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Regulation of NF-κB and HIV-1 LTR Activity in Mouse L Cells by Ultraviolet Radiation: LTR *trans*-Activation in a Nonirradiated Genome in Heterokaryons

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A mouse model system for studying the effect of ultraviolet (uv) radiation on reporter gene expression directed by the human immunodeficiency virus type 1 long-terminal repeat (HIV-LTR) has been developed to address the signals required for LTR *trans*-activation in cells with the reporter gene stably integrated into the genome. In a stable mouse L cell clone, L-15, NF-κB DNA binding activity induced by uv-C (254 nm) but not by tumor necrosis factor-α (TNF-α) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) correlated with the stimulation of HIV-LTR-directed chloramphenicol acetyltransferase (CAT) activity; uv-C was more effective than uv-B (312 nm), while uv-A (365 nm) had little effect on CAT activity. Inducers of oxidative stress, such as H_2O_2 treatment up to 200 μM or ionizing radiation up to 20 Gy, also had little effect on CAT expression. Pyrrolidine dithiocarbamate (PDTC) inhibited NF-κB DNA binding and stimulation of CAT activity by uv-C in a dose-dependent manner. Unexpectedly, PDTC induced NF-κB DNA binding that was additive with the response with TNF. In an effort to separate uv irradiation and uv-induced DNA damage from *trans*-activation of the HIV-LTR we devised a heterokaryon system. The fusion of uv-irradiated human fibroblasts with a mouse L cell clone containing the HIV-LTR-directed *lacZ* gene resulted in the activation of lacZ activity that was detected in heterokaryons at the single-cell level. These data suggest that uv-induced DNA damage in the chromosomal DNA containing the reporter gene cannot explain activation of the HIV-LTR. This finding demonstrates LTR *trans*-activation in a nonirradiated genome. © 1997 Academic Press

INTRODUCTION

The response of mammalian cells to ultraviolet (uv) radiation is complex. In addition to causing DNA damage, uv radiation induces lipid peroxidation and the formation of reactive oxygen intermediates (ROIs) that affect many signal transduction pathways and transcription factors [1–4]. The NF-κB subunits p50 (NF-κB_1) and p65 (RelA) are members of the *c-rel* family of transcription factors, which share the highly conserved Rel domain considered to be required for DNA binding and protein interaction [5, 6]. The NF-κB complex in the cytoplasm is released and translocated into the nucleus in response to agents that cause the degradation of IκB-α [7–9]. Ultraviolet, ionizing radiation, H_2O_2, TPA, and TNF are all examples of agents which generate ROIs that act as messengers in the activation of the transcription factor NF-κB [10]. Although studies using antioxidants have shown that NF-κB is an oxidative stress-responsive transcription factor, more recent evidence [11] shows that the response of NF-κB to oxidative stimuli is also cell line dependent.

Previous studies have established that the HIV-LTR is responsive to NF-κB and that mutations in the κB sequence abolish inducibility [12]. Ultraviolet irradiation has also been shown to activate NF-κB and gene expression directed by the HIV-LTR [13–16].

The molecular mechanism by which uv initiates the signaling pathway responsible for the activation of the HIV-LTR is unclear. Although transient transfection experiments have demonstrated that the uv response involves both the AP-1 and NF-κB families of transcription factors [17], in transgenic mice, the LTR deleted for the κB sites was still uv-inducible in epidermal tissue. This suggests that uv-induced changes in the chromatin state may be an additional mechanism involved in gene activation [18]. Recent studies have demonstrated that the activation of the HIV-LTR by uv radiation does not require the enhancer region containing two NF-κB binding sites, but appears to require only an intact basal promoter [19] when stably integrated into the genome. This evidence is consistent with the suggestion that uv irradiation may activate the HIV-LTR by direct uv-mediated DNA damage causing an alteration in chromatin structure [14].

To evaluate directly the role of uv-mediated DNA

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damage in LTR activation we established a reporter system operating in mouse L cells and first characterized the response of the HIV-LTR in this cellular background. Mouse L cells were used because, as shown here, they provided a cellular background that met the stringent requirements for partners in cell-fusion experiments with uv-irradiated human fibroblasts. Cell fusion and the formation of heterokaryons provided an assay system to show that uv-induced DNA damage in the chromosome containing the reporter gene cannot explain activation of the HIV-LTR. This finding demonstrates LTR *trans*-activation in a nonirradiated genome.

MATERIALS AND METHODS

Chemicals and reagents. The chemicals 12-*O*-tetra-decanoylphorbol-13-acetate (TPA), pyrrolidine dithiocarbamate (PDTC), and mitomycin C (MMC) were obtained from Sigma (St. Louis, MO). The double-stranded DNA dI-dC and bovine serum albumin (BSA) were from Pharmacia. TNF-α was from Cellular Products Inc. (Buffalo, NY). IL-1α was a gift from Hoffmann–La Roche.

Transfection of cells and culture conditions. Human MRC5 and mouse L929 fibroblasts were obtained from H. Blau's laboratory (Stanford University). Cells were cultured in BME supplemented with 10% fetal calf serum (FCS). Mouse L cells were stably transfected by the calcium/phosphate or the polybrene/dimethyl sulfoxide (DMSO) procedure [20] with pHIVCAT or pHIVlacZ with pSV2neo and selected for G418 (800 μg/ml) resistance. Individual colonies were isolated by using cloning cylinders. After expansion, clones with uv-inducible CAT activity were identified by preparing cell extracts for CAT assay [21] 24 h after uv irradiation. Clones with uv-inducible lacZ activity were identified by X-gal staining 24 h after uv irradiation.

Human MRC5 or mouse L929 fibroblasts were transiently transfected by the polybrene/DMSO procedure [20]. The cells were incubated with the polybrene/DNA mixture (10 μg of plasmid per 60-mm dish) for 8 h, then were DMSO shocked [25% for mouse L929 cells and 30% for human MRC5 cells in BMEo (without serum)] for 1 min. After the cultures were gently washed, fresh BME_{10} (supplemented with 10% FBS) was added. After a 16-h overnight incubation, the cultures were uv irradiated (30 J/m^2). Cultures were harvested 48 h later for CAT assay. In cotransfection experiments with the pcDEBtat [22] expression vector, pUC19 plasmid was used as nonspecific DNA to allow for equal amounts of the DNA added per culture. All experiments were performed in duplicate, and the results were verified in multiple experiments.

Ultraviolet irradiation. For uv-C irradiation, cells were irradiated directly in dishes by removing the medium and exposing the cells to a dose of 30 J/m^2 with a 254-nm germicidal lamp. The removed medium was replaced after irradiation. The uv dose was determined by using a radiometer (Model IL1400A, International Light, Newburyport, MA); uv-B or uv-A irradiation was performed using a Model 1840 Stratalinker uv cross-linker (Stratagene, La Jolla, CA) with 312- or 365-nm lamps, respectively. The power output generated by this device was measured by an internal photodetector and the dose generated displayed in units that are reported here in mJ. For uv-B irradiation, cells were irradiated directly in dishes after removing the medium. For uv-A irradiation, cells were rinsed, and then irradiated in phosphate-buffered saline (PBS).

