jun-NH₂-Terminal Kinase Activation Mediated by UV-induced DNA Lesions in Melanoma and Fibroblast Cells

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Abstract

jun-NH₂-terminal kinase (JNK) belongs to a family of protein kinases that phosphorylates c-Jun, ATF2, and Elk1 in response to various forms of stress including UV irradiation and heat shock. Although in previous studies we have demonstrated the importance of membrane components for JNK activation by UV irradiation, here we have elucidated the role of DNA damage in this response. We show that in vitro-irradiated or sonicated DNA that is added to proteins prepared from UV-treated cells can further induce JNK activation in a dose-dependent manner. When compared with UV-B (300 nm), UV-C (254 nm), which is better absorbed by the DNA, is significantly more potent in activating JNK. Furthermore, when wavelengths lower than 300 nm were filtered out, UV-B was no longer able to activate JNK. With the aid of melanoma and fibroblast cells, which exhibit different resistances to irradiation and require different UV doses to generate the same number of DNA lesions, we demonstrate that above a threshold level of 0.45 lesions and up to 0.75 lesions per 1875 bp, the degree of JNK activation correlates with the amount of lesions induced by UV-C irradiation. Finally, to explore the role of nuclear and mitochondrial DNA (mtDNA) in mediating JNK activation after UV irradiation, we have used cells that lack mtDNA. Although the lack of mtDNA did not impair the ability of UV to activate JNK, when enucleated, these cells had lost the ability to activate JNK in response to UV irradiation. Overall, our results suggest that DNA damage in the nuclear compartment is an essential component that acts in concert with membrane-anchored proteins to mediate c-Jun phosphorylation by JNK.

Introduction

The mammalian response to stress and DNA damage involves a large subset of cellular proteins such as p53, (1) GADD45 (2, 3), WAF1/p21/cip, (4, 5) nuclear factor-κB (6, 7), and c-Jun (8–10). The immediate response to stress involves, in most cases, posttranslational modification of preexisting proteins as shown for Fos-jun (6), nuclear factor-κB (7), p62CF (11), and ATF2 (12). Together, these stress-modulated proteins dictate whether the cell will undergo temporal growth arrest (13, 14) to enable proper repair (15) and subsequent recovery or apoptosis (16). A subset of protein kinases, including src (17), raf (18), JNK (19–21), mitogen-activated protein kinase (22), and growth factor receptors (23), as well as DNA-PK (Refs. 24 and 25), were shown to participate in the stress response. As a multi-member family (26, 27), JNK phosphorylates c-Jun on serines 63 and 73, which are important to mediate c-Jun activities in transcription, replication, and transformation (28, 29). To mediate its activity, JNK requires the ability to interact with the δ domain of c-Jun (30), a key component that is deleted in its oncogenic v-jun counterpart (31, 32).

One of the key questions toward understanding the mammalian stress response concerns the mechanisms involved in the activation of stress-related protein kinases that can mediate the respective cellular changes. To date, the response to UV-irradiation was shown to include cell surface receptors, such as epidermal growth factor receptor (23, 33, 34), and is in agreement with the fact that stress response resembles the response to mitogens (35). In the case of UV irradiation, membrane-based signals appear to depend on p21WAF1 (37, 38), which involves the activation of at least two independent signal transduction pathways, including Src, Raf-1 (36, 37) and MEK, JNKK (38, 39), both of which participate in mediating c-Jun activation (7, 39). When the integrity of the cell membrane and its associated components (receptor/anchored proteins) is modified, UV can no longer mediate JNK activation (40).

Although these studies indicate that UV-modulated membrane components are necessary to mediate JNK activation, it was our interest to examine whether DNA damage per se could also be required to mediate this response. The latter has been shown for DNA-PK, which resides in the nucleus and is activated by DNA damage (24, 25). Through the use of four independent experimental approaches, we demonstrate that UV-induced DNA lesions are also required to activate JNK. The role of DNA damage and its biological relevance to JNK activation are discussed.

