Ionizing Radiation Activates Nuclear Factor κB but Fails To Produce an Increase in Human Immunodeficiency Virus Gene Expression in Stably Transfected Human Cells†

Kristoffer Valerie,*,‡,§ William S. Laster,‡ Jean C. Kirkham,‡ and Nancy B. Kuemmerle‡

Departments of Radiation Oncology and Microbiology and Immunology, Massey Cancer Center, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0058

Received October 17, 1994; Revised Manuscript Received October 3, 1995⊗

ABSTRACT: We have investigated the differential effects of ultraviolet light (UV) and ionizing radiation (IR) on human immunodeficiency virus type 1 (HIV) and *c-jun* expression in HIVcat/HeLa cells. This cell line harbors stably integrated copies of the chloramphenicol acetyltransferase (*cat*) gene under control of the HIV promoter. Both UV and IR increased the binding of nuclear proteins to an oligonucleotide spanning the HIV enhancer region nuclear factor κB sites, but only UV increased HIVcat steady-state mRNA and CAT activity. By comparison, transcription of the cellular *c-jun* gene increased after both types of radiation, but UV was at least 5-fold more effective than IR despite the fact that protein binding to an activator protein 1 oligonucleotide increased similarly after both UV and IR. The lack of HIVcat transcriptional response after IR does not appear to be the result of a repressor binding to upstream promoter elements since cells stably transfected with different HIV promoter deletions showed a lack of response to IR indistinguishable from that of the intact promoter. While our findings indicate no correlation between increased binding of transcription factors to upstream promoter elements and increased expression of these genes after radiation, we did observe major differences in how UV and IR affected chromatin structure. UV produced extensive global chromatin decondensation, whereas IR did not, as seen in the microscope and determined by the increased susceptibility of chromatin to micrococcal nuclease digestion. Finally, although no effect of IR was detected by the CAT assay up to 24 h, a 2−3-fold increase was seen after several days, suggesting that HIVcat expression is regulated by both early and late effects after radiation; only UV produces the much more pronounced early effect. In summary, our findings are in agreement with a mechanism whereby UV exerts a positive effect on HIVcat transcription through extensive global changes in chromatin structure, perhaps associated with the DNA repair process, while bypassing "true" transcriptional activation through upstream promoter elements.

DNA damage elicits a number of stress responses in mammalian cells, including changes in transcription [see Holbrook and Fornace (1991) for review]. Many reports have shown that ultraviolet light (UV)[1] increases the transcription of specific genes, and more recently, the positive effect of ionizing radiation (IR) on gene expression has been well-documented (Holbrook & Fornace, 1991; Fuks et al., 1993). Certain genes, such as those encoding specific transcription factors (NF-κB [p50], Jun/Fos [AP-1], and EGR-1), and other genes susceptible to growth stimuli respond in a positive fashion to IR (Sherman et al., 1990; Brach et al., 1991; Hallahan et al., 1991, 1993; Chae et al., 1993; Uckun et al., 1993). UV (Devary et al., 1991; Stein et al., 1992) and IR (Hallahan et al., 1993) activate *c-jun* transcription through AP-1 binding to the phorbol ester-responsive element (TRE) in transient transfection experiments. The human immunodeficiency virus (HIV) promoter is also very sensitive to the effects of DNA-damaging agents and responds by increasing transcription after exposure to many, but not all, types of DNA damage (Valerie et al., 1988, 1994; Valerie & Rosenberg, 1990; Stanley et al., 1989; Stein et al., 1989; Cavard et al., 1990; Zmudzka & Beer, 1990; Beer et al., 1994; Morrey et al., 1991; Frucht et al., 1991; Vogel et al., 1992; Zider et al., 1993; Zmudzka et al., 1993.

Several groups have demonstrated a correlation between increased transcription of HIV after UV and enhanced binding of NF-κB to the HIV enhancer region (Stein et al., 1989; Devary et al., 1993; Zider et al., 1993), whereas others suggest that NF-κB and the enhancer play less important roles in UV activation (Zider et al., 1993; Valerie et al., 1995), perhaps depending on whether the transcription unit is integrated in the genome or not. We have shown that, when the HIV transcription unit is stably integrated in the genome, the UV response does not map to any specific upstream element in the HIV promoter, including the enhancer. Instead, we find a correlation with the overall competence

† Supported by USPHS Grant CA53199 awarded to K.V.

* Corresponding author: Department of Radiation Oncology, MCV Station Box 980058, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0058. Telephone: (804) 828-8778. Fax: (804) 828-6042. E-mail: kvalerie@gems.vcu.edu.

‡ Department of Radiation Oncology.

§ Department of Microbiology and Immunology.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1995.

[1] Abbreviations: AP-1, activator protein 1; araC, 1-β-arabinofuranosylcytosine; BER, base excision repair; CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus type 1; NF-κB, nuclear factor κB; IR, ionizing radiation; MN, micrococcal nuclease; NER, nucleotide excision repair; PBS, phosphate-buffered saline; PCC, premature chromosome condensation; LTR, long terminal repeat; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SSC, sodium salt citrate (20×; 3 M NaCl/0.3 M sodium citrate); TRE, TPA-responsive element; UV, ultraviolet light.

0006-2960/95/0434-15768$09.00/0

of the basal promoter and the extent of UV activation (Valerie et al., 1995). We have proposed previously that UV may activate HIV gene expression in stably transfected cells by producing changes in chromatin structure necessary for DNA repair (Valerie & Rosenberg, 1990; Wallace & Lasker, 1992). These changes may allow for enhanced accessibility of factors involved in basal transcription to the HIV promoter region or, alternatively, for preformed transcription complexes elongating short nascent RNA (Valerie et al., 1995). In spite of the observation that IR produces increased binding of NF-κB to its promoter element (Brach et al., 1991; Uckun et al., 1993), several studies have demonstrated that IR does not increase HIV gene expression (Valerie & Rosenberg, 1990; Beer et al., 1994; Zmudzka & Beer, 1990; Stanley et al., 1989), suggesting that additional requirements need to be fulfilled for a full HIVcat transcriptional response.

In the present study, we have investigated further the lack of response to IR by comparing and dissecting the differential effects of UV and IR on HIVcat and *c-jun* expression in stably transfected HIVcat/HeLa cells. We show that IR, similarly to UV, produces an increased binding of NF-κB to the HIV enhancer region but apparently does not elicit the cellular changes required to increase HIVcat gene expression. Furthermore, UV, but not IR, produces major structural changes in the chromatin of mitotic and interphase cells, suggesting that one reason for the effectiveness of UV in activating HIVcat gene expression may be associated with changes in chromatin structure which do not occur after IR.

MATERIALS AND METHODS

Cells and Treatments. The wild-type HIVcat/HeLa, enhancer$^+$ (−119/+80), and enhancer$^-$ (−69/+80) cells have been described (Valerie et al., 1988, 1995; Valerie & Rosenberg, 1990). UV irradiations were carried out with a calibrated (Spectroline Model DM-254N, Spectronics Corp., Westbury, NY) germicidal UV lamp. The dose rate was 1−2.5 W/m^2. Ionizing irradiations were performed at room temperature or on ice (chromatin decondensation assay) with a Model Mark I ^{137}Cs source (J. L. Shepard and Associates, Glendale, CA) at a dose rate of 2 Gy/min to the indicated total doses. CAT assays were carried out as described (Gorman et al., 1983; Valerie et al., 1988).