Formation of heterokaryons. MRC5 fibroblasts were grown in BME containing 10% FBS. As a control, transient transfection with the HIV-LTR-directed CAT construct verified that the human MRC5 fibroblasts used for the heterokaryon studies were responsive to uv-C. Cells in 1.5 ml of medium were added to 35-mm tissue culture dishes (Corning) 1 day prior to Day 0 [defined as the day of polyethylene glycol (PEG) fusion]. Because the cell density required for these studies was found to be critical, dishes were set up at 0.75 and 1.5 $\times$ 10^6 cells per dish. Dishes with the appropriate density (not sparse, but with little cell–cell contact) were identified and used. After the human cells were mock- or uv-irradiated (30 J/m^2), mouse L-23 cells (stable clone with pHIVlacZ) were added (1 $\times$ 10^6 cells in 1 ml) and allowed to attach for 1 h, and then the cultures were exposed to a 50% solution of PEG 1000 (BDH Chemicals Ltd, Poole, England) prepared as described [23]. After PEG fusion, cultures were maintained in a mitogen-poor medium composed of BME containing 1% FCS to reduce nuclear fusion and the formation of hybrids. Twenty-four hours after fusion, cultures were fixed in PBS containing 3.7% formaldehyde for 5 min and stained with X-gal to visualize lacZ activity histochemically. Controls consisted of nonirradiated MRC5 cells fused with mouse L-23 cells and uv-irradiated MRC5 fibroblasts cocultured (no PEG treatment) with mouse L-23 cells; the latter allowed us to control for the possibility of an effect by a putative uv-induced extracellular factor. Once dishes were evaluated for the number of X-gal positive cells, they were stained with 0.5 μg/ml Hoechst 33258 (which preferentially stains adenine- and thymine-rich regions of DNA found in the centromeres of mouse but not human chromosomes—mouse nuclei appear punctate and human nuclei are uniformly stained [24]). Cells were photographed by using a Zeiss Axioskop microscope with a Zeiss 25$\times$ multiimmersion objective with water immersion to allow the simultaneous detection of Hoeschst fluorescence and X-gal staining.

Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared from 10^7 cells by a rapid procedure [25]. The 3′ κB sequence from the HIV-1 LTR (5′-GATCCAGAGGGGACTTTCCAAGAGG-3′; κB site underlined) was used for the electrophoretic mobility shift assay (EMSA). Oligonucleotides were annealed with the complementary strand to create the 5′-overhanging ends (*Bam*HI), which allowed labeling by Klenow polymerase in the presence of dNTPs and with [^{32}P]dCTP (3000 Ci/mmol; Amersham) for EMSA. The labeled oligonucleotide was purified on push columns (Stratagene). The typical binding reaction of 20 μl contained 10,000 cpm of ^{32}P-labeled double-stranded oligonucleotide, 10 μg of nuclear extract in buffer C (20 m*M* Hepes-KOH, pH 7.9, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.2 m*M* PMSF, 25% glycerol), 20 μg BSA, and 2 μg poly dI-dC in gel shift-binding buffer. This resulted in final concentrations of 15 m*M* Hepes-KOH, 105 m*M* NaCl, 0.4 m*M* $MgCl_2$, 0.1% NP-40, 1.3 m*M* DTT, 0.05 m*M* PMSF, 1 mg/ml BSA, 0.1 mg/ml poly dI-dC, and 6.25% glycerol. Poly dI-dC was used to eliminate nonspecific binding. Specific binding was demonstrated by competition with a 100- to 200-fold excess of unlabeled double-stranded oligonucleotide competitor. After incubation for 20 min at room temperature, samples were analyzed on a 4% native acrylamide gel run at 170 V for 1.5 h in 0.5$\times$ TBE buffer. Gels were dried and visualized by autoradiography.

Plasmids. The construction and characterization of the pHIVCAT and pcDEBtat plasmids have been described [22]. The pHIVCAT plasmid containing mutated κB sites was obtained from G. Nabel (University of Michigan) [12]. The pHIVlacZ plasmid was obtained from J. Maio (Albert Einstein College of Medicine) [26].

X-gal histochemical staining. Cells were fixed at room temperature for 5 min in PBS containing 3.7% formaldehyde. After rinsing with PBS, cells were stained for lacZ expression by incubation of cells in PBS containing 5 m*M* potassium ferrocyanide, 5 m*M* potassium ferricyanide, 2 m*M* $MgCl_2$, and 500 μg/ml X-gal for 2 to 24 h at 37°C in a non-CO_2 incubator.

Detection of lacZ expression by flow cytometry. L-23 cells were stained with the fluorogenic substrate fluorescein-di-galactoside (FDG from Molecular Probes, Inc., Eugene, OR). A 100-μl aliquot containing 10 $\times$ 10^6 cells/ml was added to a 5-ml polystyrene tube which was then placed in a 37°C water bath for 5 min; 100 μl of prewarmed 2 m*M* FDG diluted in water was added, and the sample was mixed gently and placed in a 37°C water bath for 1 min. The

tube was then placed on ice and 1.8 ml of ice-cold medium was added with or without 1 μg/ml of propidium iodide (PI), added to stain the dead cells. Data were collected on 10,000 cells by using a live cell gate (PI-negative staining cells) with a Coulter Elite flow cytometer and plotted as log fluorescence units (as an index of lacZ activity) versus cell number. Parental (nontransfected) and nonirradiated L-23 cells served as controls for the basal level of lacZ expression.

RESULTS

HIV-LTR Activity Is Poorly Responsive to Ultraviolet-C Irradiation in Transient Transfections of Mouse L Cells

Our attempts to use transient transfections to introduce the HIV-LTR-directed CAT reporter gene into mouse L cells in order to evaluate the uv-C response were not successful. This failure was not due to the transfection procedure used. Extracts prepared from cells 24 h after uv-C irradiation (30 J/m^2) showed little response to uv-C regardless of transfection by calcium phosphate precipitation, polybrene/DMSO, or electroporation (data not shown). Because we were unable to demonstrate a significant response with uv-C in mouse L cells by using transient transfections, we next evaluated the possibility that stable integration of the reporter gene may be required in this cellular background.

Discordance between NF-κB DNA Binding and HIV-LTR Activity in Stable Clones of Mouse L Cells

Mouse L cells were transfected with a 1:5 ratio of pSV2neo and pHIVCAT and G418 resistant clones were selected. Of 30 clones only 10% were found to contain inducible CAT activity after uv-C irradiation. This number of clones with an inducible phenotype is consistent with results from other studies of similar design [27].

Three L cell clones were next treated for 24 h with various agents, and CAT activity was measured. All clones responded strongly to uv-C, less to MMC, and little to TNF or TPA (Fig. 1A). For example, in clone L-15, uv and MMC induced CAT activity 43- and 13-fold, respectively. The clone designated L-15 was chosen for further study. Although TNF treatment failed to stimulate CAT activity, TNF induced NF-κB DNA-binding activity in a dose- (data not shown) and time-dependent manner (Fig. 1C). TNF was more potent than uv-C (Fig. 1B) in activating NF-κB (Figs. 1B, 1C) in nuclear extracts prepared at various times posttreatment. Nuclear and CAT extracts were prepared from L-15 cells 24 h after TNF, TPA, or uv-C treatment. All agents activated NF-κB DNA binding activity (data not shown), but only uv-C treatment increased the level of CAT gene expression.

Induction of HIV-LTR-Directed CAT Activity Is Dose-Dependent by Ultraviolet-C and -B but Is Unresponsive to Ultraviolet-A Irradiation or Oxidative Stress Induced by H_2O_2 or Ionizing Radiation

L-15 cells were irradiated with uv-C (254 nm), uv-B (312 nm), or uv-A (365 nm) radiation and the effect on HIV-LTR-directed CAT activity was measured in cell extracts 24 h after exposure. HIV-LTR-directed CAT activity was strongly induced by 15 J/m^2 and increasing doses of uv-C (Fig. 2A). The time course of the uv response showed a slight increase in CAT activity at 10 h which increased and reached an apparent maximum, measured at 24–48 hr postirradiation (Fig. 2B). CAT activity increased in a dose-dependent manner, measured at 24 h postirradiation, with uv-C (Fig. 2A) or uv-B (Fig. 2C), but uv-A (Fig. 2C) exposure had little effect. The dependence of the HIV-LTR response on wavelength is in agreement with another study that examined the action spectrum for uv-induced DNA damage in human cells [15]. Ultraviolet-A radiation, which is rather ineffective in damaging DNA but is known to cause oxidative stress, was also ineffective in stimulating CAT activity. This result, combined with the lack of response with TPA and TNF, suggested that the HIV-LTR may be unresponsive to oxidative stress in the mouse L929 cellular background.