Results

In Vitro-damaged DNA Is Capable of Activating JNK. To elucidate the role of DNA damage in JNK activation, we used DNA that had been damaged in vitro. To this end, genomic DNA prepared from nontreated 3T3-4A cells (41) was irradiated in vitro with different doses of UV-C (254 nm). Equal amounts of the irradiated DNA were then added to the kinase reaction, which consisted of pGEX-jun as well as protein extracts from either nontreated or UV-treated

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mouse fibroblasts as the source for JNK. When added to nontreated protein extract, damaged DNA was not able to activate JNK. However, when the irradiated DNA was added to protein extracts prepared from 3T3-4A cells that were exposed to 5 J/m² of UV-C, which by themselves exhibit only a low level of JNK activation, a 2–3-fold increase in JNK activation was observed (data not shown). Similarly, in vitro-damaged DNA that was added to a diluted portion (1 of 10) of protein extracts prepared from UV-C-treated 3T3-4A cells (20 J/m²) caused a 2–3-fold increase in JNK activity (Fig. 1A). Since undiluted extract mediates a 20–30-fold increase in JNK activity, we attribute the weaker degree of JNK activation in the diluted extracts to cellular components required at a certain threshold level to mediate this high degree of activation and which may have not been available upon their dilution. The ability of in vitro-damaged DNA to activate JNK was dose dependent and reached a plateau at the UV dose of 20 J/m². When untreated DNA was added to these reactions, it was not capable of activating JNK (Fig. 1A).

Although the type of lesions produced by UV irradiation (pyrimidine dimers, 6:4 photoproducts) lead to the formation of single-strand nicks as part of the excision repair mechanism, we have also tested the ability of double-strand breaks to mediate JNK activation. To this end, genomic DNA from 3T3-4A cells was sonicated for varying time lengths before it was incubated with a diluted portion of protein extracts prepared from UV-treated cells as a source for JNK. To verify that this treatment did indeed yield double-strand breaks, a portion of the sonicated DNA was analyzed on ethidium bromide-stained gels to reveal a characteristic smear, which was proportional to the length of sonication (not shown). As shown in Fig. 1B, strand breaks caused by the sonication were also capable of activating JNK in a pattern that resembled the effect of in vitro-irradiated DNA, i.e., using diluted proteins (1:10) prepared from cells exposed to 20 J/m². Since the degree of JNK activation is limited in these extracts due to the dilution, we have also performed this experiment using protein extracts prepared with 13 min after UV irradiation (80 J/m²). These extracts already contain activated JNK (see below), yet the degree of JNK activation is still low. When sonicated DNA was added, a 6-fold increase in JNK activation was noticed (data not shown). The inability of in vitro-damaged DNA to mediate JNK activation in extracts that were prepared from sham-treated cells can be understood in light of the essential requirement for cytosolic components to mediate JNK activation by UV, as was demonstrated in our recent studies (40).

**UV-B and UV-C as Inducers of JNK.** The finding that JNK can be activated in vitro via the addition of damaged DNA led to the design of studies aimed to test this possibility in vivo. To this end, three independent experiments were performed. We have: (a) compared the effects of UV-B and UV-C as mediators of JNK activation; (b) elucidated the possible correlation between number of lesions and JNK activation in two cell systems that exhibit different degrees of resistance to UV irradiation; and (c) evaluated the role of mitochondrial and nuclear DNA in this response. Although both UV-C and UV-B damage DNA, UV-C is substantially more efficient (42), as reflected by the ratios of pyrimidine dimers and (6-4) photo products, two of the major lesions formed in response to UV irradiation (43). Thus, it was of interest to compare the ability of each of these two wave-lengths to activate JNK. As shown in Fig. 2A, a dose of more than 500 J/m² UV-B (corresponding to 20 min of exposure) administered to 3T3-4A cells was less efficient in activating JNK than 20 J/m² of UV-C, suggesting that DNA damage

![Image](https://example.com/image.png)