Gel Shift Assays. Nuclear extracts of HIVcat/HeLa cells were prepared essentially as described (Stein et al., 1989). Briefly, cells from a confluent 100 mm dish were washed in phosphate-buffered saline (PBS), scraped off the dish, and transferred to a microfuge tube. Cell nuclei were then produced in 100 μL of lysis buffer containing 10 mM Hepes (pH 7.8), 60 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5% NP-40, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mM Na_3VO_4, 1 μg/mL aprotinin, and 1 μg/mL leupeptin on ice for 5 min. After centrifugation (2000 rpm for 5 min), the supernatant was removed, and the nuclei were washed once with 1 mL of lysis buffer lacking NP-40. After centrifugation, the nuclei were resuspended in 50 μL of nuclear extraction buffer containing 250 mM Tris-HCl (pH 7.8), 60 mM KCl, 1 mM DTT, 1 mM PMSF, and 0.5 mM Na_3VO_4 and freeze−thawed three times (−70 to +37 °C). The samples were then centrifuged at 7000 rpm for 15 min; the supernatant constitutes the nuclear extract. Protein concentrations were determined by the BCA copper-chelating assay (Pierce Chemical Co.) using bovine serum albumin as standard. The HIV NF-κB 33-mer (5′-$^{-111}$GCTACAAGGGACTTTCC-GCTGGGGACTTTCCAG^{-79}-3′; only the top strand is shown) (Gimble et al., 1988) was made on a Cyclone DNA synthesizer (Biosearch, Inc.). In the mutant NF-κB oligonucleotide, the GGG nucleotides were replaced with CTC. The consensus AP-1 27-mer (5′-GATCCATGACTCA-GAGGAAAACATACG-3′; only the top strand is shown) (Kouzarides & Ziff, 1989) was made as above for NF-κB. In the mutant AP-1 oligonucleotide, the underlined Ts were replaced with Cs. The oligonucleotides were purified on 20% urea polyacrylamide gels and the complementary strands annealed (Sambrook et al., 1989). The oligonucleotides were labeled with [γ-^{32}P]ATP (7000 Ci/mmol; ICN) using T4 polynucleotide kinase (Sambrook et al., 1989). Labeled oligonucleotide was mixed with 5 μg of nuclear extract in a total of 10 μL of nuclear extraction buffer supplemented with 0.5 μg of poly(dI-dC)−poly(dI-dC) (Pharmacia, Inc.). The reaction mixtures were incubated at room temperature for 20 min prior to electrophoresis on 6% polyacrylamide:Bis (30:1) gels in 0.25 × Tris-borate buffer (Sambrook et al., 1989). The gels were dried and exposed to X-ray film.

RNA Analysis. Total cytoplasmic RNA was extracted as described (Favoloro et al., 1980; Sambrook et al., 1989). Northern analyses were carried out on 1% agarose/2.2 M formaldehyde gels and transferred to GeneScreen (New England Nuclear) membranes by capillary blotting (Sambrook et al., 1989). The RNA was covalently bound to the membrane by UV cross-linking, and the transfer of RNA was confirmed by methylene blue staining (Herrin & Schmidt, 1988). Hybridization probes were gel-purified from low-melting agarose (FMC, Inc.), labeled by random priming (Feinberg & Vogelstein, 1984), and purified over spin columns. The *cat* probe was an ~0.7-kilobase (kb) *Hin*dIII-*Xba*I fragment from pIBI20-cat (Valerie et al., 1988), and the human β-actin probe was a ~2 kb *Bam*HI fragment from pHFAβ1 (Gunning et al., 1983). A ~1 kb human *c-jun* probe was generated by PCR. Membranes were hybridized to [^{32}P]-labeled probes (>10^9 dpm/μg of DNA) in 50% formamide, 1 M NaCl, 1% SDS, 50 mM Na_xPO_4 (pH 7.0), 10 mM EDTA, 10% dextran sulfate, and 100 μg/mL denatured salmon sperm DNA overnight at 45 °C with constant agitation. The membranes were then sequentially washed with 2 × SSC at room temperature (2 × 10 min), 2 × SSC/1% SDS at 65 °C (2 × 30 min), and finally with 0.1 × SSC at room temperature (1 × 15 min) and then exposed to X-ray film with an intensifying screen.

Mitotic Synchronization and Chromosome Decondensation Assay. HIVcat/HeLa cells were synchronized in mitosis with nocadazole (0.2 μg/mL) overnight (~16 h). Mitotic cells were obtained by shakeoff and processed for decondensation assay as described (Mullinger & Johnson, 1985). Briefly, mitotic cells in suspension and in nocadazole were exposed to 0.1 mM 1-β-arabinofuranosylcytosine (araC) and 10 mM hydroxyurea for 30 min prior to irradiation with 10 and 30 J/m^2 of UV, 5 and 20 Gy of ^{137}Cs, or a combination of the two. The cells were centrifuged briefly in a microfuge (2000 rpm for 2 min), and the cell pellet was resuspended in 50 μL of PBS and irradiated with UV (2.5 W/m^2) or IR (2 Gy/min) to the total final dose. The conditioned medium,

including supplements, was added back, and cells were incubated for another 1−2 h at 37 °C. At this time, the cells were centrifuged briefly, and the medium was removed. After hypotonic swelling in 0.25 × Hank's Buffered Salt Solution at 37 °C for 10 min, the cells were centrifuged, fixed in Carnoy's fixative (3:1 v:v of methanol/acetic acid), spread on prewetted glass slides, and stained with a 1:10 dilution of Giemsa. Stained chromosomes were visualized with a standard oil-immersion microscope (Nikon Diaphot) at 400× magnification. Representative chromosome spreads were then photographed. Routinely, mitotic indices of untreated cells were estimated to be >95%.

Micrococcal Nuclease Digestion of Isolated Nuclei. Micrococcal nuclease (MN) digestions of isolated nuclei were performed using a modification of previously described protocols (Smerdon et al., 1978; Walker & Sikorska, 1986). Briefly, cells were metabolically labeled with [*methyl*-^{3}H]-thymidine (78 Ci/mmol; ICN, Riverside, CA) and [^{14}C]amino acids (35 mCi/microatom of carbon; Moravek Biochemicals, Inc., Brea, CA) at 0.27 and 0.013 μCi/mL, respectively, for 48 h, followed by a 24 h chase with conditioned medium. Hydroxyurea and araC were added to the medium to final concentrations of 10 and 0.1 mM, respectively, 30 min prior to irradiation. The inclusion of these inhibitors promotes chromatin decondensation by preventing resealing of DNA breaks (Mullinger & Johnson, 1985). The cells were irradiated with 30 J/m^2 of UV or 10 Gy of IR or left untreated. They were harvested 1 or 2 h later by scraping into digestion buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose. To prepare nuclei, cells were lysed in digestion buffer supplemented with 0.5% Triton X-100 and briefly centrifuged (600*g* for 5 min); nuclei were resuspended in buffer without detergent by vigorous Dounce (B pestle) homogenization and digested with MN (50 000 units/mL; Worthington) at a final concentration of 50 units/mL in the presence of 0.1 mM $CaCl_2$. Aliquots were removed at different times and terminated by the addition of EDTA to 10 mM. Perchloric acid (70%) was added to a final concentration of 7%, and the samples were held on ice for 20 min. Acid-soluble radioactivity was obtained as the supernatant after a 16000*g* centrifugation for 10 min. A portion of the supernatant was diluted with water and counted in liquid scintillation fluid. The acid-soluble DNA [^{3}H] was normalized to protein content [^{14}C] and to the untreated sample of the control group.