H_2O_2 and ionizing radiation are well-known mediators of oxidative stress. L-15 cells were treated with increasing doses of H_2O_2 or ionizing radiation, and NF-κB DNA binding and HIV-LTR-directed CAT activity were determined 24 h after treatment. No stimulatory effect on the level of NF-κB was seen when these samples were compared to 24-h samples irradiated with uv; interestingly, the gel-shift analysis demonstrated an increase in the level of the lower band (p50 subunit) that was dependent on the dose of H_2O_2 or ionizing radiation. As shown in Fig. 3, increasing H_2O_2 to 200 μ*M* or ionizing radiation to 20 Gy had little effect on the level of CAT activity. In other studies with the human promonocytic cell line U937, increasing H_2O_2 to 200 μ*M* or ionizing radiation to 20 Gy activated NF-κB but not HIV-LTR activity (Miller, unpublished observations). The significance of these results is that an oxidative stress-generated signal was not sufficient for stimulating HIV-LTR activity in the mouse L cell background.

The Antioxidant PDTC Inhibits the Effect of Ultraviolet-C on HIV-LTR Activity and Stimulates NF-κB Activity That Is Additive with NF-κB Induced by TNF

To evaluate the role of oxidative stress and ROIs in the response to uv irradiation, we used the antioxidant PDTC which has been shown to inhibit the degradation of IκB-α [28]. We examined the effect of increasing PDTC concentration on NF-κB- and HIV-LTR-directed

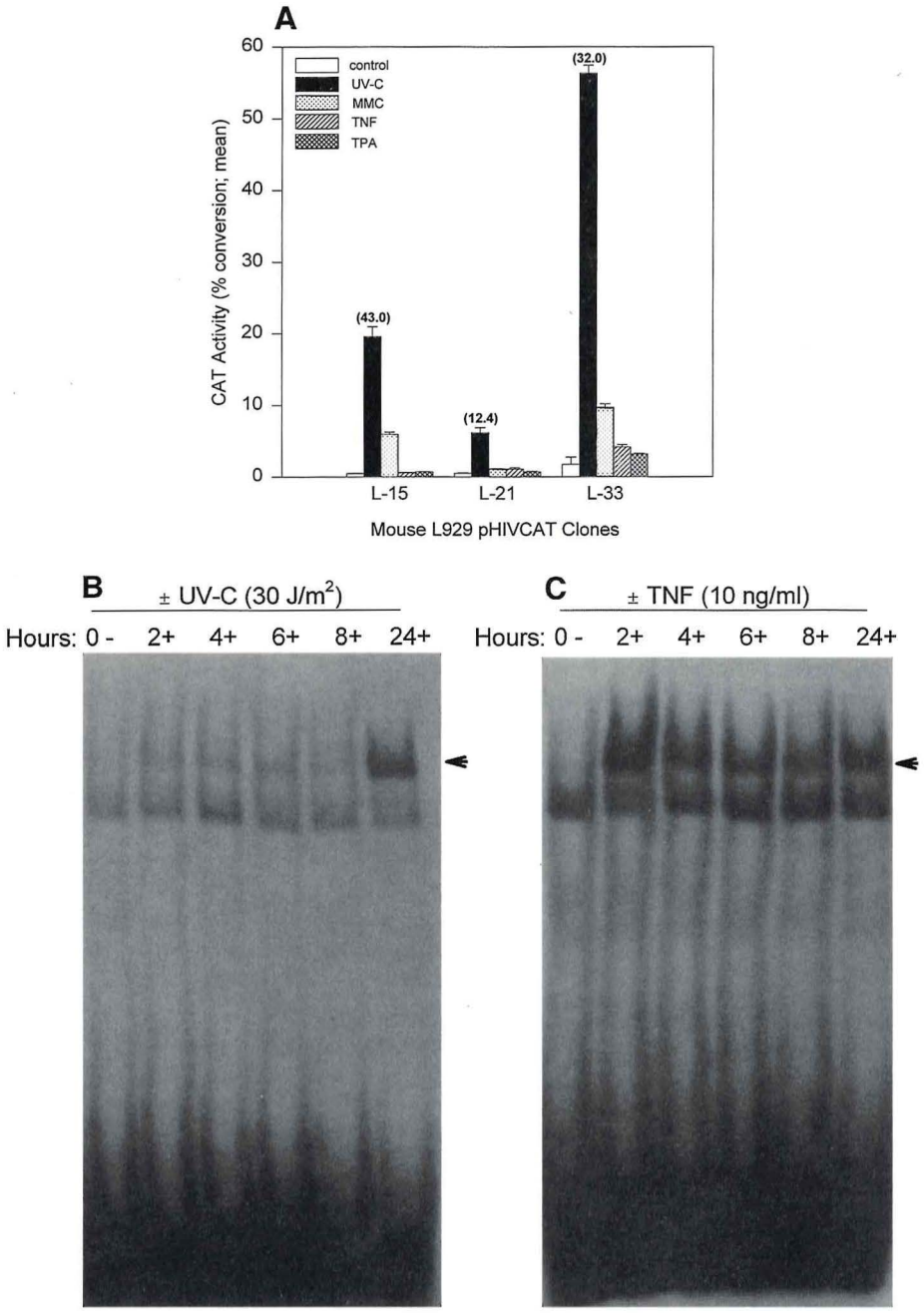

FIG. 1. (A) Regulation of HIV-LTR-directed CAT activity in three stable clones of mouse L929 cells. Cells were treated with uv-C irradiation (30 J/m^2) or no irradiation (control), or with MMC (20 μg/ml), TNF (100 ng/ml), or TPA (100 ng/ml). Equal amounts of protein were assayed for CAT activity 24 h after treatment. The CAT activity results are expressed as the percentage conversion (mean of duplicate samples; error bars indicate sample standard deviations). The numbers in parentheses represent the fold increase in the uv-C-treated samples relative to the control sample. (B) Electrophoretic mobility-shift assay (EMSA) of NF-κB DNA-binding activity (arrow indicates the position of the inducible NF-κB DNA complex) in nuclear extracts prepared from clone L-15 cells at various times after uv-C irradiation (30 J/m^2) (B) or incubated for the indicated times with 10 ng/ml TNF (C).

CAT activity in L-15 extracts prepared 24 h postirradiation. As shown in Figs. 4A and 4B, PDTC inhibited the stimulation of CAT activity by uv-C in a dose-dependent manner. Unexpectedly, PDTC on its own induced NF-κB DNA binding activity (Figs. 4C and 4D), without altering the basal level of CAT activity. Significantly, PDTC inhibited the stimulation of NF-κB DNA binding activity (Figs. 4C and 4D) induced by uv-C treatment to the level observed with PDTC alone. This decrease in NF-κB DNA binding activity corre-

