-lacZ control plasmid on parallel plates. CAT activities were normalized to protein concentrations of cell extracts.

**In Vivo Phosphorylation Assay.** Cells transfected with an expression plasmid for HA-IRF7 were incubated in phosphate-free DMEM for 30 min, and [<sup>32</sup>P]P<sub>i</sub> was added (330  $\mu$ Ci/ml) for 2 h upon treatment with UV or Adriamycin. Extracts were precleared on protein A/G Sepharose. HA-IRF7 was then immunoprecipitated with anti-HA antibody and separated on SDS-PAGE for detection of phosphorylated IRF7 using a PhosphorImager (12).

**GFP Assay.** HeLa cells were transfected with an expression plasmid for GFP-IRF7. The subcellular localization was analyzed with a fluorescence microscope after treatment, as indicated in the figures.

## Results and Discussion

To examine the potential role of genotoxic stress in the activation of IRF7, we treated cells with various DNA-damaging agents and then examined the transcriptional activity of a GAL4-IRF7 fusion protein with a reporter plasmid containing GAL4 binding sites (Fig. 1A). Expression of GAL4-IRF7 resulted in a low level of reporter gene expression that was markedly increased by viral infection (Fig. 1A, Lane 3), but not by IFN- $\gamma$  treatment (Fig. 1A, Lane 4). Under these conditions, the transcriptional activity of GAL4-IRF7 was also strongly stimulated by UV irradiation (Fig. 1A, Lane 5). UV induces several forms of DNA damage, including thymine dimer formation.

We also tested DNA-damaging chemotherapeutic agents, including Adriamycin, mitomycin C, cisplatin, and etoposide. These agents can cause DNA damage through the following distinct mechanisms; Adriamycin is a DNA intercalating agent that binds to topoisomerase II and causes DNA strand breaks, mitomycin C alkylates DNA, cisplatin generates various DNA adducts through platinum-DNA complex formation, and etoposide triggers DNA strand breaks through a ternary complex with DNA and topoisomerase II (13). The transcriptional activity of GAL4-IRF7 was markedly stimulated by treatment with Adriamycin, mitomycin C, cisplatin, and etoposide, at levels compa-

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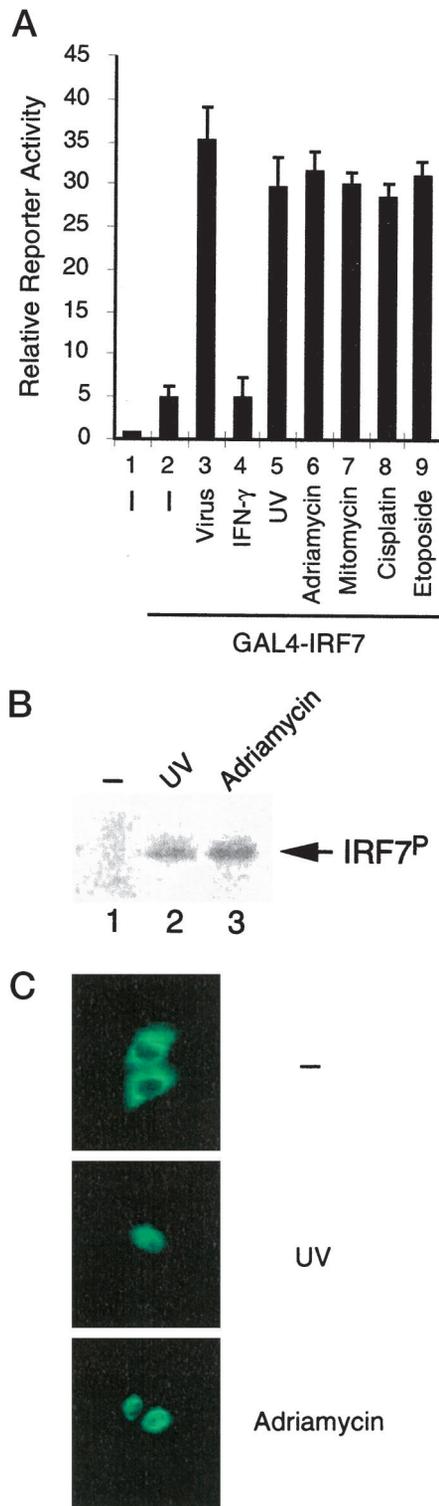
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<sup>3</sup> The abbreviations used are: IRF, IFN regulatory factor; JNK, c-Jun NH<sub>2</sub>-terminal kinase; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; MKK4, mitogen-activated protein kinase kinase-4.

