Activation of Human Immunodeficiency Virus Gene Expression by Ultraviolet Light in Stably Transfected Human Cells Does Not Require the Enhancer Element†

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ABSTRACT: Ultraviolet light (UV) exposure of cells infected with human immunodeficiency virus type 1 (HIV) or transfected with HIV reporter genes increases virus-directed gene expression. Here we report the mapping of the UV response on the long terminal repeat (LTR) by using human cells stably transfected with HIV promoter plasmids harboring different mutations and controlling the expression of the chloramphenicol acetyltransferase (*cat*) reporter gene. Promoter mutation analysis revealed that no specific upstream region of the LTR was associated with UV activation, although a significant decrease was observed with mutations in the basal promoter elements Sp1 and TATA. Most importantly, UV activation was not diminished by removal of the −119 to −69 region encompassing the LTR enhancer region or, more specifically, by point mutations in the NF-κB binding elements. Consistent with this result, we found that the phorbol ester (PMA) response, which is known to act through the enhancer, occurred independently and was synergistic with the UV response. Removal of the −119 to −69 region did not affect UV activation; however, it resulted in total abrogation of the PMA response. These results suggest that UV activation is distinct from NF-κB activation and does not act through the enhancer in stably transfected cells. This is in dramatic contrast to what is found with transient expression analysis of these responses. Lastly, RNA protection experiments revealed that UV may act on preassembled basal transcription complexes by allowing elongation of nascent short mRNAs generated from the LTR. We conclude that neither the enhancer nor any other single upstream LTR element is necessary for UV activation of HIVcat expression in stably transfected cells. Instead, UV activation appears to require only an intact basal promoter.

Viruses have often been used to assess the effects of genotoxic agents on DNA repair, mutability, and other DNA damage-induced phenomena [for reviews, see Friedberg (1985) and Herrlich et al. (1986)]. Recently, several groups have reported the efficient activation of human immunodeficiency virus type 1 (HIV)[1] gene expression initiated from the long terminal repeat (LTR) by certain types of DNA damage and various genotoxic drugs (Beer et al., 1994; Cavard et al., 1990; Devary et al., 1993; Frucht et al., 1991; Morrey et al., 1991; Sadaie et al., 1990; Stanley et al., 1989; Stein et al., 1989; Valerie et al., 1988, 1990; Vogel et al., 1992; Zider et al., 1993; Zmudzka & Beer, 1990; Zmudzka et al., 1993). The HIV LTR can be divided into three major regions (+1 indicates the transcriptional start point): the core or basal promoter elements (−78/−1) encompassing a TATA box (−28/−24) and three tandem Sp1 sites (−78/−46), a modulatory or upstream region (−485/−78) with an enhancer encompassing two NF-κB binding sites (−104/−81) and other elements which bind inducible transcription factors, and the TAR region (+1/+60) which is essential for Tat trans activation (Gaynor, 1992).

Previous work demonstrated in transient transfection experiments that the pleiotropic transcription factor NF-κB and the HIV enhancer are important for UV activation of HIV gene expression (Devary et al., 1993; Stein et al., 1989; Zider et al., 1993). However, one of these studies also demonstrated that when an HIVβgal reporter gene was stably integrated in the genome, the NF-κB elements were not important for UV activation (Zider et al., 1993). These apparently conflicting findings raise the question as to why a stably integrated expression unit exhibited NF-κB independence whereas one based on transient DNA transfection showed clear dependence. Perhaps when the transcription unit is stably integrated into the genome, other factors such as changes in chromatin structure associated with the DNA repair process overshadow the effect of specific transcription factors on UV-activated gene expression observed transiently. For example, on the basis of the differential response of various DNA-damaging agents on HIV gene expression and the effects of drugs known to affect chromatin structure, we have suggested previously that changes in chromatin structure may be important for UV activation of HIV gene expression (Valerie & Rosenberg, 1990; Valerie et al., 1994). These

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus type 1; LTR, long terminal repeat; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PK-C, protein kinase C; PMA, phorbol 12-myristate 13-acetate; UV, ultraviolet light.

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changes may occur simultaneously with the nucleotide excision repair process (Valerie & Rosenberg, 1990; Wallace & Lasker, 1992) through alterations in chromatin structure necessary for DNA repair (Smerdon, 1991).