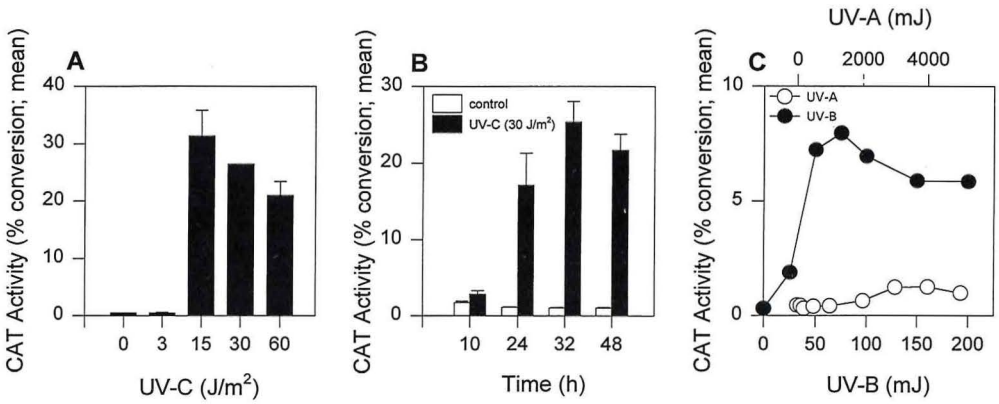

FIG. 2. (A) Dose of uv-C irradiation required for the activation of HIV-LTR-directed CAT activity in L-15 cells. Duplicate cultures were uv-C-irradiated at the indicated doses and CAT activity was determined 24 h postirradiation. The results represent the mean of duplicate samples; error bars indicate sample standard deviations. (B) Time course of CAT activity in uv-irradiated (30 J/m^2) and nonirradiated cultures. The results represent the mean of duplicate samples, measured at various times postirradiation; error bars indicate sample standard deviations. (C) Induction of HIV-LTR activity in L-15 cells by irradiation is dose-dependent for uv-B, but not for uv-A. L-15 cells were irradiated with uv-B (312 nm) or uv-A (365 nm) light for increasing amounts of time to give the indicated dose as determined by an internal photodetector in the Stratalinker uv cross-linker used for these studies. After a 24-h incubation, CAT activity was determined in duplicate cultures. The results represent the mean.

lated directly with changes in HIV-LTR activity (Figs. 4A and 4B).

In an effort to characterize the response of NF-κB to PDTC, we next evaluated the effect of PDTC on the activation of NF-κB by TNF. PDTC was added for 1 h before the addition of TNF. Nuclear extracts were prepared 1 h post-TNF treatment and analyzed for NF-κB activity by EMSA. The results shown in Figs. 4E and 4F show that PDTC induced NF-κB DNA binding activity which in a dose-dependent manner enhanced the level of NF-κB induced by TNF in an additive fashion. Similar results were obtained with samples prepared 24 h post-TNF treatment (data not shown). These results are consistent with recent evidence that PDTC has oxidant activity [29].

Mouse L Cells Are Unresponsive to Extracellular Factor Produced by Ultraviolet-Irradiated Human MRC5 Fibroblasts

A cell fusion strategy to form heterokaryons was developed to investigate the ability to separate uv-irradiation of human cells from the response of the HIV-LTR stably integrated in mouse cells. Because a uv-inducible extracellular factor [16, 30, 31] has been shown to stimulate HIV-LTR-directed gene expression by activating NF-κB [16] and the presence of an extracellular factor secreted from uv-irradiated human fibroblasts acting on mouse L cells would prevent the use of heterokaryons, we used multiple approaches to evaluate whether such a factor would stimulate HIV-LTR activity in L-15 cells. First, we examined whether the conditioned medium from uv-irradiated human MRC5 fibroblast cells could activate HIV-LTR-directed CAT activity in L-15 cells. MRC5 cells were irradiated with 30 J/m^2 of uv, the conditioned medium was collected after 48 h and added to L-15 cells, and CAT activity was measured 24 h later. The uv-conditioned medium had no effect on the level of HIV-LTR-directed CAT activity or on NF-κB DNA-binding activity in mouse L-15 cells (data not shown). Second, experiments with uv-irradiated human MRC5 fibroblasts cocultured with L-15 cells also failed to demonstrate an induction of CAT activity (results not shown). Thus, the L cell model system was uniquely appropriate for heterokaryon studies with uv-irradiated human cells. HIV-LTR activity in L-15 cells was not regulated by cell–cell contact, or by exposure to a uv-induced extracellular mediator.

Detection of Ultraviolet-Induced HIV-LTR-Directed lacZ Reporter Gene Expression at the Single-Cell Level

To detect reporter gene activity at the single-cell level, mouse L929 cells were transfected with pHIV-lacZ (HIV-LTR driving the *lacZ* reporter gene). Of 70 stable clones selected for G418 resistance, only seven clones were identified as uv-C responsive by X-gal staining. LacZ activity was undetectable by X-gal staining in the absence of uv irradiation. Similar studies, done in parallel with human HT1080 fibrosarcoma stable clones, resulted in a much higher baseline lacZ activity by X-gal staining in human cells when compared to that in the mouse cellular background (Miller, unpublished observations). Unexpectedly, all uv-inducible mouse clones were heterogeneous in lacZ expression when evaluated by X-gal staining 24 h after uv-C irradiation. One uv-inducible mouse clone, designated L-23, was chosen for

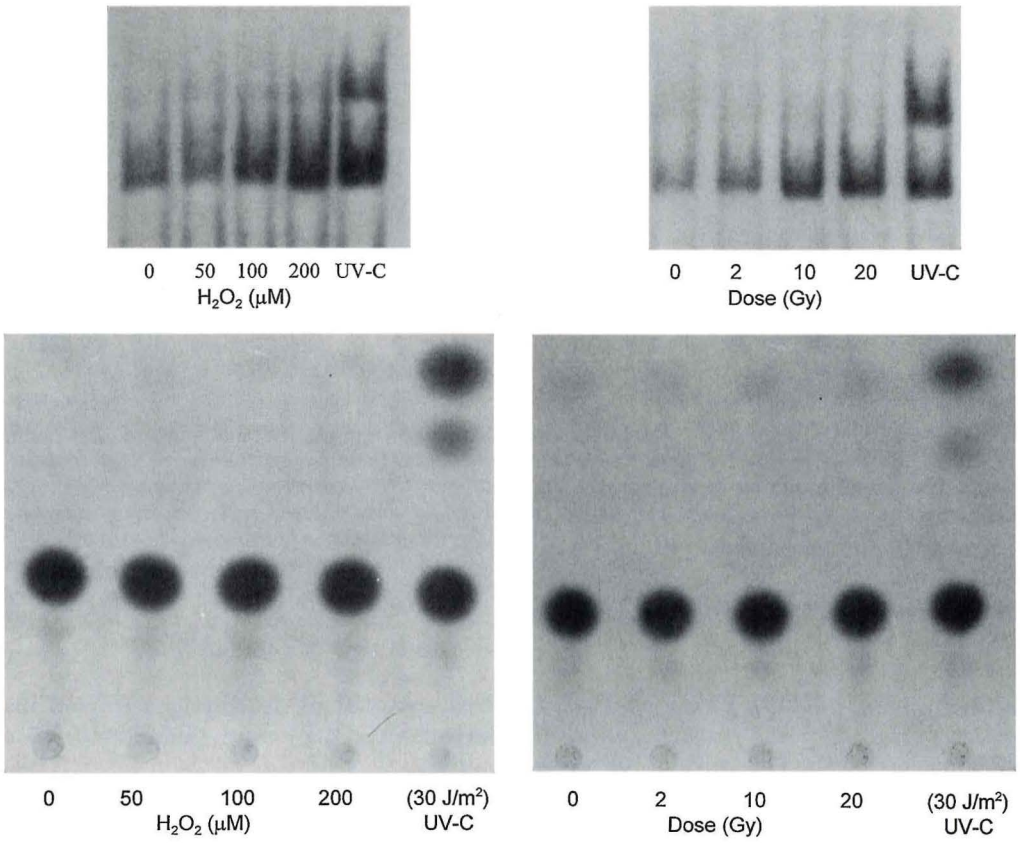

FIG. 3. HIV-LTR activity in L-15 cells is unresponsive to oxidative stress induced by H_2O_2 or ionizing radiation. (left) L-15 cells were treated with 0, 50, 100, or 200 μM H_2O_2 and NF-κB (top), and CAT activity (bottom) was measured 24 h after treatment. (right) L-15 cells were exposed to ionizing radiation at 0, 2, 10, or 20 Gy, and NF-κB (top) and CAT activity (bottom) were measured 24 h after irradiation. In parallel, one set of cells was uv-C-irradiated (30 J/m^2).

further study. When cultures were uv irradiated, only some cells responded with lacZ expression that was detectable by X-gal staining 24 h postirradiation. Cells were plated at clonal density to examine further the heterogeneity in uv-inducible lacZ activity. Single cells were identified, allowed to form small colonies before uv irradiation, and then stained for lacZ expression 24 h postirradiation. Clones derived from single cells also showed the heterogeneity in lacZ activity. An example of this heterogeneity is shown in Fig. 5A. One clone had 32 cells, but only 16 (50%) expressed lacZ after uv irradiation at a level detectable by X-gal staining (Fig. 5A; solid arrows point to the lacZ positives and open arrows point to negatives; Fig. 5B shows the bright-field image to emphasize the X-gal staining).