RESULTS

Transcription Factors NF-κB and AP-1 Are Activated to a Similar Extent after UV and IR Exposure. Previous work has demonstrated that NF-κB is activated post-translationally by UV (Stein et al., 1989; Devary et al., 1993) and IR (Brach et al., 1991; Uckun et al., 1993) in HeLa and leukemic myeloid and B-cell precursor cells. However, neither of the studies involving IR investigated the effect of NF-κB activation on subsequent expression of responsive genes. Because we have previously reported that IR does not increase HIV gene expression (Valerie et al., 1990), it was of interest to examine whether increased NF-κB binding could be demonstrated in cells that have the bacterial *cat* gene under control of the HIV promoter region stably integrated in the genome. HIVcat/HeLa cells were irradiated with either UV (30 J/m^2) or IR (5 and 10 Gy), and nuclear extracts were prepared after 0.5, 1, and 3 h. Gel retardation

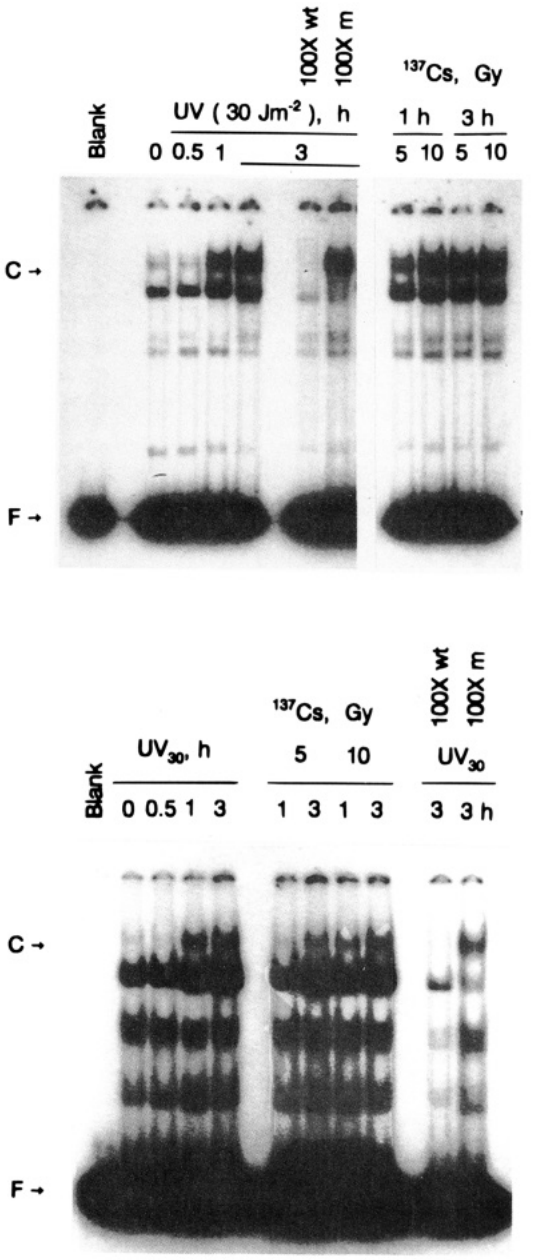

FIGURE 1: Activation of NF-κB and AP-1 after UV and IR exposure. HIVcat/HeLa cells were irradiated with UV (30 J/m^2) or IR (5 and 10 Gy). (Top) Nuclear extracts were prepared at 0.5, 1, and 3 h (UV) and 1 and 3 h (IR) postirradiation and incubated with a [^{32}P]-labeled 33-mer spanning the HIV enhancer region. Incompetition experiments, a 100-fold excess of the unlabeled wild-type (100× wt) or mutant (100× mt) oligonucleotide was included in the binding reactions. After electrophoresis on a 6% polyacrylamide gel, it was exposed to X-ray film. (Bottom) Similarly, the same nuclear extracts were incubated with a radioactive AP-1 oligonucleotide and processed as above: no nuclear extract added (Blank), free oligonucleotide (F), and oligonucleotide complexed with protein (C).

of a [^{32}P]-labeled oligonucleotide identical to the HIV enhancer region was then carried out by polyacrylamide gel electrophoresis (PAGE) followed by autoradiography (Figure 1, top). We found that UV produced a time-dependent increase in NF-κB binding which was first detected at 1 h and became more pronounced at 3 h after irradiation. Similarly, both 5 and 10 Gy of IR resulted in increased binding at 1 h which was less with 5 Gy than with 10 Gy, while at 3 h postirradiation, both doses produced similar levels of binding (Figure 1, top). A 100-fold excess of

unlabeled wild-type NF-κB oligonucleotide effectively competed out the binding, while a mutant oligonucleotide did not, demonstrating specific protein binding to the NF-κB oligonucleotide.

A cellular gene, *c-jun*, responds to both UV (Stein et al., 1989; Devary et al., 1991, 1992) and IR (Hallahan et al., 1991, 1993; Sherman et al., 1990; Uckun et al., 1993) and appears to be autoregulated by the binding of AP-1 to the TRE (Angel & Karin, 1991). Even though the HIVcat/HeLa cell construct was generated in order to facilitate the analysis of DNA damage-induced gene expression, it is, of course, an artificial construct. To correlate the effects of UV and IR on HIVcat transcription with the effects on a cellular gene, we also examined AP-1 binding to a consensus AP-1 oligonucleotide. We found that specific binding to the AP-1 oligonucleotide increased with similar kinetics and to a similar extent after UV and IR (Figure 1, bottom).

Transcription of HIVcat Parallels That of c-jun after Treatment with UV and Phorbol Ester. To investigate the transcriptional effects of UV and phorbol ester (PMA) on the HIVcat/HeLa cells, we first established the time at which maximum steady-state *cat* mRNA accumulates after UV. HIVcat/HeLa cells were irradiated with UV (10−40 J/m^2) and mRNA levels determined by Northern analysis between 1 and 5 h post-UV. We found that the HIVcat mRNA levels peaked between 3 and 5 h postirradiation (Figure 2A). A dose of 30−40 J/m^2 produced the strongest signal for both HIVcat and *c-jun*. Furthermore, PMA and UV produced a synergistic effect on *c-jun* transcriptional activation similar to the combined effect of these two agents on HIVcat transcription (Figure 2A).

IR Fails To Increase HIVcat Transcription and Produces Relatively Small Increases in c-jun mRNA. We have previously shown that doses as large as 14 Gy of IR do not stimulate HIVcat gene expression measured by CAT assays (Valerie & Rosenberg, 1990). To investigate at what level (transcriptional or post-transcriptional) this effect was operating, we irradiated (2, 10, and 30 Gy) the cells and extracted RNA at 1, 3, and 5 h postirradiation. For comparative purposes, cells were also exposed to 10 and 30 J/m^2 of UV. A Northern blot of these RNA samples is shown in Figure 2B. No HIVcat mRNA was detected at any dose or time after IR, whereas UV at 10 and 30 J/m^2 produced significant increases in HIVcat mRNA. Maximum *c-jun* levels were detected 1 h after UV, while IR produced a smaller but still significant increase (~20% of the UV level) at 1 h after 30 Gy (Figure 2B). A much smaller response was observed at 3 and 5 h with both UV and IR, consistent with previous reports (Sherman et al., 1990; Devary et al., 1991; Uckun et al., 1993). A portion of the irradiated cells was taken at 5 h, and in a separate set of reactions at 20 h after irradiation, to determine CAT activity levels after each treatment (data not shown). IR did not give any increase in CAT activity at any dose or time. On the other hand, UV produced approximately 3- and 22-fold increases in CAT activity after 10 and 30 J/m^2, respectively, at 5 h post-UV, and at 20 h, a 26-fold increase was obtained with 15 J/m^2.