<sup>4</sup> W. G. Lee and T. K. Kim, unpublished data.



**Fig. 1.** Transcriptional activation, phosphorylation, and nuclear translocation of IRF7 in response to DNA-damaging agents. **A**, CAT activities were measured in extracts of cells cotransfected with 2  $\mu$ g of a reporter plasmid containing five GAL4 binding sites and 0.5  $\mu$ g of an expression plasmid for mock (Lane 1) or GAL4-IRF7 (Lanes 2–9). Cells were irradiated with 50 J/m<sup>2</sup> UV or incubated with 200 hemagglutinin unit/ml Sendai virus, 500 units/ml recombinant IFN- $\gamma$ , 1  $\mu$ g/ml Adriamycin, 1  $\mu$ g/ml mitomycin C, 0.5  $\mu$ g/ml cisplatin, or 1  $\mu$ g/ml etoposide. Bars, SD. **B**, HeLa cells were transfected with an expression plasmid for HA-tagged IRF7. Transfected cells were incubated in phosphate-free DMEM for 30 min, and [<sup>32</sup>P]P<sub>i</sub> was added (330  $\mu$ Ci/ml) for 2 h upon treatment with UV or Adriamycin. HA-IRF7 was immunoprecipitated with anti-HA antibody and separated on SDS-PAGE for detection of phosphorylated IRF7 using a PhosphorImager. **C**, HeLa cells were transfected with 1  $\mu$ g of an expression plasmid for GFP-IRF7 (–). The subcellular localization was analyzed with a fluorescence microscope after treatment with UV or Adriamycin as indicated.

rable with those from UV irradiation (Fig. 1A, Lanes 5–9). Thus, all of the tested inducers of DNA damage, despite their different induction mechanisms, potentiated the transcriptional activity of IRF7.

In response to viral infection, IRF7 is activated by phosphorylation (7–10). To confirm that DNA-damaging agents activate IRF7 in a similar manner, we examined the phosphorylation of IRF7 in response to UV and Adriamycin treatments. Cells treated with UV or Adriamycin were labeled with radioactive P<sub>i</sub>. Then HA-IRF7 was immunoprecipitated from <sup>32</sup>P-labeled cell extracts with anti-HA antibody. Immunoprecipitates of IRF7 showed that an IRF7 phosphoprotein was induced in response to Adriamycin or UV radiation (Fig. 1B). Thus, IRF7 is phosphorylated by UV and Adriamycin treatments.

Viral infection also induces nuclear translocation of IRF7 for its activation (7–10). Thus, we investigated the subcellular localization of IRF7 in response to UV and Adriamycin. Cells were transfected with IRF7 linked to the GFP and then examined for UV/Adriamycin-induced changes in subcellular localization (Fig. 1C). In uninduced cells, GFP-IRF7 was localized almost exclusively to the cytoplasm. Treatment with UV or Adriamycin resulted in the translocation of GFP-IRF7 into the nucleus (Fig. 1C). Taken together with data from Fig. 1, these results indicate that, in response to DNA-damaging agents, IRF7 is phosphorylated and translocated into the nucleus for transcriptional activation of promoters containing its binding sites.

Because JNK is known to be involved in responses to genotoxic stresses (14–17), we tested its potential role in the activation of IRF7 after treatment with UV and Adriamycin (Fig. 2A). Increasing amounts of an expression plasmid for JNK1 (a JNK isoform) were cotransfected into HeLa cells with a GAL4-IRF7 expression plasmid and a GAL4 reporter plasmid. Expression of JNK1 itself did not significantly stimulate the transcriptional activity of IRF7 (Fig. 2A, Lanes 3 and 4). As in Fig. 1A, treatment with UV or Adriamycin dramatically induced reporter gene expression by GAL4-IRF7 (Fig. 2A, Lanes 6 and 10). Significantly, this induction further increased when UV or Adriamycin treatment was given in the presence of JNK1 (Fig. 2A, Lanes 7–8 and 11–12). These results are consistent with the idea that JNK is involved in the activation of IRF7 by DNA-damaging agents.