Because the detection of lacZ activity by X-gal staining is limited by its sensitivity, we used a flow cytometric fluorescence procedure (FACS-FDG assay), which is a more sensitive method for measuring lacZ activity in single cells [32]. L-23 cells were uv irradiated and assayed for lacZ expression by flow cytometry. Parental L929 and nonirradiated L-23 cells were used as controls. The results demonstrated that the X-gal and FACS-FDG assays (Fig. 5C) defined approximately the same amount of lacZ expression detectable at the clonal (Figs. 5A and 5B) or population (Fig. 5C) level after uv irradiation. The bimodal expression of lacZ activity by X-gal staining has been commonly observed by different investigators [32–34]. Most importantly, HIV-LTR activity in L-23 was similar to that found with L-15 cells, i.e., not regulated by cell–cell contact, or by exposure to a uv-induced extracellular mediator (data not shown). Thus, L-23 cells provided a cellular background that met the stringent requirements for partners in cell-fusion experiments with uv-irradiated human fibroblasts.

HIV-LTR Activity Is Activated by trans-Acting Factors in Heterokaryons Formed from the Fusion of Ultraviolet-Irradiated Human Cells with Mouse L Cells

We next examined the ability of uv-irradiated human MRC5 fibroblasts to activate lacZ expression after cell fusion with nonirradiated mouse clone L-23 (containing

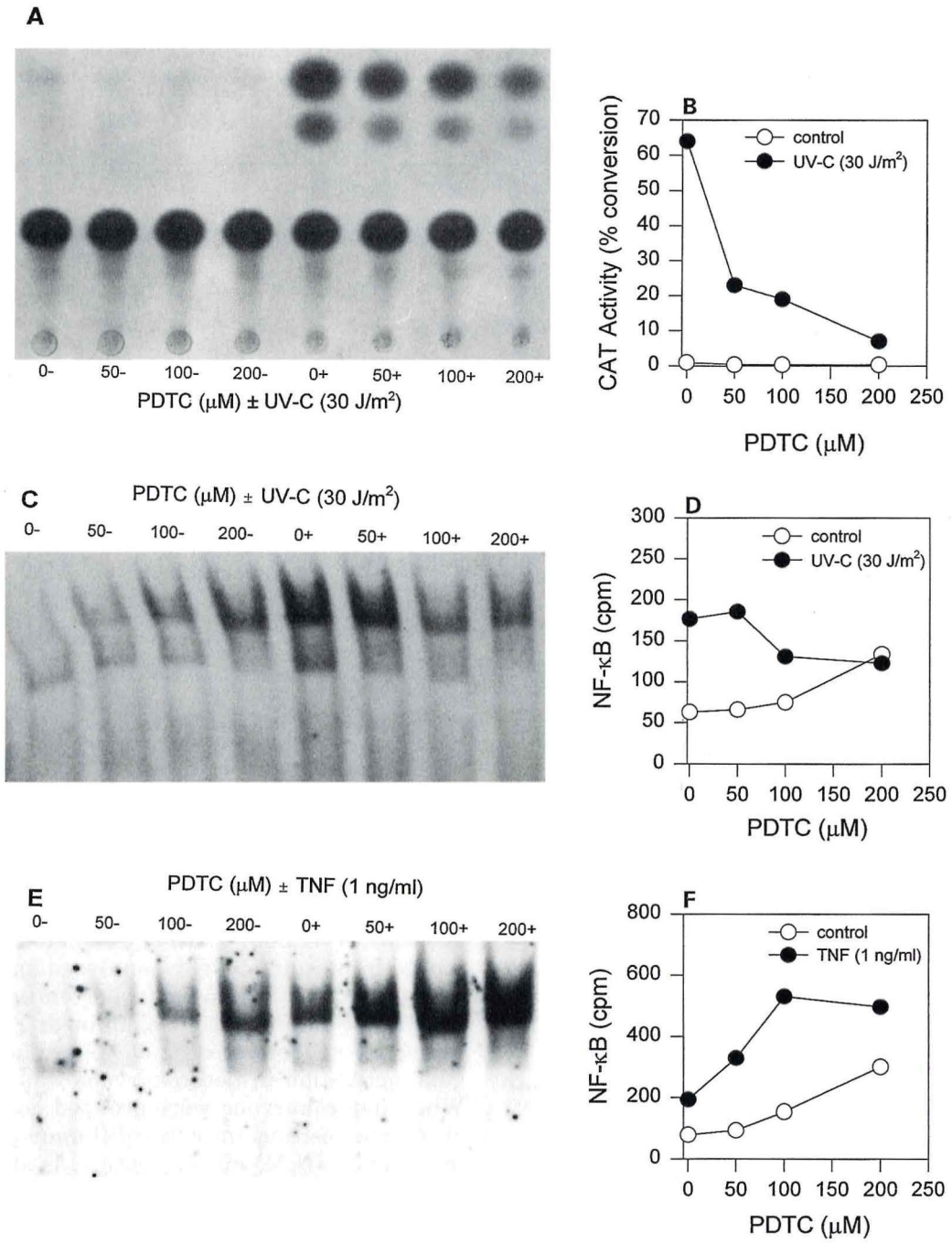

FIG. 4. The dithiocarbamate PDTC defines multiple sites for regulation of NF-κB and HIV-LTR activity. PDTC inhibits HIV-LTR activity (A, B) and NF-κB (C, D) induced by uv-C. L-15 cells were pretreated with PDTC (the numbers shown represent the PDTC concentration ± treatment) for 1 h, the cells were uv-C-irradiated, and NF-κB- and HIV-LTR-directed CAT activity was measured 24 h postirradiation. The upper NF-κB band was cut out and counted by liquid scintillation counting. (E, F) PDTC stimulates NF-κB activity that is additive with NF-κB induced by TNF. L-15 cells were pretreated with PDTC (the numbers shown represent the PDTC concentration ± treatment) for 1 h and TNF (1 ng/ml) was added. After 1 h, nuclear extracts were prepared and NF-κB DNA binding activity was measured by EMSA. The upper NF-κB band was cut out and counted by liquid scintillation counting. The results shown are representative of multiple experiments showing a similar pattern of results.

the HIV-LTR-directed *lacZ* gene). The human cells were uv irradiated and mouse clone L-23 cells were added directly to the human MRC5 cells and allowed to attach for 1 h, and then the mouse and human cells were fused by PEG treatment. The controls for these experiments are described in Table 1. All cultures were fixed and stained with X-gal 24 h after PEG fusion. The results (Table 1) demonstrated the induction of

lacZ activity only in heterokaryons formed between mouse L-23 cells and uv-irradiated human MRC5 cells. Examples of heterokaryons in two different fields are shown in Figs. 6A–6F. X-gal staining followed by staining with the fluorescent DNA dye Hoechst 33258 demonstrated that cells staining positive by X-gal could be identified as heterokaryons by their nuclear staining pattern (Figs. 6B and 6E; mouse nuclei appear punctate and human nuclei stain uniformly). LacZ expression as detected by X-gal staining was not activated in all heterokaryons (solid arrows point to heterokaryons staining lacZ positive and open arrows to examples of heterokaryons staining negative for lacZ expression). Thus, heterokaryons activated lacZ expression in a bimodal manner similar to that found with uv-irradiated mouse L-23 cells (i.e., not all cells responded).