These results demonstrate that IR did not produce any increase in HIVcat steady-state mRNA levels with doses and at times that produced increases in NF-κB and AP-1 binding, whereas UV resulted in large increases in both assays. Even though IR at an optimal dose and time produced a significant increase in *c-jun* steady-state mRNA, this increase was substantially lower (~20%) than that after UV. Furthermore, concordant with the lack of HIVcat mRNA accumulation after IR, no increase in CAT activity was detected, suggesting that IR fails to activate HIVcat transcription. Thus, no correlation exists between the extent of NF-κB and AP-1 binding and the expression of the HIVcat and *c-jun* transcription units after IR.

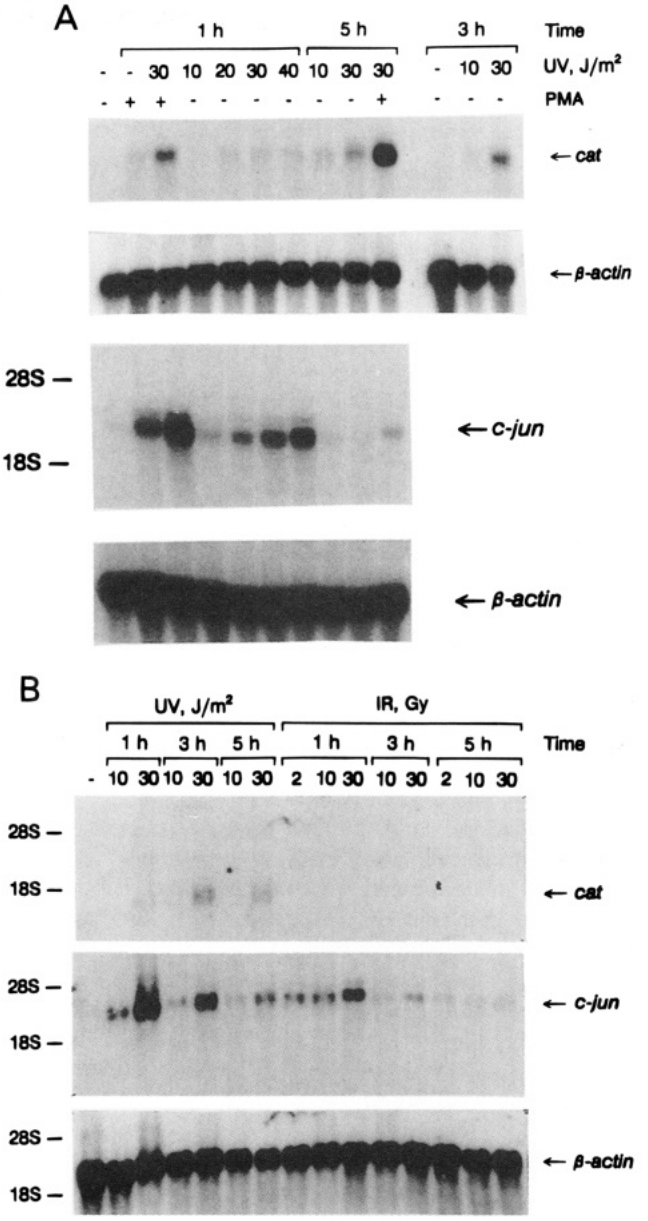

FIGURE 2: IR fails to activate HIVcat transcriptionally and produces relatively small increases in *c-jun* mRNA. (A) UV dose response and time course of HIVcat and *c-jun* transcriptional activation. HIVcat/HeLa cells were treated with PMA (20 nM), UV (10−40 J/m^2), or the combination of UV and PMA. Cytoplasmic RNA was isolated between 1 and 5 h post-treatment and processed for Northern analysis. Hybridization probes were *cat* and *c-jun* with β-actin as control. (B) UV and IR dose response and time course of HIVcat and *c-jun* transcriptional activation.

IR Does Not Suppress Transcription through Any Upstream LTR Promoter Elements. It is conceivable that the reason for the lack of increase in HIVcat transcription after IR is due to suppressive effects at the level of the HIV promoter. Suppressive effects of AP-1 protein complexes

Table 1 [a]

treatment	fold activation: wild-type (−485/+80)	enhancer+ (−119/+80)	enhancer⁻ (−69/+80)
control	1.0	1.0	1.0
UV (15 J/m²)	16.4	19.0	12.0
UV + PMA (20 nM)	57.7 } 3.5×	54.0 } 2.8×	16.0 } 1.3×
1 Gy	1.2	1.3	1.2
5 Gy	1.2	1.1	1.0
10 Gy	1.0	1.0	1.1

[a] IR does not suppress transcription at the level of the HIV LTR. Wild-type, enhancer⁺ (119/+80), and enhancer⁻ (−69/+80) HIVcat/HeLa cells were treated with UV (15 J/m²), IR (1, 5, or 10 Gy), or the combination of UV (15 J/m²) and PMA (20 nM). CAT assays were performed after 20 h. The percent conversion of unacetylated to acetylated [^{14}C]chloramphenicol species was taken as a measure of CAT activity. Fold activation was calculated by division of the percent conversion of extracts from treated cells by the percent conversion of extracts from untreated control cells.

on the expression of other genes have been described previously (Angel & Karin, 1991). Because putative AP-1 binding sites have been identified in the −357 to −316 region of the HIV promoter (Franza et al., 1988) and AP-1 DNA binding increases after IR, one can perhaps envision a situation whereby binding of AP-1 or some other factor to the upstream LTR elements could inhibit HIVcat transcription. In order to address this question, we made use of three cell populations: (1) the wild-type (−485/+80) HIVcat/HeLa cell line, (2) one which has the HIV basal promoter elements and the enhancer (−119/+80), and (3) one which has the basal promoter elements but not the enhancer (−69/+80) upstream of the *cat* gene integrated in their genomes. The putative AP-1 binding sites at position (−357/−316) is present in the wild-type promoter construct but not in the two deletion constructs, and the enhancer is present in the two former but not in the latter.

The different cell populations were treated with IR, UV, or a combination of UV and PMA to examine any differences in how they responded to these treatments (Table 1). The wild-type (−485/+80) cells responded to UV alone (~16-fold) and produced a 3.5-fold synergistic response to UV and PMA, but no significant increase in CAT activity was observed after IR. Similarly, the enhancer⁺ (−119/+80) cells responded to UV (19-fold enhancement) and showed a synergistic response in combination with PMA (2.8-fold above UV levels) but produced no significant increase after IR. The enhancer⁻ (−69/+80) cell population responded well to UV (12-fold enhancement) but did not exhibit synergism with PMA (1.3-fold above UV levels), concordant with previous work showing that the PMA response maps to the enhancer (Nabel & Baltimore, 1987) and the UV response is not associated with any upstream LTR elements (Valerie et al., 1995). In a manner similar to that of the other two cell populations, the enhancer⁻ cell population also failed to respond to IR. All combined, it is unlikely that promoter elements upstream from position −69 in the LTR are involved in suppressing HIVcat expression after IR.