**Fig. 1.** In vitro irradiation or sonication of DNA activates JNK. JNK assays were carried out as described in “Materials and Methods.” The GST-jun fusion protein was used as a substrate bound to glutathione-Sepharose beads and incubated with the test protein extracts prepared 30 min after UV treatment (20 J/m²), which was diluted 1:10 (A and B). To these reactions, nondenatured DNA (2 μg, +DNA), DNA damaged by UV (A), or sonication (B) was added. The beads were pelleted, washed, and boiled in SDS sample buffer. The eluted proteins were run on a 15% SDS-polyacrylamide gel. The gel was dried, and phosphorilation of the specific M, 50,000 band, reflecting pGEX-jun, was determined by autoradiography and quantified with the aid of a computerized radiography device. Each of the experiments was performed at least four times; bars, SD.
may indeed play a role in JNK activation. Additional experiments were performed using a filter that was placed under the UV lamp to eliminate all wavelengths below 300 nm. This filter was used since the UV-B lamps used in the present study were found to emit about 5% of UV-C. Since this filter decreased the amount of UV-B that reached the cells, we have adjusted the exposure time necessary to mediate 300 J/m². As shown in Fig. 2B, when wavelengths below 300 nm were filtered out, JNK was no longer activated in UV-B-treated cells. These findings provide additional support for the role of DNA damage in mediating JNK activation.

**JNK Activation in UV-treated Melanomas and Fibroblasts.** As a third independent approach for elucidating the role of DNA damage in JNK activation, we evaluated the possible correlation between JNK activation and the amount of DNA lesions caused by UV-C, using melanoma and fibroblast cell lines which exhibit different resistances to irradiation. To measure the degree of resistance to UV irradiation, we have counted viable cells 24, 48, and 72 h after their exposure to increasing doses of UV irradiation. Although about 80% of the melanoma cells survived a dose of 10 J/m² 48 and 72 h after UV treatment, only 20% of the mouse fibroblast cells survived at the 48-h time point and less than 10% at the 72-h time point. The difference was even more striking when higher doses were used (Fig. 3).

We next established the kinetics of JNK activation upon UV-C irradiation of human melanoma and mouse fibroblast cells and compared the effects with those in HeLa cells that...
are known to exhibit JNK activation after UV irradiation as well as after heat shock treatment (30, 44). In HeLa cells, a dose of 40 J/m² is sufficient to transiently activate JNK more than 3-fold above its basal level within 1 min and more than 10-fold within 10 min. Analyses of human melanoma revealed similar patterns of activation, with minor changes in the time frame of inducibility. Although a similar pattern was also observed in mouse fibroblasts, they required a significantly lower dose (20 J/m²) to mediate JNK activation (data not shown).

Subsequently, we compared the ability of melanoma and fibroblasts to activate JNK in response to increasing doses of UV-C irradiation. Although the fibroblasts showed an 8-fold increase in JNK activation after UV-C exposure at a dose of 20 J/m², this dose did not activate JNK in the human melanoma cells. The melanoma cells required 60 J/m² to yield a 6-fold increase in JNK activity (Fig. 4). When higher doses were applied, an additional increase in JNK activation was noticed in the fibroblasts, reaching a plateau at 40–60 J/m², which declined when higher doses were used (Fig. 4). In contrast, the degree of JNK activation in the melanoma cells further increased at doses up to 120 J/m² (data not shown).

In both the melanoma and fibroblasts, doses of up to 10 J/m² did not activate JNK, pointing to a possible threshold in the ability to mediate JNK activation. In all, these results indicate that fibroblasts are more susceptible to JNK activation than melanoma cells at low doses of UV-C.

**JNK Isozymes in WM35 and 3T3-4A Cells.** To identify the JNK isozymes expressed in the cells used in the present study, we have performed IP and Western blot analysis with the aid of antibodies to JNK. Fig. 5 depicts the different forms of JNK that are expressed in WM35 and 3T3-4A cells. While the M, 46,000 and M, 54,000 proteins (which represent JNK1 and JNK2, respectively) are expressed in the 3T3-4A cells, at least two additional higher molecular weight forms (M, 60,000 and M, 68,000) are expressed in the WM35 cells (Fig. 5A). The slower migration of the M, 46,000 form in 3T3-4A cells could be either due to its phosphorylation status or may represent a new JNK isozyme. We have then tested the ability of the material precipitated with antibodies to JNK to phosphorylate pGEX-Jun. As shown in Fig. 6B immunoprecipitated material phosphorylated NH₂-terminal c-Jun in a solid phase kinase assay using pGEX-jun as a substrate. Since multiple isoforms were recognized in Western blots (Fig. 5A), it was of interest to identify those that, in fact, bind to c-Jun. Therefore, protein extracts from WM35 cells were incubated with the pGEX-jun as in the kinase reactions. After extensive washings, bound proteins were eluted with the aid of 3% octyl β-D-glucopyranoside and were analyzed on Western blots with the aid of antibodies to JNK. Interestingly, all forms of JNK that were recognized after IP (data not shown) were also identified after elution (Fig. 5A).