To ascertain the requirements for gene activation in this heterokaryon system, the results were analyzed in three ways. First, the fields shown in Fig. 6 were analyzed for the total number of human or mouse nuclei, the number of human and mouse nuclei contained in heterokaryons, and the number of heterokaryons scored as X-gal positive (Table 2). In these two fields 16–21% of the mouse and 25–32% of the human nuclei scored were in heterokaryons. Second, to determine the percentage of heterokaryons that were X-gal positive, 104 heterokaryons were scored from random fields, analyzed for nuclear composition, and tabulated as a percentage of the total number of heterokaryons examined. Thus, as determined by random scoring of fields, the efficiency of gene activation in heterokaryons was 8% (Table 2). When heterokaryons were grouped according to nuclear composition into five different groups of human:mouse (H:M) nuclear ratios, as shown in Table 2, the percentage of heterokaryons with different nuclear compositions gave a Gaussian distribution with the 1:1 ratio most frequent (67%). In addition, when heterokaryons were analyzed for X-gal staining, no heterokaryons with an excess of mouse nuclei were scored as X-gal positive (Table 2). Third, to determine if lacZ activation in heterokaryons was dependent on gene dosage, the relationship between nuclear ratio and the level of X-gal staining was evaluated selectively only in heterokaryons staining positive for lacZ (Table 2). The 1:1 ratio of human to mouse nuclei was the most frequent nuclear ratio (39%) observed, then 2:1 (38%), and >2:1 (23%). As shown in Table 2, heterokaryons with equivalent or 2:1 nuclear input had similar distributions of heterokaryons when scored

TABLE 1

UV-C-Irradiated Human Fibroblasts *trans*-Activate HIV-LTR Activity in Human × Mouse Heterokaryons

Sample[a]	± PEG fusion	No. X-gal-positive cells
MRC5 X L-23	–	0
	+	0
UV + MRC5 X L-23	–	0
	+	89 ± 5
	+	76 ± 13
	+	76 ± 11
L-23	–	0
	+	0
UV + L-23	–	$>10^4$
	+	$>10^4$

[a] Controls consisted of nonirradiated MRC5 cells with mouse L-23 cells (MRC5 X L-23) ± PEG fusion, and uv-irradiated MRC5 fibroblasts cocultured (no PEG treatment) with mouse L-23 cells (UV + MRC5 X L-23); cells cocultured without PEG treatment provided a control for detecting the effect of a putative uv-induced extracellular factor produced by MRC5 cells. The uv + L-23 sample was a control for monitoring the effect of PEG fusion on the level of lacZ activity induced after uv irradiation (scored as $>10^4$ because of large number of cells staining X-gal positive). Once dishes were evaluated for the number of cells staining positive with X-gal (numbers represent the mean ± SD from counting each dish three times), they were stained with 0.5 μg/ml Hoechst 33258 to identify heterokaryons by nuclear staining (see Materials and Methods). Examples of heterokaryons, X-gal, and nuclear staining are shown in Fig. 6. The analyses of nuclear composition of uv-irradiated MRC5 fibroblasts X mouse heterokaryons and X-gal staining are shown in Table 2.

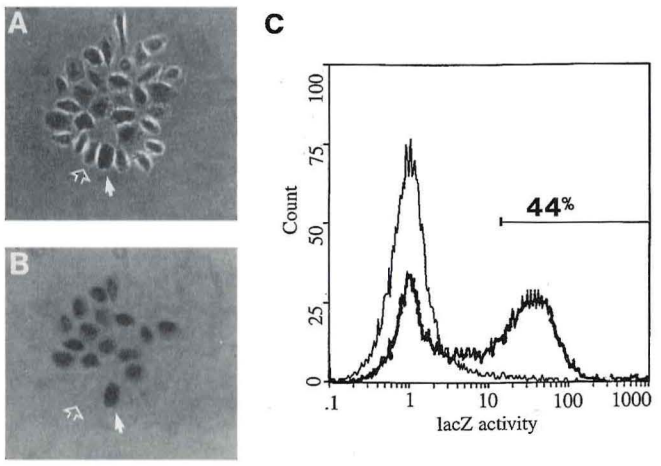

FIG. 5. uv-C irradiation activates HIV-LTR-directed lacZ expression in single cells. Mouse L-23 cells (containing pHIVlacZ) were plated at clonal level, grown until clones were visible, uv-irradiated at 30 J/m^2, and stained with X-gal to detect lacZ expression 24 h postirradiation. (A) Phase-contrast image, (B) bright-field image to emphasize the X-gal staining; solid arrows point to cells staining X-gal (lacZ) positive and open arrows point to those staining negative. (C) Flow cytometry analysis of lacZ expression in L-23 cells after uv-C irradiation. Cells were uv-C-irradiated (30 J/m^2) and stained for FACS-FDG analysis 24 h postirradiation. The results from nonirradiated clone L-23 and uv-irradiated L-23 cells (heavy line) are shown. The *X* axis is designated lacZ activity and represents the relative amount of fluorescence per cell. A total of 10,000 cells were analyzed. The *Y* axis represents the number of cells. An analysis region was set at the 2% level for nonirradiated L-23 cells to define the level of fluorescence observed after uv irradiation (44%). Other experiments demonstrated that the level of fluorescence in the parental population (no *lacZ* gene) was not altered by uv irradiation.