To further address the role of the JNK pathway in IRF7 activation, we analyzed the effects of MKK4, an upstream activator of JNK, on the transcriptional activity of IRF7 (Fig. 2B). Increasing amounts of an expression plasmid for MKK4 were cotransfected into HeLa cells with a GAL4-IRF7 expression plasmid and a reporter plasmid containing GAL4 binding sites. Expression of MKK4 potentiated the transcriptional activity of IRF7 in a dosage-dependent manner, even in the absence of UV or Adriamycin treatment (Fig. 2B, Lanes 3 and 4). This high level of induction of IRF7 activity by MKK4 was almost comparable with that by UV (Fig. 2B, Lane 5). Thus, the MKK4-JNK pathway may play a role in the activation of IRF7 in response to DNA-damaging agents.

To confirm that IRF7 activation involves the MKK4-JNK pathway, we examined the effects of MKK4 on the localization of IRF7 in cells (Fig. 2C). In mock-transfected cells, GFP-IRF7 was localized almost exclusively in the cytoplasm. In contrast, expression of MKK4 dramatically induced the nuclear localization of GFP-IRF7. Expression of JNK1 also induced the nuclear translocation of GFP-IRF7 with much lower efficiency. The physiological significance of this JNK1-mediated translocation is not clear, because JNK is known to be inactive in the absence of inducing stimuli (see also Fig. 2A, Lanes 3 and 4). Taken together with data from Fig. 2, these results suggest that the MKK4-JNK kinase pathway activates and causes the nuclear localization of IRF7 in response to DNA-damaging agents.

In the present study, we have demonstrated that transcription factor IRF7 is activated, in concert with its phosphorylation and nuclear