In the present investigation, we have examined whether UV activation of HIV gene expression in cells containing integrated copies of an LTR reporter gene is associated with any specific LTR promoter element. By using human cells stably transfected with reporter gene constructs, we have attempted to more closely mimic the latent proviral state of HIV than can be achieved by transient transfection experiments. We demonstrate that, in contrast to the results obtained with transient transfections, UV activation of an integrated HIV gene expression unit does not map to the HIV enhancer, or any other single upstream element in the LTR. Instead, we find that efficient UV activation correlates with intact basal promoter elements. Most importantly, we find that the UV response is separable and synergistic with the phorbol ester response which does map to the NF-κB enhancer region. Furthermore, UV appears to activate already assembled transcription complexes by elongating short nascent transcripts initiated from the LTR.

MATERIALS AND METHODS

Chemicals. Phorbol 12-myristate 13-acetate (PMA) and acetyl coenzyme A were purchased from Sigma Chemical Co., St. Louis, MO. [α-^{32}P]UTP and [α-^{32}P]GTP (3000 Ci mmol^{-1}) were from ICN, Riverside, CA, and D-*threo*-[*dichloroacetyl*-1-^{14}C]chloramphenicol (54 mCi mmol^{-1}) was from Amersham Corp., Arlington Heights, IL.

Cells and Culture Conditions. The HIVcat/HeLa (clone A5) cells have the bacterial chloramphenicol acetyltransferase (*cat*) gene under control of the HIV LTR (−485/+80) stably integrated in the genome and have been described previously (Valerie et al., 1988). The HeLa/tat cell line was generated by transfection of HeLa cells with pSVtat-SVneo, a derivative of pRSVtat-SVneo (Valerie et al., 1989), with the RSV LTR replaced by the SV40 early promoter. One G418-resistant clone, which was able to trans activate HIVcat/HeLa cells after PEG fusion, was found to produce *tat* RNA and Tat protein by RNA protection and immunoblotting, respectively (Valerie & Rosenberg, 1989). The cell line HIVcat/tat was made by transfection of a second plasmid, with the HIV *tat* gene under control of the SV40 early promoter, into HIVcat/HeLa cells by selection of mycophenolic acid resistance conferred by the bacterial *gpt* gene (Mulligan & Berg, 1981). Plasmids were introduced into HeLa cells by standard calcium phosphate DNA precipitation followed by a 15% glycerol shock (Southern & Berg, 1982). When the plasmid pHIVcat-SVneo, or derivative thereof, was transfected, 20 μg of DNA per 100 mm dish was used. Cotransfections with HIVcat plasmids and pSV2-neo (Southern & Berg, 1982) were carried out with a 4:1 DNA ratio (20 and 5 μg) per 100 mm dish. Resistant cells were selected by addition of G418 to 1 mg/mL (50% active) after 2 days. We routinely obtained between 10 and 50 drug-resistant colonies per dish. For the experiments described here, we used pooled colonies from one tissue culture dish to even out possible response fluctuations between individual isolates which may result from different genomic integration sites. The cells were maintained in Dulbecco's Modified Essential Medium (high glucose) with 10% fetal bovine serum, penicillin G (100 u/mL), streptomycin (100 μg/mL), and G418 (500 μg/mL) when stably transfected with a *neo* gene plasmid. Tissue culture reagents were purchased from GIBCO-BRL, Grand Island, NY, and Sigma Chemical Co., St. Louis, MO.

Treatment of Cells. Cells were irradiated with ultraviolet light (254 nm) from an 8 W Sylvania germicidal G8T5 lamp (GTE) calibrated with a Spectroline Model DM-254N (Spectronics Corp., Westbury, NY) short-wave UV meter. Routinely, a dose of 10−30 J/m^2 (2.5−3 W/m^2) was given to the cell monolayer after the medium was aspirated off, which was then added back to the cells after irradiation. A stock of PMA (20 μM) was made in sterile water and diluted in medium to 20 nM. To test for Tat trans activation of HIV gene expression, HIVcat/HeLa cells were mixed with HeLa/tat cells after trypsin treatment and centrifuged together to form a cell pellet. The medium was aspirated off, and 50 μL of a 50% PEG-1000/DMEM mixture was added, mixed thoroughly with the cells, and left at room temperature for 2 min. Standard tissue culture medium supplemented with gentamycin (50 μg/mL) was then added to the cells which were subsequently incubated for 20 h prior to harvest and CAT assay. Chloramphenicol acetyltransferase (CAT) assays were carried out as described previously and were normalized to total protein content by the Bio-Rad or BCA (Pierce Chemical Co.) protein assay kits with bovine serum albumin as standard (Gorman et al., 1982; Valerie et al., 1988). Statistical analysis was performed on the fold CAT activity levels after UV.