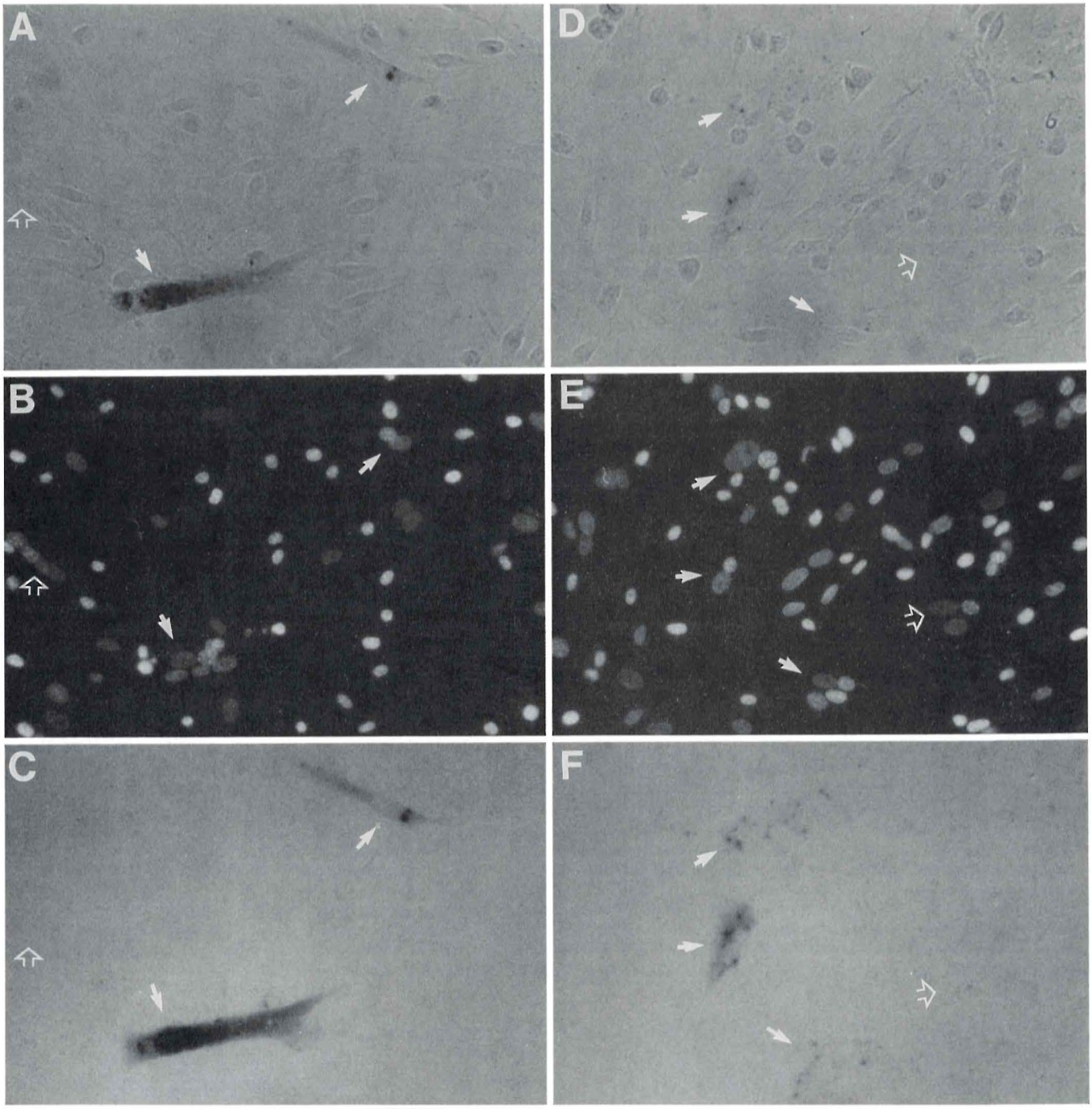

FIG. 6. Transactivation of HIV-LTR activity in heterokaryons formed from the fusion of uv-C-irradiated human fibroblasts with mouse L-23 cells. (A–F) Heterokaryons formed by fusing uv-irradiated human MRC5 fibroblasts with mouse L cell clone L-23. (A, D) Phase-contrast images, (B, E) nuclear staining obtained with Hoeschst 33258 fluorescent DNA dye (mouse nuclei appear punctate and human nuclei stain uniformly), (C, F) the bright-field images to emphasize the detection of lacZ expression by X-gal staining. Open arrows in figures point to heterokaryons that fail to stain with X-gal. Solid arrows point to heterokaryons staining positive with X-gal. Cells were photographed using a Zeiss Axioskop microscope with a Zeiss 25× multiimmersion objective with water to allow the simultaneous detection of Hoeschst fluorescence and X-gal staining.

by the intensity of X-gal staining. The percentage of heterokaryons scored as medium (++) X-gal staining with a nuclear ratio of 1:1 (29%) or 2:1 (28%) increased to 42% in the >2:1 group. Considering that the >2:1 nuclear group consisted of 4% of all heterokaryons and 23% of the X-gal positive heterokaryons, it is possible that the activation of lacZ activity in heterokaryons containing an equivalent or excess uv-

TABLE 2

Nuclear Composition of Human X Mouse Heterokaryons and X-gal Staining of lacZ Activity

Field score[a]	Nuclei scored[b]		Heterokaryons scored	Heterokaryons scored X-gal$^+$ (%)	Nuclear ratio (H:M)[c]	% Heterokaryons	% of Human or Mouse Nuclei in heterokaryons	
	Human	Mouse					Human	Mouse
A–C[d]	22	37	4	2 (50)	1:1	75	32	16
					2:1	25		
D–F[e]	32	48	7	3 (43)	1:1	100	25	21
Heterokaryons scored in random[f]								
							% Heterokaryons scored X-gal$^+$	
			135	11 (8)	1:>2	3	0	
					1:2	10	0	
					1:1	67	6	
					2:1	16	23	
					>2:1	4	17	
Heterokaryons scored X-gal^{+g}								
							% Heterokaryons scored X-gal^{+h}		
							+	++	+++
			104	104 (100)	1:>2	0	0	0	0
					1:2	0	0	0	0
					1:1	39	29	29	41
					2:1	38	21	28	51
					>2:1	23	13	42	46

[a] Heterokaryons produced by the fusion of uv-irradiated human MRC5 fibroblasts with mouse clone L-23 clone (containing the HIV-LTR-directed *lacZ* gene) were scored by nuclear and X-gal staining (see Materials and Methods).

[b] Total number of human or mouse nuclei scored from fields shown in Figs. 6A–6F.

[c] Heterokaryons scored were grouped according to nuclear composition representing the number of human and mouse nuclei they contained. The five different groups were defined as the following ratios of human to mouse (H:M) nuclei: 1:>2; 1:2 (1:x, where $1 < x \leq 2$); 1:1; 2:1 (x:1, where $1 < x \leq 2$); and >2:1.

[d] Scored for the total number of human or mouse nuclei, heterokaryons, X-gal staining, and nuclear composition.

[e] Scored for the total number of human or mouse nuclei, heterokaryons, X-gal staining, and nuclear composition.

[f] Random fields were scored for heterokaryons, X-gal staining, and nuclear composition.

[g] Heterokaryons staining positive for lacZ activity (X-gal$^+$) were scored by intensity of X-gal staining and for nuclear composition.

[h] Heterokaryons scored as X-gal$^+$ were grouped according to nuclear composition and by intensity of X-gal staining defined as weak (+), medium (++), or strong (+++). An example of the (+) level of X-gal staining is shown by two of the three heterokaryons in Figs. 6D–6F; the middle heterokaryon was scored (++). The lower heterokaryon in Figs. 6A–6C was scored (+++).

irradiated human nuclei involved a mechanism influenced by the human gene dosage. More studies are needed to further address this issue.

DISCUSSION

Mouse L Cells Provide a Cellular Background Permissive for HIV-LTR Stimulation by Ultraviolet but Not by TNF, TPA, or Oxidative Stress Induced by H_2O_2 or Ionizing Radiation

This research has addressed the question of the signal(s) induced by uv radiation that are required for the activation of the HIV-LTR stably integrated into the mouse L cell genome. The mouse L cell model system was developed specifically to evaluate the ability to separate uv irradiation of human cells from activation of the HIV-LTR-directed reporter gene stably integrated into the mouse cell genome. Recent evidence that the activation of the HIV-LTR by uv radiation does not require the enhancer region containing two NF-κB binding sites, but appears to require only an intact basal promoter in stable transfectants [19], suggests that the mechanism involved may be separated from NF-κB regulation and be dependent on uv-induced DNA damage in the chromosome containing the LTR transcription unit. Most importantly, our experimental approach of using cell fusion to form heterokaryons has shown directly that DNA damage in the chromosome containing the reporter gene cannot explain activation of the HIV-LTR.

The uv inducibility of the HIV-LTR varied among L

cell clones (10% were found to be inducible), a finding in agreement with results of other investigators using a similar approach with another promoter in L cells [27]. The basal and inducible HIV-LTR-directed CAT activities are heritable and stable properties of these clones. Our results in mouse L cells are in agreement with other studies in human HeLa cells [13] showing that uv induction of the HIV-LTR is more efficient when stably integrated [19].

The L-15 clone was used to characterize the signal transduction pathways acting on NF-κB and HIV-LTR activity. Treatment with TPA or TNF stimulated NF-κB but not HIV-LTR activity. Results obtained in T cell clones have also demonstrated that TNF treatment leads to a dissociation between NF-κB activity and stimulation of HIV-LTR activity [35]. Our results show that this effect may be relevant to a variety of cell types. In contrast to TNF, uv-C induced both NF-κB and HIV-LTR activity. Similar results were found with three independently derived clones. Moreover, the responsiveness of L-15 cells to various agents appears to be remarkably similar to that described for a stable HeLa cell clone containing the HIV-LTR-directed CAT gene [13, 14]; agents that produce "bulky" DNA lesions, such as uv-C and MMC stimulated HIV-LTR activity, while agents that cause DNA breaks, such as H_2O_2 or ionizing radiation, had little effect.