**Quantification of UV-induced DNA Lesions.** Since JNK activation required different doses of UV irradiation when melanoma and fibroblasts were compared, we next determined the number of lesions present in the DNA of each of these two cell types after UV-C irradiation. For this purpose, genomic DNA was prepared from the fibroblasts and melanoma cells at different time points after UV irradiation. This DNA was then used as a template for a quantitative PCR, which enabled us to determine the relative number of lesions based on the efficiency of the amplification. This analysis was performed on a 1875-bp fragment of the K-ras gene, which was amplified with respective primers. These reactions were carried out in the presence of radioactive deoxynucleotide triphosphates, thus enabling us to identify and quantify the amplified product at the expected size (see "Materials and Methods" for details). Fig. 6A demonstrates that the efficiency of the amplification of this fragment is dependent on the amount of DNA present in the reaction. The optimal amount of DNA (1.0–1.6 µg) was then treated in vitro with increasing doses of UV irradiation. Immediately after the UV exposure, the DNA was taken for PCR amplification. Since lesions induced by UV light are capable of blocking/halting the DNA polymerase (45, 46), we were able to determine the relative number of lesions based on the yield of the full-sized amplified DNA fragment.
Smaller-sized fragments are also generated due to the presence of lesions, yet since they will not be amplified exponentially, they are poorly visualized (data not shown). Based on a quantitative analysis of the 1875-bp DNA fragment, an inverse correlation was obtained between the dose of UV irradiation and the amount of amplified fragment (Fig. 6B).

The dose-dependent decrease in the amplification of DNA that had been damaged in vitro served as a reference to quantify the lesions present in DNA that was irradiated in vivo. Quantification of lesions present in DNA prepared from cells within 30 min after exposure to a UV dose of 20 J/m² revealed a greater number of lesions in the DNA of mouse fibroblasts (0.61/1875 bp) than in the DNA of human melanoma (0.4/1875 bp; Fig. 7A). These differences were also noted at higher UV doses, i.e., 0.74 lesions in DNA of fibroblasts versus 0.52 lesions in DNA of melanoma cells per 1875 bp at 40 J/m². A similar number of lesions were reported previously in the UV-irradiated DHFR gene (46).

As a second independent approach for determining the relative amounts of lesions within UV-irradiated DNA, we have used in vitro-irradiated, ³²P-labeled plasmid DNA that served as a template for T4 DNA polymerase activity. The fragments generated by T4 exonuclease activity were separated on a sequencing gel, autoradiographed, and quantified. The latter revealed the same relative number of le-
sions per a given dose of UV as the PCR-based approach (data not shown).

The amount of lesions shown here correlates with the degree of JNK activation, as indicated in Fig. 7B. Within the range of 0.5 to 0.75 lesions per 1875 bp, there appears to be linear correlation between the degree of JNK activation and amount of lesions. A threshold level of up to about 0.45 lesions per 1875 bp appear to exist as well (Fig. 7B). This correlation is true for both mouse fibroblasts and human melanoma studied here, in spite of the fact that different doses of UV were required to create the same number of lesions (Fig. 7A). When the number of lesions is higher than 0.75, there is a decrease in ability to activate JNK (Figs. 4 and 7B), indicative of other cellular factors that are required to mediate this response, and which may be modulated above certain amount of lesions.

UV Irradiation Requires Nuclear DNA to Activate JNK.