UV, but Not IR, Produces Extensive Decondensation of Chromatin. Because no correlation could be demonstrated between increased DNA binding of NF-κB and AP-1 and transcription of HIVcat and *c-jun*, respectively, it appears that some other mechanism plays a more important role in UV activation of transcription.

We have proposed that the DNA repair process itself may be involved in UV activation of HIVcat gene expression through changes in chromatin structure (Valerie & Rosenberg, 1990). Repair of DNA damage generated by UV and IR involves two different pathways, the nucleotide excision repair (NER) and base excision repair (BER) pathways, respectively (Hoeijmakers, 1993; Lindahl, 1982). DNA incision at base damage is generally accomplished by small DNA glycosylases, whereas repair of bulky UV damage is initiated by a large protein complex which presumably requires changes in chromatin structure in order to gain access to the DNA damage (Smerdon, 1991). It is believed that incision by small DNA glycosylases may not require extensive changes in chromatin structure (Lindahl, 1982). Some twenty years ago, experiments using the premature chromosome condensation (PCC) assay (Johnson & Rao, 1970) demonstrated that UV produces extensive chromatin decondensation in interphase cells after fusion to mitotic cells while IR does not (Waldren & Johnson, 1974; Johnson & Collins, 1978). The same biological effects were observed when mitotic cells were UV irradiated directly without any cell fusion (Mullinger & Johnson, 1985). The cellular mechanism involved in chromatin decondensation is not known, but these and other findings (Smerdon, 1991) are concordant with the idea that NER, but not BER, is associated with a transient decondensation of chromatin. This notion is consistent with our earlier observation that DNA damage repaired by the NER pathway, but not damage most likely repaired by the BER pathway, activates HIVcat gene expression (Valerie & Rosenberg, 1990; Valerie et al., 1994).

To investigate the difference in chromatin structure after UV and IR treatment of HIVcat/HeLa cells, we prepared mitotic cells by overnight synchronization with nocodazole. Mitotic cells were irradiated with either UV (10 and 30 J/m²) or IR (5 and 20 Gy), spread on glass slides at either 1 or 2 h postirradiation, and then stained with Giemsa (Figure 3). We found that, without any treatment (panel A) or after IR (panels B and C), individual chromosomes stained darkly and appeared condensed and relatively unaltered. However, at the larger dose of IR, the chromosomes were considerably fragmented (panel C). In contrast, UV treatment resulted in extensive chromosome decondensation which was more pronounced after the larger dose (compare panels E and F) and more at 2 h than at 1 h after UV (compare panels D and E). No individual chromosomes could be identified 2 h after the larger UV dose (panel F). Instead, the chromosomes appeared in the microscope as one large mass with less intense staining compared to chromosome spreads from untreated cells. The processes through which UV induces these extensive chromatin changes have not been established, but the result is consistent with the notion that changes in chromatin structure may be involved in UV activation of HIVcat gene expression. The fact that IR, which does not activate HIVcat transcription, does not produce these changes in chromatin further supports a role for changes in chromatin structure as a possible mechanism of transcriptional activation by UV.

The UV Effect Overrides the IR Effect at the Level of CAT Activity and Chromatin Decondensation. If increased binding of NF-κB to the HIV enhancer is not sufficient to increase HIVcat gene expression after IR, something else is needed to activate transcription or perhaps override a suppressive effect on transcriptional activation. Suppression could

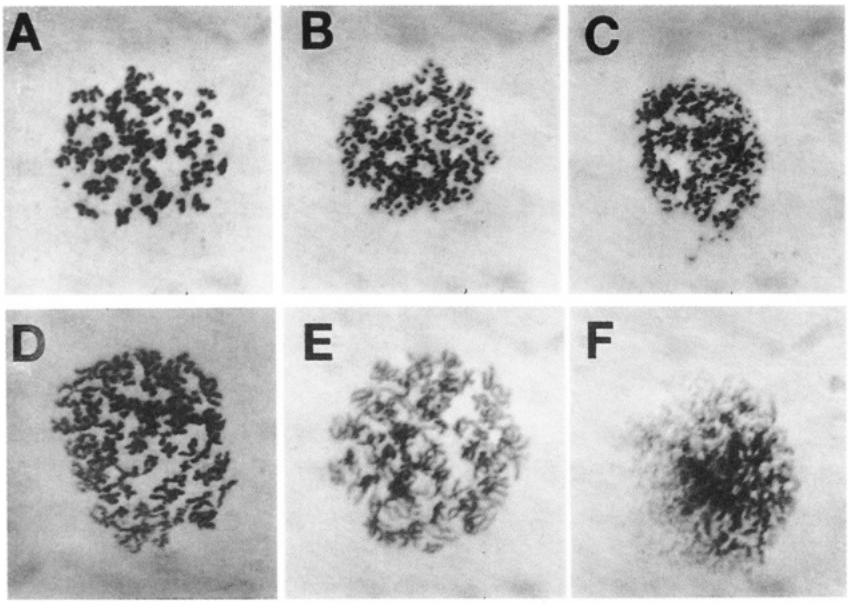

FIGURE 3: UV, but not IR, produces extensive chromatin decondensation. Mitotic HIVcat/HeLa cells were left untreated or irradiated with UV (10 and 30 J/m^2) or IR (5 and 20 Gy). After incubation for 1 or 2 h, chromosomes were spread on glass slides and stained with Giemsa. Stained chromosomes were visualized by phase contrast microscopy (Nikon Diaphot) and photographed: (A) control, no irradiation; (B) 5 Gy, 2 h; (C) 20 Gy, 2 h; (D) 10 J/m^2, 1 h; (E) 10 J/m^2, 2 h; and (F) 30 J/m^2, 2 h.

involve the inhibition of RNA polymerase and/or some basal transcription factor, or perhaps IR does not produce the changes in chromatin structure which are conducive to transcription. To see if UV could override a possible general suppressive effect of IR on HIVcat gene expression and chromatin decondensation, we treated HIVcat/HeLa cells with UV or IR and with a combination of the two. We found that UV acts in a dominant fashion over IR in the chromosome decondensation assay, since treatment with both UV and IR resulted in decondensation indistinguishable from the effect of UV alone (data not shown). Similarly, we found no significant differences in the CAT activity levels with the combination of UV and IR compared to UV treatment alone, irrespective of whether the cells received IR before or after UV (data not shown). These results demonstrate that the positive effect of UV on HIVcat gene expression and chromatin decondensation overrides the lack of effect by IR.

UV, but Not IR, Increases DNA Accessibility to Micrococcal Nuclease. Micrococcal nuclease (MN) preferentially digests internucleosomal DNA, a property which has been used in the past to determine DNA accessibility in nuclei isolated from UV-treated cells (Smerdon et al., 1978). To examine if the global chromatin changes observed in mitotic cells after UV could also be detected in transcriptionally active interphase cells, we irradiated metabolically labeled HIVcat/HeLa cells with UV or IR or left them untreated. Nuclei were isolated 1 and 2 h later using physiological salt concentrations to minimize alterations in chromatin structure (Walker & Sikorska, 1986). The nuclei were then digested with MN for increasing periods of time, followed by precipitation of acid-insoluble material. The digested DNA recovered as the acid-soluble portion was taken as a measure of DNA accessibility and chromatin decondensation. The result of this experiment is shown in Figure 4. UV produced a significant increase in the susceptibility to MN compared to that of untreated cells. IR, on the other hand, did not produce any increases above control levels at either time.