Our results are also consistent with other evidence demonstrating that uv-C and -B irradiation induced HIV-LTR-directed CAT expression [13–16] and production of infectious virus [13, 36]. In contrast, uv-A irradiation has been shown to activate the HIV-LTR only in combination with the drug psoralen [14]. Similar results have been shown in transgenic mice carrying the HIV-LTR-directed luciferase and lacZ reporter genes [37]. Oxidative stress induced by H_2O_2, uv-A, or ionizing radiation is not sufficient to stimulate HIV-LTR activity in L-15 cells. These results are in agreement with other studies that have shown that ionizing radiation does not increase HIV-LTR-directed gene expression in stables [38].

The Dithiocarbamate PDTC Defines Multiple Sites for NF-κB Regulation

It is well established that TNF activates HIV-LTR activity in an NF-κB-dependent manner [41] through a PKC-independent pathway referred to as the sphingomyelin signal transduction pathway [42, 43]. The dithiocarbamate PDTC has been shown by a number of laboratories to inhibit the activation of NF-κB, by a variety of stimuli, including TNF, by preventing the loss of IκB-α [5–9]. More recently, PDTC has been shown to inhibit the appearance of hyperphosphorylated IκB-α, an observation suggesting an inhibition of the IκB kinase [28].

Our results obtained using PDTC suggests that uv radiation induced a particular mechanism that regulates NF-κB and HIV-LTR activity and is inhibited by PDTC. Moreover, in mouse L cells PDTC had other functional activities. It inhibited the activation of NF-κB by the uv-induced pathway and stimulated NF-κB that functionally is like that induced by TNF, i.e., unable to activate HIV-LTR-directed gene expression. Although dithiocarbamates such as PDTC are well known to exert prooxidant [29] and antioxidant [42] effects, the ability of PDTC to induce NF-κB DNA binding activity has not been reported. In mouse L cells PDTC has an oxidant effect that resulted in the activation of NF-κB DNA binding activity which was additive to that induced by TNF. The conclusion that this is an oxidant effect is in agreement with other evidence showing a strong prooxidant effect in thymocytes [29]. This suggests that the biochemical activity of dithiocarbamates, such as PDTC, may be cell line dependent and more complex than other antioxidants [43; Miller, unpublished observations]. These results are also consistent with other evidence for oxidant-initiated and redox-regulated mechanisms controlling the activation of NF-κB in a cell-line-dependent manner [11].

Mouse L Cells Provide a Cellular Background Nonpermissive for a Ultraviolet-Induced Extracellular Factor

A uv-induced extracellular factor [30, 31] has been shown to stimulate HIV-LTR-directed gene expression by activating NF-κB in human cells [16]. We evaluated our system for the presence of such a secreted factor by multiple approaches. First, we examined the effect of conditioned medium from uv-irradiated human MRC5 fibroblast cells on HIV-LTR-directed CAT activity in clone L-15. The results demonstrated no effect on CAT or NF-κB (data not shown). Second, experiments with uv-irradiated human MRC5 fibroblasts cocultured with L-15 cells had no effect on CAT activity (results not shown). Third, experiments with L-23 cells (containing the HIV-LTR directed *lacZ* gene) cocultured with uv-irradiated human MRC5 fibroblasts had no effect on lacZ activity as measured by X-gal staining at the single cell level. Finally, the cytokines TNF and IL-1α induced NF-κB without stimulating HIV-LTR activity in L-15 cells (Miller, unpublished results). These experiments established that cell–cell contact or exposure to an extracellular mediator is not involved in gene activation in this system. Mouse L cells provided the cellular background that met the stringent requirements for partners in heterokaryon experiments with uv-irradiated human fibroblasts.

HIV-LTR Activity Is Activated by Ultraviolet-Induced trans-Acting Factors in Heterokaryons

Evidence for the ability to separate uv irradiation and uv-induced DNA damage from the gene response

has previously been suggested by results from cell-fusion and heterokaryon experiments [44, 45]. Our heterokaryon system was developed to directly evaluate whether uv irradiation *trans*-activated HIV-LTR-directed *lacZ* gene expression at the single-cell level. Although the conditions required for evaluating the effect of uv-irradiated human cells as a donor for the uv-induced signal were not developed for efficient heterokaryon formation, the procedure allowed small numbers of cells expressing lacZ to be readily visualized as heterokaryons by X-gal staining. The activation of lacZ as detected by X-gal staining required PEG fusion of the mouse L cell clone L-23 with the uv-irradiated human cells. Because the nuclei of the human and mouse cells remain separate in heterokaryons, this finding is evidence of *lacZ* gene activation mediated by *trans*-acting molecules stimulating HIV-LTR activity in a nonirradiated genome. Thus, DNA damage in the chromatin containing the HIV-LTR cannot be involved in activation of gene expression, considering that the mouse genome containing the HIV-LTR reporter gene was not exposed to uv irradiation. However, our experiments do not rule out the involvement of some structural mouse chromatin changes [14, 19] mediated by *trans*-acting factors induced by uv irradiation of the human cells.

In this report, the results obtained from this heterokaryon system were analyzed in three ways to ascertain the requirements for gene activation. First, the analyses of the same fields shown in Figs. 6A–6F demonstrated that 16–21% of the mouse and 25–32% of the human nuclei scored were in heterokaryons. Second, as determined by random scoring of fields, the efficiency of gene activation in heterokaryons was 8% (Table 2). When heterokaryons were grouped according to nuclear composition into five different groups of H:M nuclear ratios, the percentage of heterokaryons with different nuclear compositions gave a Gaussian distribution with the 1:1 ratio most frequent (67%). Most importantly, no heterokaryons with an excess of mouse nuclei were scored as X-gal positive, raising the possibility of a mechanism influenced by the human gene dosage. Third, an examination of the relationship between nuclear ratio and the level of X-gal staining, selectively evaluated in the X-gal positive heterokaryons, indicated that the 1:1 ratio of human to mouse nuclei was the most frequent nuclear ratio (39%) observed, followed by 2:1 (38%) and >2:1 (23%). The fact that the percentage of heterokaryons scored as medium (++) X-gal staining with a nuclear ratio of 1:1 (29%) or 2:1 (28%) increased to 42% in the >2:1 group is consistent with the hypothesis that the activation of the lacZ gene in heterokaryons containing an equivalent or excess uv-irradiated human nuclei involved a mechanism influenced by the human gene dosage. Our results showing that the >2:1 nuclear group consisted of 4% of all heterokaryons and represented 23% of the X-gal positive heterokaryons are consistent, but do not test the hypothesis directly. The development of this heterokaryon gene activation system indicates that it should now be possible to test this hypothesis directly. Moreover, this system provides an assay for the evaluation of the uv-responsive biological state (such as DNA repair, or phase of the cell cycle) of the human fibroblast that is required for efficient activation of the HIV-LTR transgene in the mouse L cell genome.

Recent evidence that the activation of the HIV-LTR by uv radiation does not require the enhancer region containing two NF-κB binding sites, but appears to require only an intact basal promoter in an integrated HIV gene expression unit [19], indicates that: (1) different mechanisms are involved in the activation of gene expression from episomal versus integrated reporter constructs, and (2) uv radiation may activate already assembled transcription complexes by the elongation of short nascent transcripts. The ability to use cell fusion to separate uv irradiation from gene activation may prove to be a useful method to further define the signaling pathway controlling the *trans*-acting factors involved.

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