The ability to activate JNK via damaged DNA led us to test whether mitochondrial or nuclear DNA contribute to JNK activation after UV irradiation. To this end, we have measured JNK activation after UV irradiation in cells that lack mtDNA (47, 48). We show that in spite of the lack of mtDNA, the cells exhibited proper ability to activate JNK after UV irradiation (Fig. 8A). Surprisingly, however, the cells lost their ability to mediate JNK activation after heat shock, indicating that heat shock and UV mediate JNK activation via alternate cellular pathways (Fig. 8A). In related studies, we have identified different cellular components that are necessary to mediate JNK activation after UV and heat shock, respectively (40). To determine whether nuclear DNA is required for JNK activation, we have enucleated the cells that lacked mtDNA. When tested by itself, cytochalasin B, which was used to enucleate the cells, did not have any significant effects on JNK activation (Fig. 8B). As shown, cytoplasts that lacked both mitochondrial and nuclear DNA did not mediate JNK activation after UV irradiation (Fig. 8B). To confirm the role of nuclear DNA in mediating this response, we have repeated this experiment using 3T3-4A cells. Upon their enucleation, 3T3-4A cells have also lost the ability to mediate JNK activation (Fig. 8B). Enucleation of HeLa cells also abolished their ability to mediate JNK activation (data not shown). Interestingly, UV-treated 3T3-4A cells (prior to their enucleation) retained their high level of JNK activity, as well as activated src-related tyrosine kinases, suggesting that the enucleation process does not impair on the activity of these kinases. When in vitro-damaged DNA was added to sham-treated cells after their enucleation, a mild increase (up to 30%) in JNK activity was noticed (data not shown).

Discussion

The present study demonstrates, via the use of four independent approaches, the role of DNA damage in mediating JNK activation. In vitro experiments provide evidence for the ability of damaged DNA (by UV or sonication) to activate JNK. Both damaged DNA and cytoplasmic proteins (from treated cells that were diluted or obtained after low dose of irradiation) show only a weak level of JNK activity and cannot mediate JNK activation when tested by themselves. However, when combined, they are able to activate JNK. The degree of JNK activation is directly correlated with the number of lesions and is not seen with nondamaged DNA. Although the 2–3-fold activation of JNK seen in our in vitro reaction is attributed to the experimental conditions used (in which protein extracts were selected based on their weak JNK activity), a similar degree of JNK activation was also observed 3–12 h after exposure of human fibroblasts to tumor necrosis factor α (51). That this response can be observed only in extracts prepared from UV-treated cells can be explained by the need for UV-mediated changes at the membrane level and is likely to involve receptors (i.e., epidermal growth factor receptor) or anchored proteins (i.e., p214). We have shown recently that pretreatment of cultured cells with Triton X-100 abolishes the ability of UV (but not of heat shock) to activate JNK. When added to cells after UV treatment, Triton X-100 has only minor effects on JNK activity (40). The ability of damaged DNA to activate JNK in UV-derived extracts is attributed to changes mediated by such lesions in one of the following three potential mechanisms. The first is that DNA lesions attract the repertoire of proteins involved in excision repair, some of which are by themselves transcription factors (i.e., ERCC3) or subunits of DNA protein kinases (i.e., Ku 70, 54), altering the balance and potential phosphorylation of proteins such as c-Jun. A second possibility relates to the possible presence of JNK-associated proteins that may act as inhibitors that are dissociated upon UV irradiation and may be affected by cellular changes elicited by a certain number of lesions. Finally, nicked DNA, which is expected to be part of the excision repair process, may provide additional negatively charged phosphorus groups that could alter the overall cellular charge, which by itself has been shown to be sufficient for mediating JNK activation (30).

The second independent assessment of the role of DNA damage in JNK activation revealed that the ability of UV-B to activate JNK was abolished when wavelengths lower that 300 nm are filtered out. This is consistent with the action spectrum of UV-B, which is less efficient than UV-C as per DNA damage. It is important to emphasize that our results are UV-C related, as it is apparent that alternate cellular pathways which do not require membrane components, nor do they rely on DNA damage (i.e., heat shock, TNFα), are potent inducers of JNK (36, 40). That it is the UV-C component which mediates this activation questions possible physiological relevance because wavelengths below 270 nm are not known to penetrate the ozone layer. This important question is presently addressed in our ongoing studies that identify relevant physiological conditions that could also mediate potent JNK activation.5