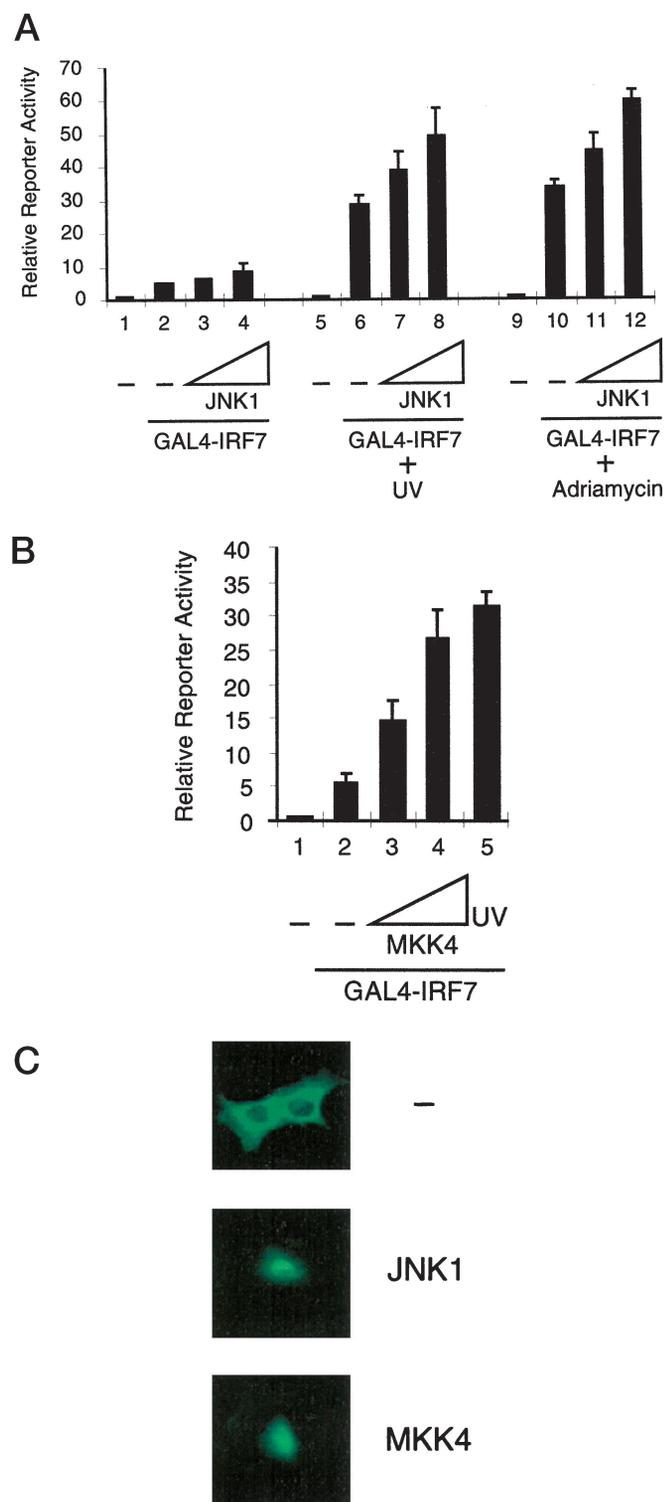


Fig. 2. Involvement of the JNK pathway in the activation of IRF7 in response to DNA-damaging agents. A, CAT activities were measured in extracts of cells cotransfected with 2  $\mu$ g of a reporter plasmid containing five GAL4 binding sites and an expression plasmid for mock (0.5  $\mu$ g; Lanes 1, 5, and 9) or GAL4-IRF7 (0.5  $\mu$ g; Lanes 2–4, 6–8, and 10–12) in the absence (Lanes 1, 2, 5, 6, 9, and 10) or presence (2  $\mu$ g in Lanes 3, 7, and 11; 4  $\mu$ g in Lanes 4, 8, and 12) of a JNK1 expression plasmid. Bars, SD. B, CAT activities were measured in extracts of cells cotransfected with 2  $\mu$ g of a reporter plasmid containing five GAL4 binding sites, along with 0.5  $\mu$ g of an expression plasmid for mock (Lanes 1) or GAL4-IRF7 (Lanes 2–5) in the absence (Lanes 1, 2, and 5) or presence (1  $\mu$ g in Lane 3; 3  $\mu$ g in Lane 4) of an MKK4 expression plasmid. Transfected cells were irradiated with 50 J/m<sup>2</sup> UV (Lane 5). Bars, SD. C, HeLa cells were transfected with 1  $\mu$ g of an expression plasmid for mock (–), MKK4, or JNK1 in the presence of 1  $\mu$ g of a GFP-IRF7 expression plasmid. The subcellular localization of GFP-IRF7 was analyzed with a fluorescence microscope.

localization, after treatment with UV and Adriamycin. Furthermore, we have shown that the JNK pathway is involved in IRF7 phosphorylation, nuclear translocation, and transcriptional activation events.

Because various chemotherapeutic drugs can stimulate its transcriptional activity, IRF7, like p53, may be a mediator of DNA damage signaling pathways. Interestingly, JNK has also been shown to phosphorylate and activate 53 (18). It is possible that these transcription factors elicit defensive responses through induction of overlapping and nonoverlapping target genes. In this regard, it will be important to identify the target genes of IRF7 to gain further insights into cellular responses to chemotherapeutic DNA-damaging agents. These target genes may provide a means to modulate the chemosensitivity of cells during chemotherapy. For example, up-regulation of specific target genes of IRF7 might provide the unique opportunity to induce apoptosis and/or growth arrest in p53-deficient tumor cells.

The JNK signaling pathway can be activated by a variety of DNA-damaging agents (19–21) and is an important signaling pathway for these DNA damage responses (22–24). Consistent with its implicated role in DNA damage responses, ectopic expression of IRF7 dramatically inhibited tumor cell growth, although the mechanism for that inhibition remains to be determined.<sup>5</sup> We also noted that cell growth was significantly reduced when IRF7 was activated in response to UV radiation or Adriamycin in the present study.<sup>5</sup> Related to these observations, other members of the IRF family, IRF1 and IRF3, were also induced by genotoxic stresses and implicated to be important for cell cycle arrest and apoptosis (25–27). Thus, interplay of these IRF family members may provide tight control mechanisms for the protective responses to DNA-damaging agents. In conclusion, understanding IRF signaling pathways as p53-independent pathways for DNA damage responses will be critical to circumvent the resistance to chemotherapeutic agents in human cancers lacking functional p53.

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