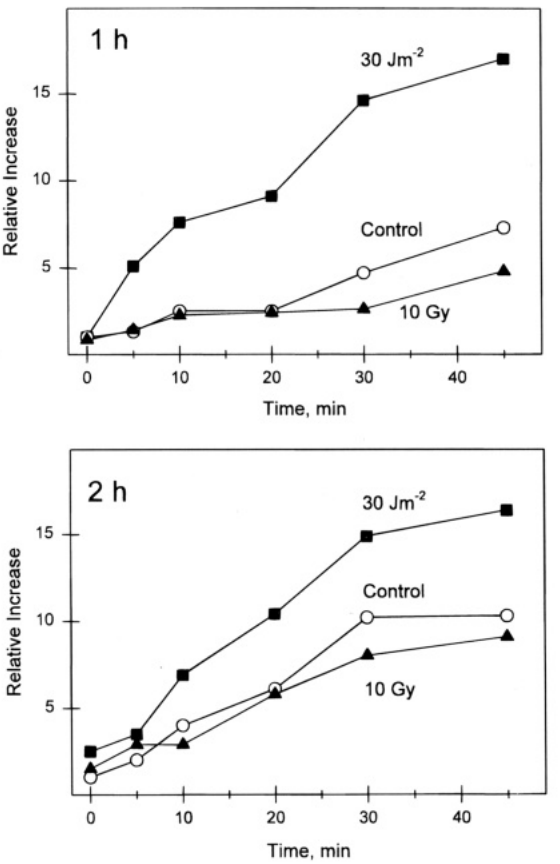

FIGURE 4: Increased susceptibility of DNA to micrococcal nuclease after UV. HIVcat/HeLa cells were metabolically labeled with [*methyl*-^{3}H]thymidine and [^{14}C]-labeled amino acids and irradiated with UV (30 J/m^2) or IR (10 Gy) or left untreated. Nuclei were prepared at 1 h (top) and 2 h (bottom) post-treatment and digested with MN for the indicated times. Acid-soluble [^{3}H] radioactivity normalized to [^{14}C] was taken as a measure of DNA accessibility and chromatin decondensation. All samples were normalized to the untreated (no MN) sample of the control group. The relative level of released acid-soluble counts per minute is shown as a function of MN digestion time.

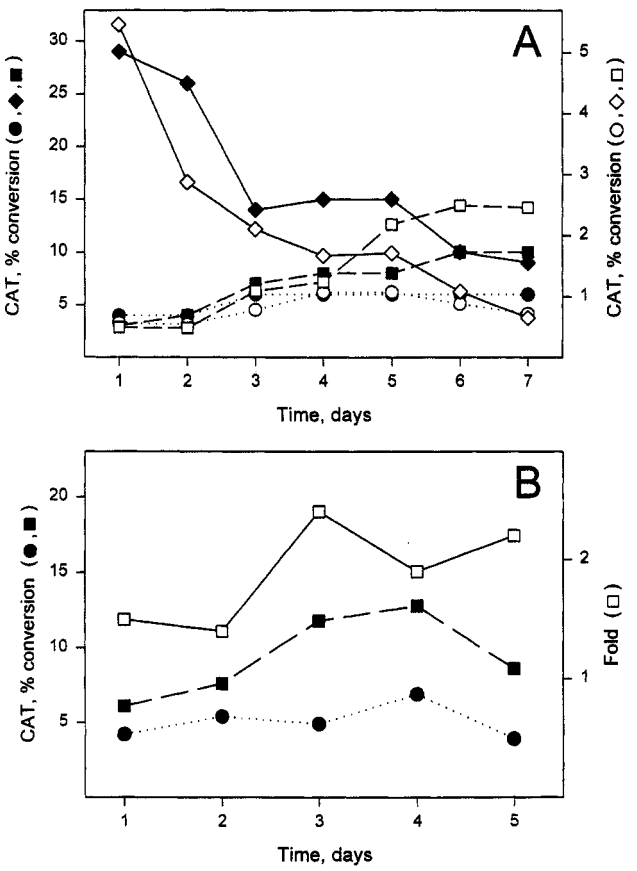

FIGURE 5: IR produces an increase in HIVcat gene expression after several days in culture. (A) HIVcat/HeLa cells were irradiated with 10 J/m^2 of UV or 5 Gy of IR or left untreated. At 24 h postirradiation, and every day thereafter, CAT assays were carried out. Cells received conditioned medium on days 2 and 4 postirradiation. Symbols: untreated (○ and ●), UV (◇ and ◆) and IR (□, and ■). Solid and open symbols represent two separate experiments. (B) HIVcat/HeLa cells were irradiated with 2 Gy of IR or left untreated. At 24 h postirradiation, and every day thereafter, CAT assays were carried out. Symbols: untreated (●), IR (■), and fold activation (□).

This result demonstrates that exposure of cells to UV increases DNA accessibility to MN, which is indicative of chromatin decondensation in interphase cells at times when transcription activation occurs (see Figure 2). IR did not produce any increase in the extent of acid-soluble radioactivity, and the chromatin in these nuclei is therefore not likely decondensed. This result is concordant with the result obtained with mitotic cells (Figure 4).

IR Increases HIVcat Gene Expression after Several Days. Because we did not detect any increases in HIVcat gene expression up to 24 h after IR, we investigated the effects of IR after longer periods of time. A time course experiment was conducted after the cells were exposed to either 10 J/m^2 of UV or 5 Gy of IR. These doses produce similar cell killing (~95%) of the HIVcat/HeLa cells (Beer et al., 1994). Irradiated cells and untreated cells were harvested each day for up to 7 days, and CAT assays were performed (Figure 5). Again, IR did not produce any increase in CAT activity within 24 h postirradiation, whereas UV produced a large increase. However, a reproducible increase in CAT activity which was sustained up to 7 days at 2−3-fold above control levels was observed 3−4 days after IR. The CAT activity obtained from the UV-irradiated cells peaked at 24 h and then declined. To see if the IR effect could be detected at a lower dose with less cell killing, we repeated the above experiment with a dose of 2 Gy (>50% survival; Beer et al., 1994). Again, we observed a 2-fold increase in CAT activity above basal level. These results suggest that at least two different mechanisms, one which occurs early and one which occurs late, regulate HIVcat gene expression after UV and ionizing radiation.

DISCUSSION

During the last few years, it has become evident that chromatin structure and the packaging of promoter sequences by nucleosomes can affect transcription initiation by modulating the accessibility of DNA to RNA polymerase and transcription factors (Wolfe, 1984; Workman & Buchman, 1993). DNA repair, NER in particular, may also be modulated by DNA accessibility; in order for a presumably large repair protein complex to gain access to damaged DNA, a transient decondensation of chromatin is necessary (Smerdon, 1991). We show in the present study that both UV and IR activate NF-κB DNA binding to a similar degree and with similar kinetics, while only the former increases HIVcat gene expression at doses that increase NF-κB binding, suggesting that binding cannot be the determining factor for transcriptional activation. Instead, our results suggest that UV may create alterations in chromatin structure conducive to transcriptional activation. UV can accomplish this efficiently, while IR does not elicit changes in chromatin structure and does not activate HIVcat gene expression (Valerie & Rosenberg, 1990; Beer et al., 1994). This differential transcriptional response is not likely to be attributable to an experimental artifact involving the HIVcat/HeLa cells since an infectious HIV cell clone responds in a similar way (Stanley et al., 1989). Furthermore, monkey CV-1 cell clones stably transfected with an HIVcat construct also show a similar differential response to these types of radiation (Cheng and Valerie, unpublished results). We have shown recently that the HIV enhancer is not important for UV activation of HIVcat gene expression in stably transfected cells and that basal transcription factors are likely to be bound to the HIV promoter prior to UV activation (Valerie et al., 1995). Thus, it is possible that a significant portion of the HIVcat UV response is produced by this complex, mediated through changes in chromatin structure. However, other mechanisms are also likely to be involved in increasing steady-state mRNA after UV, such as increased mRNA stability (Jackman et al., 1994).