Our third approach relied on in vivo studies using cultures of melanoma and fibroblasts and provided correlative support for the role of DNA damage in JNK activation. Although each of these cell types exhibits different sensitivities to irradiation, the number of lesions produced in their nuclear DNA correlates with the degree of JNK activation. With a threshold of about 0.45 lesions/1875 bp, a linear correlation was found for both melanoma and fibroblasts between the number of lesions and degree of JNK activation within the range of 0.5–0.75 lesions/1875 bp (Fig. 7B). The number of lesions measured 30 min after UV irradiation is likely to reflect the amount of lesions than the rate of DNA repair, because the latter should have been reflected in greater JNK activation at earlier time points, which was not observed. Although it is tempting to specu--

4 V. Adler, A. Schaffer, and Z. Ronai, unpublished observations.
5 V. Adler, J. Kim, L. Dolan, R. Davis, and Z. Ronai, unpublished observations.
late that the difference between melanoma and fibroblasts in the number of UV-induced lesions formed with the same dose of irradiation may be attributed to the presence of melanin, which protects the melanoma DNA, additional studies are required to provide a better understanding of these striking differences.

The fourth approach to elucidate the role of DNA damage in JNK activation relied on the cells that were depleted of their mitochondrial or nuclear DNA, or both. Although the lack of mtDNA does not impair the ability to activate JNK by UV irradiation, enucleation of these cells abolished this ability. That it is nuclear rather than mtDNA that is required to mediate JNK activation was confirmed from the use of 3T3-4A cells, which upon enucleation also lost their ability to activate JNK in response to UV irradiation. Our results are in contrast to studies of Devar et al. (7), who have demonstrated that enucleation of HeLa cells do not impair JNK inducibility by UV irradiation. At this time, we cannot resolve this discrepancy.

Our present understanding of the ability of UV to mediate JNK activation and c-Jun phosphorylation relies on the following observations: (a) DNA damage per se is essential for UV-C-mediated JNK activation (this study); (b) membrane-anchored components are a prerequisite for UV irradiation-mediated JNK activation (19, 23, 40); (c) wavelengths above 270 nm fail to activate JNK (this study); (d) UV irradiation abolishes an inhibitor of JNK activity; and (e) JNK and Raf-1 associates with p21<sup>WAF1</sup> via alternate regions on ras protein itself (52, 53). When taken together, the following scheme is proposed. UV irradiation (<270 nm) modulates cell surface receptors (23) as well as other membrane components, including p21<sup>WAF1</sup> (37) which recruits JNK (52) and Raf-1 (53), enabling their activation and possible translocation to the nuclear compartment. Translocation of kinases to the nuclear compartment to phosphorylate their substrates was shown for ERK and was proposed recently for JNK (reviewed in Ref. 54). The presence of DNA lesions may modulate JNK-associated inhibitor<sup>4</sup> which enables efficient phosphorylation of JNK substrates c-Jun, ATF2, or Elk1 (54, 55). Important to note is that kinase activation by DNA damage is not confined to JNK because it has been shown for DNA-PK (24, 25). Interestingly, Ku 70, one of DNA-PK subunits, was shown capable of phosphorylating c-Jun on its COOH-terminal domain (amino acid 249; Ref. 56), making c-Jun a substrate for two independent kinases that are activated by DNA damage.

Because JNK is a multi-member family, the results shown here may reflect the activity of different JNK isoforms. Since our assays measured phosphorylation of the c-Jun NH<sub>2</sub>-terminal region, we cannot exclude the possibility that

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**Fig. 7.** A, quantification of lesions in DNA from UV-C-treated melanoma or fibroblast cells. Cells were irradiated with the indicated doses and, 30 min after treatment, the DNA (1.6 μg) was used as a template for the amplification. Bars, SD calculated from three independent experiments. B, ratio between number of lesions and JNK activation fold increase in JNK activation is plotted against number of lesions monitored within 1875 bp after UV-C irradiation at doses of 5, 10, 20, 40, and 80 J/m² it data used to generate this figure was obtained from Figs. 5 and 7A.