Plasmid Constructs. The plasmid pHIVcat-SVneo is a derivative of pHIVcatBGH (Valerie et al., 1988) with the *gpt* gene replaced by the *neo* gene, resulting in two tandemly positioned expression cartridges: the *cat* gene under control of the HIV LTR (−485/+80) and a positive selection cartridge consisting of the bacterial *neo* gene under control of the SV40 early promoter. The LTR deletion plasmids pHIV(−485/+80)cat-SVneo, pHIV(−340/+80)cat-SVneo, pHIV(−139/+80)cat-SVneo, pHIV(−119/+80)cat-SVneo, pHIV(−69/+80)cat-SVneo, and pHIV(−485/+80)cat-SVneo were made by replacement of the wild-type LTR from pHIVcat-SVneo by digestion with *Hpa*I and *Eco*RI, which will cut out the entire LTR and a portion of the *cat* gene, and insertion of the *Eco*RV-*Eco*RI (−340/+330), *Sca*I-*Eco*RI (−139/+330), *Taq*I(DNA ends filled in with Klenow fragment of poll)-*Eco*RI (−119/+330), *Hae*III-*Eco*RI (−69/+330) fragments, respectively, by ligation. The plasmid pHIV(−485/+23)cat-SVneo (ΔTAR) was generated by fusion of the +23 *Bgl*II site to the +80 *Hin*dIII site in pHIVcat-SVneo after the ends were made flush with Klenow fragment, followed by ligation. The NF-κB, ΔSpl, and TATA plasmids (kindly provided by G. Nabel, University of Michigan, Ann Arbor, MI) have been described previously (Nabel et al., 1988). These plasmids have a different DNA backbone than the pHIVcat-SVneo plasmids, most notably the polyA signal sequence which is derived from SV40 (Nabel et al., 1988), while the former plasmids have the bovine growth hormone polyA signal sequence. It was therefore important to construct a wild-type LTR plasmid, WT, which has the intact HIV LTR (−485/+80) in the same plasmid backbone as the NF-κB, ΔSp1, and TATA plasmids. This plasmid was made by ligation of the *Dra*III-*Eco*RI (−256/+330) LTR fragment from pHIVcat-SVneo into the large fragment of *Dra*III-*Eco*RI-digested NF-κB plasmid. The pGEM3Z-HIVcat

plasmid producing T7 antisense HIVcat RNA has a *Pst*I-*Eco*RI fragment, spanning the entire HIV LTR (−485/+80) and 250 base pairs of the *cat* gene (Valerie et al., 1988), cloned into pGEM3Z (Promega Corp., Madison, WI). All plasmid constructs were sequenced by primer extension (Chen & Seeberg, 1985).

RNA Isolation and Analysis. Total cytoplasmic RNA was isolated from cells as described (Favoloro et al., 1980). Radioactive antisense RNA was produced with T7 RNA polymerase using a kit and following a protocol supplied by Promega Corp., Madison, WI. Briefly, 1 μg of pGEM3Z-HIVcat plasmid DNA linearized with *Pst*I or pGEM3Z-SVneo linearized with *Bam*HI was used in standard *in vitro* transcription reactions with 50 μCi of [α-^{32}P]UTP or [α-^{32}P]-GTP. The protected RNA was electrophoresed on 5 or 8% urea polyacrylamide gels (Sambrook et al., 1989). The predominant HIVcat mRNA protected is ~330 nucleotides (nt). Dried gels were exposed to X-ray film with an intensifying screen. Autoradiograms were scanned, and quantitation was accomplished by a laser densitometer (Shimadzu CS-9000).

RESULTS

UV Activation Does Not Depend on Any Specific Upstream LTR Element, Including the Enhancer Region, while the Basal Promoter Elements Appear Important. In order to map the UV response on the HIV LTR promoter, we constructed different promoter deletion plasmids, transfected these into HeLa cells, and selected for stably transfected cells. Pooled cell populations were then used for promoter deletion analysis (Figure 1). First, to see if these cell populations were functioning as expected, we fused them to HeLa/tat cells which produce the HIV Tat protein (Figure 1A). Whereas the cells having deletions up to position −119 (the transcriptional start point is defined as +1) in the LTR responded well to Tat, fold CAT activity decreased substantially (~10-fold) with the (−485/+23) cells having a deletion of the Tat-responsive element TAR. This result demonstrates that our cell constructs respond to Tat as expected; *i.e.*, Tat maps to the TAR sequence (+18 to +44), in line with similar mapping studies using transient transfection assays (Berkhout & Jeang, 1992; Rosen et al., 1985). The enhancer located between −104 and −81 is required to achieve a full effect of Tat (Berkhout & Jeang, 1992), which likely explains the 3-fold decrease in CAT activity seen with the (−69/+80) cells.