Transcription of the cellular *c-jun* gene also differs in its response to these two types of radiation; UV activates transcription more effectively than IR at optimum doses and times. The major UV effector of *c-jun* transcription is the AP-1 transcription factor (Devary et al., 1991). Our investigation suggests that *c-jun* transcriptional activation is unlikely to be the result of increased AP-1 binding to the *c-jun* promoter alone because both UV and IR increase AP-1 binding to a similar extent, whereas UV is at least 5-fold more effective than IR in promoting transcription. Therefore, there is little or no correlation between the activation of either NF-κB or AP-1, as determined by gel shift assay, and the level of HIVcat and *c-jun* expression, respectively. It is interesting to note that PMA and UV increase NF-κB binding to a similar extent and with similar kinetics (Stein et al., 1989; Devary et al., 1993), but UV produces an increase 1 order of magnitude larger in HIVcat gene expression than

does PMA (Valerie et al., 1995). The uncoupling of increased binding of NF-κB to the HIV enhancer from increased gene expression has been reported earlier (Doppler et al., 1992; Mahadevan et al., 1990) and is not specific for DNA damage-induced responses. Our findings suggest that some other mechanism is more important for UV activation of gene expression than the binding of transcription factors to upstream promoter elements when the transcription unit is stably integrated in the genome.

We have shown here that the lack of an effect of IR on HIVcat gene expression does not involve any suppressive factor acting through upstream HIV promoter elements. However, it is possible that IR may cause inhibition of transcription in a more general way. For example, relaxation of torsional stress by IR-induced single-stranded DNA breaks has been reported as one possible way of inhibiting cellular transcription (Villeponteau & Martinson, 1987; Roi & Sauerbier, 1989). This possibility seems unlikely in our study since no significant differences in CAT activity were observed after UV irrespective of irradiation with IR before or after UV. Another possibility for the lack of effect of IR on HIVcat gene expression may be that IR causes post-translational modification of existing proteins, which may inhibit transcriptional activation. For example, poly(ADP)-ribosylation of nuclear proteins is extensive after IR (Cleaver & Morgan, 1991) and appears to modulate also UV activation of HIV gene expression in stably transfected cells (Yamagoe et al., 1991). Interestingly, the modulating effects of poly-(ADP)ribosylation on UV-B-induced *c-fos* expression differ depending upon whether the DNA is episomal or integrated; only expression from the integrated transcription unit is affected by an inhibitor of poly(ADP)ribosylation (Gosh et al., 1993). Regardless of the reason for the inefficiency of IR in activating HIVcat in stably transfected cells, our study demonstrates that the UV effect overrides the effect by IR measured by steady-state mRNA, CAT activity, and changes in chromatin structure.

Chromatin structure appears to be an integral part of HIV (Verdin et al., 1993) and cellular transcriptional activation (Wolfe, 1994). Some cellular genes may be poised for transcription and therefore respond very swiftly to UV through changes in chromatin structure. Because only a fairly limited number of genes are induced by DNA damaging (Holbrook & Fornace, 1991), how could one reconcile global changes in chromatin conformation with activation of specific transcription after DNA damage? One possibility is that genes that are activated by DNA damage have transcription complexes already associated with their promoter regions. This appears to be the case for *c-jun* (Rozek & Pfeiffer, 1993), *c-fos* (Konig, 1991), and HIV (Demarchi et al., 1993), all of which are activated well by UV and other DNA-damaging agents (Holbrook & Fornace, 1991). After UV, a rapid surveillance system may scan the genome for damage (Smerdon, 1991), at which time pre-formed transcription complexes bound to the promoter region of UV-inducible genes may activate transcription. Possible candidates for "surveillance factors" include DNA helicases, which are believed to unwind the DNA helix during the DNA repair process (Hoeijmakers, 1993) and, perhaps simultaneously, produce sufficient structural changes in transcription factor/chromatin complexes to activate transcription of specific genes. Alternatively, increased acetylation of chromatin (Ramanathan & Smerdon, 1989), which is associated with enhanced DNA repair (Smerdon, 1991) and transcription (Reeves et al., 1985), may also be involved.

Though we do not observe any effect of IR on HIVcat gene expression at early times (≤24 h) after irradiation, we can reproducibly identify a response after 3−4 days. The difference in time and extent of activation between the early UV response and the late IR response suggests the involvement of at least two different mechanisms. The reason for the delayed effects of IR on gene expression has not been delineated, but a similar phenomenon has been described after fractionated irradiation of human mammary epithelial cells (Schmidt-Ullrich et al., 1992). Furthermore, HIV is activated in human lymphocytes by low doses of IR after several days in culture (Xu et al., 1995).

This study offers a mechanism for transcriptional activation by UV which may be coupled to other cellular processes induced by DNA damage, such as DNA repair and the accompanying changes in chromatin structure. This transcriptional process may operate independently, in parallel, or together with responses generated at the plasma membrane (Devary et al., 1993) and may only be evident when the transcription unit is stably integrated. The findings made here raise the possibility that, when the transcription unit is stably integrated in the genome, activation of NF-κB may not be indicative of increased HIVcat gene expression after DNA damage.

REFERENCES

Angel, P., & Karin, M. (1991) *Biochim. Biophys. Acta 1072*, 129−157.

Beer, J. Z., Olvey, K. M., Lee, W., & Zmudzka, B. Z. (1994) *Photochem. Photobiol. 59*, 643−647.

Brach, M. A., Hass, R., Sherman, M. L., Gunji, H., Weichselbaum, R., & Kufe, D. (1991) *J. Clin. Invest. 88*, 691−695.

Cavard, C., Zider, A., Vernet, M., Bennoun, M., Saragosti, S., Grimber, G., & Briand, P. (1990) *J. Clin. Invest. 86*, 1369−1374.

Chae, P. H., Jarvis, L. J., & Uckun, F. M. (1993) *Cancer Res. 53*, 447−451.

Cleaver, J. E., & Morgan, W. F. (1991) *Mutat. Res. 257*, 1−18.

Demarchi, F., D'Agaro, P., Falaschi, A., & Giacca, M. (1993) *J. Virol. 67*, 7450−7460.

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

Devary, Y., Gottlieb, R. A., Smeal, T., & Karin, M. (1992) *Cell 71*, 1081−1091.

Devary, Y., Rosette, C., DiDonato, J. A., & Karin, M. (1993) *Science 261*, 1442−1445.

Doppler, C., Schalasta, G., Amtmann, E., & Sauer, G. (1992) *AIDS Res. Hum. Retroviruses 8*, 245−252.