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**Fig. 8.** JNK activation in cells lacking mtDNA and nuclear DNA. A, 141BT cells representing the parent cells with mtDNA (+mtDNA) and 141B20k cells in which mtDNA is depleted (--mtDNA). Cells were sham treated or UV-irradiated at 40 or 80 J/m² as indicated; same treatments were performed on cells which were grown for 2 h with cytochalasin B (added to their growth media; 20 μg/ml) as indicated. **B**, protein extracts were prepared from 141B20k cells that were enucleated and then exposed to the specified doses of UV (J/m²). Lower panel, points to a similar analysis performed on intact 3T3-4A cells or on their cytoplasts (enuc).
other kinases may have also been part of this reaction. Through IPs, Western blots, and elution from pGEX-Jun, followed by Western blots, we have demonstrated that multiple JNK isoforms are, in fact, expressed and bound to their substrate. On the basis of the assays performed here, we cannot identify which JNK isoform(s) phosphorylates c-Jun. It is likely, however, that different isoforms exhibit varying degrees of sensitivity to UV irradiation and may be involved in diverse signaling pathways.

Finally, the biological significance of JNK activation to the immediate UV response has yet to be identified. Although the transcriptional activation of c-Jun, ATF2, and Elk1 after their phosphorylation by JNK has been shown (19, 20, 28, 45, 55), emerging alternate possibilities include nuclear localization and stability, which could also contribute to the overall transcriptional activities mediated by JNK substrates.

Materials and Methods

Cell Lines. Cultures of melanoma WM35 cells were kindly provided by Dr. Yaakov Ben David (University of Toronto, Toronto, Canada) and maintained in RPMI supplemented with 10% fetal bovine serum and antibiotics. Mouse fibroblast cell line 3T3-A4 (carries an integrated copy of temperature-sensitive polyoma virus; Ref. 40) was kindly provided by Dr. Claudio Basilico (New York University, New York, New York) and was maintained in DMEM supplemented with 10% calf serum and antibiotics. Cells that were depleted of mtDNA (143B206) as well as their parental wild-type 143BT cells were characterized previously (47, 48). These cells were maintained in DMEM, supplemented with 5% FBS; in the case of 143B206, uridine (50 μg/ml) was also added. In all cases, cells were grown at 37°C with 5% CO₂.

UV Irradiation and Heat Shock Treatment. Cells were exposed to UV irradiation as indicated previously (49). Briefly, prior to irradiation the cells were washed with PBS, and with the lids off, they were placed in marked areas in the tissue culture hood, which were precalibrated for the amount of UV using the germicidal lamp (254 or 300 nm, respectively) with the aid of a UV-B or UV-C probe (UVP, San Diego, CA). Immediately after irradiation, the medium that was removed before UV exposure was added back, and the cells were harvested at the indicated time points. Heat shock treatments were performed by placing the tissue culture cells in a 42°C incubator for 1 h. When filters to exclude wavelengths below 300 nm were used, the membrane (M-Kodak) was placed just below the UV-B lamp; then the actual UV-B and UV-C output were recalibrated.

Cell Survival Studies. Cells (2 x 10⁶) of mouse fibroblasts (4A) or human melanoma (WM35) were plated/well in a 6-well plate (Corning, Corning, NY). The cells were sham or UV irradiated at the specified doses (5-40 J/m²) 48 h after plating. Twenty-four, 48, and 72 h after irradiation, cells were washed twice with PBS, trypsinized, and resuspended in a total volume of 2 ml, of which 0.5 ml was taken for counting with a Coulter counter. The ratio between the number of UV-treated and sham-treated cells was used as the survival criteria. For each dose, two independent experiments were used.

Cell Enucleation. Enucleation of 143B206 and 3T3-A4 cells was performed using cytochalasin B and discontinuous Percoll gradients as described previously (50). Briefly, cell cultures were incubated in cytochalasin B (20 μg/ml; Sigma Chemical Co.) for 2 h, washed with PBS, and detached from the culture dish with the aid of Versene (0.1%, pH 7.6). Cells were then resuspended in Percoll (1.05 g/ml; Sigma) in PBS containing cytochalasin B (5 μg/ml). After centrifugation for 1 h at 28°C and 28,000 rpm (72,000 x g) in a Beckman rotor SW-28, enucleated cells were recovered from the midpoint of the gradient, whereas whole cells were recovered from the bottom. Both fractions were washed twice with PBS, resuspended in culture medium containing 10% fetal bovine serum, and then replated onto 10-mm plates and allowed to recover for 90 min at 37°C before they were irradiated as indicated in "Results." In each of the experiments, efficiency of enucleation was monitored by viewing cells under a phase contrast microscope.

Protein Preparation. Whole cellular protein extract was prepared from the indicated cells as described previously (30). Briefly, 10⁶ cells grown in 100-mm plates were lysed with the aid of a lysis buffer that consists of kinase buffer [20 mM HEPES (pH 7.6), 1 mM EGTA, 2 mM MgCl₂, 2 mM MnCl₂, 5 mM NaF, 1 mM NaVO₃, and 50 mM NaCl] and 350 mM KCI, 2 mM NaVO₃, and a mix of protease inhibitors (1 μg/ml each of aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). In the experiments in which these proteins were diluted, they were mixed 1:10 with 1× kinase buffer.

JNK Assays. Protein kinase assays were carried out using a fusion protein between GST and c-Jun (amino acids 5–89) as a phosphotransferase acceptor. The protein kinase assay was carried out as described previously (30, 44). The GST-c-Jun fusion proteins were bound to glutathione-Sepharose beads and incubated with cellular protein extracts, [γ-²³²P]ATP (50 cpm/μmol), and kinase buffer at 30°C for 5 min. The beads were pelleted and washed extensively with PBST [150 mM NaCl, 16 mM sodium phosphate (pH 7.5), 1% Triton X-100, 2 mM EDTA, 0.1% β-mercapto, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine] and boiled in SDS sample buffer. The eluted proteins were run on a 15% SDS-polyacrylamide gel. The gel was dried, and phosphorilation of the GST-c-Jun substrate was determined by autoradiography. The radioactive signal was quantified using a computerized radioimaging blot analyzer (AMBIS, San Diego, CA). The term JNK activation used throughout our studies relates to c-Jun phosphorylation by JNK, the kinase known to have the highest affinity to bind and phosphorylate c-Jun on its NH₂-terminal region under conditions used in the present studies. As such, we cannot exclude the possibility that other kinases may have also been part of this reaction.

Quantitative PCR for Determination of DNA Lesions. A PCR-based assay was used to quantify lesions present after UV irradiation within a specific DNA sequence. Based on the methodology developed by Govan et al. (45), which relies on the principle that DNA lesions block the polymerase activity, it is possible to quantify the amplified DNA that has a specific size. Although this approach was successfully used for the dihydrofolate reductase and mtDNA fragments (46), we have adopted a similar approach to determine the mutation rate in a 1875-bp fragment of the human K-ras gene. Typical amplification consisted of 1.6 μg of genomic DNA and 50 μmol of primers, 250 μM of deoxynucleotide triphosphates, 2 μM of 1²³²PdCTP (3000 Ci/ mMol), 2.5 units of Taq polymerase, and the respective Taq buffer, supplemented with 0.01% gelatin (w/v) and 0.1% Triton X-100. Typical amplification used an initial step of 4
min denaturation, followed by 25 cycles composed of three steps which were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. After the last cycle, samples were extended at 72°C for an additional 10 min. Following the amplification, the radiolabeled amplified material was separated on a 4% nondenaturating polyacrylamide gel by electrophoresis. The amplified fragment was quantified via a computerized blot imaging analyzer (AMBIGS). To ensure that the limiting factor for amplification was not nondenamated template, the amount of nondenamated DNA was titrated from 0.1–3.2 μg. This allowed us to determine whether the amplified signal linearly correlated with the amount of template DNA. To evaluate the relative number of lesions present in a 1875-bp DNA fragment, the following formula was used: number of lesions = 1 – cpm damaged/cpm control.

**IP and Western Blot Analysis.** Protein extracts (50 μg) were immunoprecipitated with antibodies to JNK (kindly provided by PharrMingen) for 12 h at 4°C (1:2000), followed by incubation with protein A/G beads (Oncogene Science) for 30 min at room temperature. Precipitates were then washed and analyzed on either SDS-PAGE, followed by Western blot with the same antibodies (1:3000) and subsequently detected with the aid of a chemiluminescence kit (ECL, Amersham), or used for the solid phase kinase assay. Alternatively, as indicated in “Results,” protein extracts were incubated with pGEX jun, as for the kinase reaction, and after extensive washing, bound proteins were eluted with the aid of 3% octyl-β-D-glucopyranoside and analyzed on Western blot with antibodies to JNK. Each of the experiments was reproduced at least three times.

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**References.**