Favoloro, J., Treisman, R., & Kamen, R. (1980) *Methods Enzymol. 65*, 718−749.

Feinberg, A. P., & Vogelstein, B. (1984) *Anal. Biochem. 137*, 266−267.

Franza, B., Rauscher, F., Josephs, S., & Curran, T. (1988) *Science 239*, 1150−1153.

Frucht, D. M., Lamperth, L., Vincenzi, E., Belcher, J. H., & Martin, M. A. (1991) *AIDS Res. Hum. Retroviruses 7*, 729−733.

Fuks, Z., Haimovitz-Friedman, A., Hallahan, D. E., Kufe, D. W., & Weichselbaum, R. R. (1993) *Radiat. Oncol. Invest. 1*, 81−93.

Gimble, J. M., Duh, E., Ostrove, J., Gendelman, H. E., Max, E. E., & Rabson, A. B. (1988) *Mol. Cell. Biol. 62*, 4104−4112.

Gorman, C. M., Moffat, L. F., & Howard, B. H. (1982) *Mol. Cell. Biol. 2*, 1044−1051.

Gosh, R., Amstad, P., & Cerutti, P. (1993) *Mol. Cell. Biol. 13*, 6992−6999.

Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H., & Kedes, L. (1983) *Mol. Cell. Biol. 3*, 787−795.

Hallahan, D. E., Spriggs, D. R., Beckett, M. A., Kufe, D. W., & Weichselbaum, R. R. (1989) *Proc. Natl. Acad. Sci. U.S.A 86*, 1014−1017.

Hallahan, D. E., Sukhatme, V. P., Sherman, M. L., Virudachalam, S., Kufe, D. W., & Weichselbaum, R. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2156−2160.

Hallahan, D. E., Gius, D., Kuchibhotla, J., Sukhatme, V., Kufe, D. W., & Weichselbaum, R. R. (1993) *J. Biol. Chem. 268*, 4903−4907.

Herrin, D. L., & Schmidt, G. W. (1988) *BioTechniques 6*, 196−200.

Hoeijmakers, J. H. J. (1993) *Trends Genet. 9*, 211−217.

Holbrook, N. J., & Fornace, A. J., Jr. (1991) *New Biol. 3*, 825−833.

Jackman, J., Alamo, I., Jr., & Fornace, A. J., Jr. (1994) *Cancer Res. 54*, 5656−5662.

Johnson, R. T., & Rao, P. N. (1970) *Nature (London) 226*, 717−722.

Johnson, R. T., & Collins, A. R. S. (1978) *Biochem. Biophys. Res. Commun. 80*, 361−369.

Konig, H. (1991) *Nucleic Acids Res. 19*, 3607−3611.

Kouzarides, T., & Ziff, E. (1989) *Nature (London) 340*, 568−571.

Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 61−87.

Mahadevan, L. C., Wills, A. J., Hirst, E. A., Rathjen, P. D., & Hearth, J. K. (1990) *Oncogene 5*, 327−335.

Morrey, J. D., Bourn, S. M., Bunch, T. D., Jackson, M. K., Sidwell, R. T., Barrows, L. R., Daynes, R. A., & Rosen, C. (1991) *J. Virol. 65*, 5045−5051.

Mullinger, A. M., & Johnson, R. T. (1985) *J. Cell Sci. 73*, 159−186.

Nabel, G., & Baltimore, D. (1987) *Nature (London) 326*, 711−713.

Ramanathan, B., & Smerdon, M. J. (1989) *Carcinogenesis 7*, 1087−1090.

Reeves, R., Gorman, C., & Howard, B. (1985) *Nucleic Acids Res. 13*, 3599−3615.

Roi, C. P., & Sauerbier, W. J. (1989) *J. Cell. Physiol. 141*, 346−352.

Rozek, D., & Pfeiffer, G. P. (1993) *Mol. Cell. Biol. 13*, 5490−5499.

Sambrook, J., Fritsch, W. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schmidt-Ullrich, R. K., Valerie, K., Chan, W., Wazer, D. E., & Lin, P.-S. (1992) *Int. J. Radiat. Biol. 61*, 405−415.

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

Smerdon, M. J. (1991) *Curr. Opin. Cell Biol. 3*, 422−428.

Smerdon, M. J., Tlsty, T. D., & Lieberman, M. W. (1978) *Biochemistry 17*, 2377−2386.

Stanley, S. K., Folks, T. M., & Fauci, A. S. (1989) *AIDS Res. Hum. Retroviruses 5*, 375−384.

Stein, B., Rahmsdorf, H. J., Steffen, A., Litfin, M., & Herrlich, P. (1989) *Mol. Cell. Biol. 9*, 5169−5181.

Stein, B., Angel, P., van Dam, H., Ponta, H., Herrlich, P., van der Eb, A., & Rahmsdorf, H. J. (1992) *Photochem. Photobiol. 55*, 409−415.

Uckun, F. M., Schieven, G. L., Tuel-Ahlgren, L. M., Dibirdik, I., Myers, D. E., Ledbetter, J. A., & Song, S. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 252−256.

Valerie, K., & Rosenberg, M. (1990) *New Biol. 2*, 712−718.

Valerie, K., Delers, A., Bruck, C., Thiriart, C., Rosenberg, H., Debouck, C., & Rosenberg, M. (1988) *Nature (London) 333*, 78−81.

Valerie, K., Laster, W. S., Kirkham, J. C., & Kuemmerle, N. B. (1994) in *Proceedings of the International Symposium on Charge and Field Effects in Biosystems/4*, pp 520−527, World Scientific Publishing Co. PTE. Ltd.

Valerie, K., Singhal, A., Kirkham, J. C., Laster, W. S., & Rosenberg, M. (1995) *Biochemistry 34*, 15760−15767.

Verdin, E., Paras, P., Jr., & Van Lint, C. (1993) *EMBO J. 12*, 3249−3259.

Villeponteau, B., & Martinson, H. G. (1987) *Mol. Cell. Biol. 7*, 1917−1924.

Vogel, J., Cepeda, M., Tschachler, E., Napolitano, L. A., & Jay, G. (1992) *J. Virol. 66*, 1−5.

Waldren, C. A., & Johnson, R. T. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1137−1141.

Walker, P. R., & Sikorska, M. (1986) *Biochemistry 25*, 3839−3845.

Wallace, B. M., & Lasker, J. S. (1992) *Science 257*, 1211−1212.

Wolfe, A. (1994) *Cell 77*, 13−16.

Workman, J. L., & Buchman, A. R. (1993) *Trends Biochem. Sci. 8*, 90−95.

Xu, Y., Conway, B., Montaner, J. S. G., O'Shaughnessy, & Greenstock, C. L. (1995) *Rev. Med. Virol.* (in press).

Yamagoe, S., Kohda, T., & Oishi, M. (1991) *Mol. Cell. Biol. 11*, 3522−3527.

Zider, A., Mashhour, M., Fergelot, P., Grimber, G., Vernet, M., Hazan, U., Couton, D., Briand, P., & Cavard, C. (1993) *Nucleic Acids Res. 21*, 79−86.

Zmudzka, B. Z., & Beer, J. Z. (1990) *Photochem. Photobiol. 52*, 1153−1162.

Zmudzka, B. Z., Strickland, A. G., Miller, S. A., Valerie, K., Dall'Acqua, F., & Beer, J. Z. (1993) *Photochem. Photobiol. 58*, 226−232.

BI942416T