We then irradiated the different cell populations with UV and measured CAT activities 20 h later. The full-length (−485/+80) LTR produced a 27-fold increase in CAT activity after UV (Figure 1B). Upon deletion of progressively more of the LTR from the 5′ end, the basal CAT activity and concomitantly the UV activation levels decreased 75−80%, but the fold activation was not significantly affected. Similarly, a deletion from the 3′ end of the LTR to position +23 produced no significant decrease in UV activation (Figure 1B). All combined, no significant difference in UV activation was observed with these deletion mutants ($p = 0.4$).

In order to more precisely assess the effects of individual promoter elements on the level of UV activation, plasmids with point mutations and small deletions were used (Figure 2). Cells transfected with the wild-type (WT), NF-κB, and

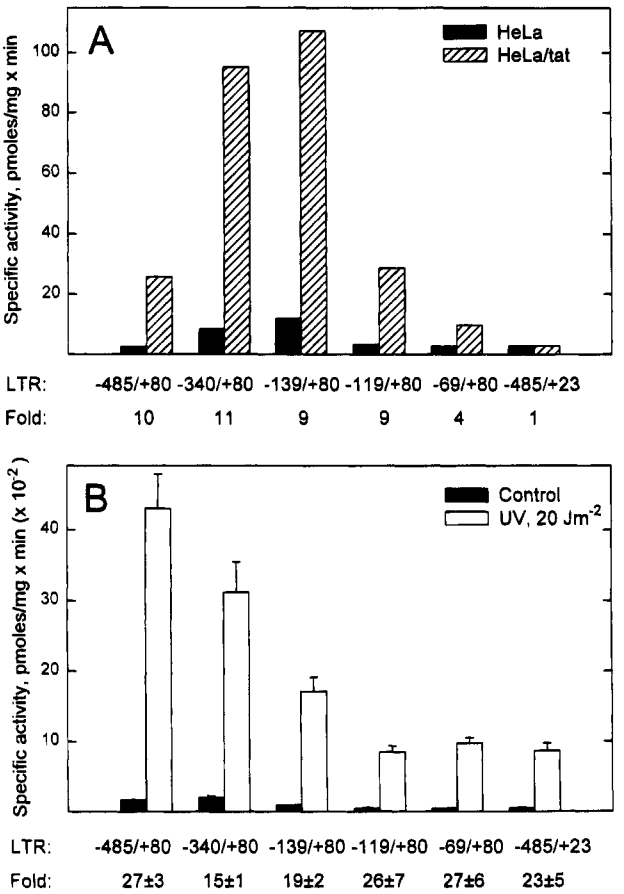

FIGURE 1: Deletion analysis of the UV response on the HIV LTR. Cell populations stably transfected with various LTR deletions upstream of the *cat* gene were fused to HeLa or HeLa/tat cells to determine Tat activation levels (A) or treated with 20 J/m^2 of UV (B). The plasmids used were (−485/+80), (−340/+80), (−139/+80), (−119/+80), (−69/+80), and (−485/+23). CAT assays were normalized for protein content, and specific activity was expressed as picomoles per milligram of protein per minute. Fold activation was calculated by division of the CAT activity obtained from UV-treated cells by the value obtained from untreated cells. UV activation values were generated from three separate experiments ($n = 7$). The standard error of the mean is indicated by error bars. Similarly, fold Tat activation was obtained by division of the CAT activity value from cells fused to HeLa/tat cells by that of the same cells fused to HeLa. Statistical analysis was performed on the fold UV activation values. No significant difference was found ($p = 0.4$).

ΔSp1, but not TATA, plasmids responded positively to Tat stimulation, but the NF-κB and ΔSp1 cells did so at reduced levels (Figure 2A). Cells transfected with the WT and NF-κB LTR plasmids were activated 7- and 8.8-fold by UV, respectively (Figure 2B), with no statistical difference ($p = 0.2$). The cell population stably transfected with a plasmid having a small deletion spanning the three Sp1 sites, ΔSp1, produced a 3.3-fold increase, while cells transfected with a TATA mutation plasmid, TATA, produced no increase in CAT activity after UV. These UV activation levels were significantly different from those obtained with the WT cells (ΔSp1, $p = 0.015$; TATA, $p = 0.0014$).

All combined, these results indicate that no single upstream element in the LTR is responsible for the entire increase in CAT activity resulting from UV. Mutations in basal promoter elements, such as Sp1 and TATA decrease (2−3-fold) or eliminate UV activation. The enhancer does not appear to be important for UV activation in stably transfected cells, contrary to the findings with transient transfection

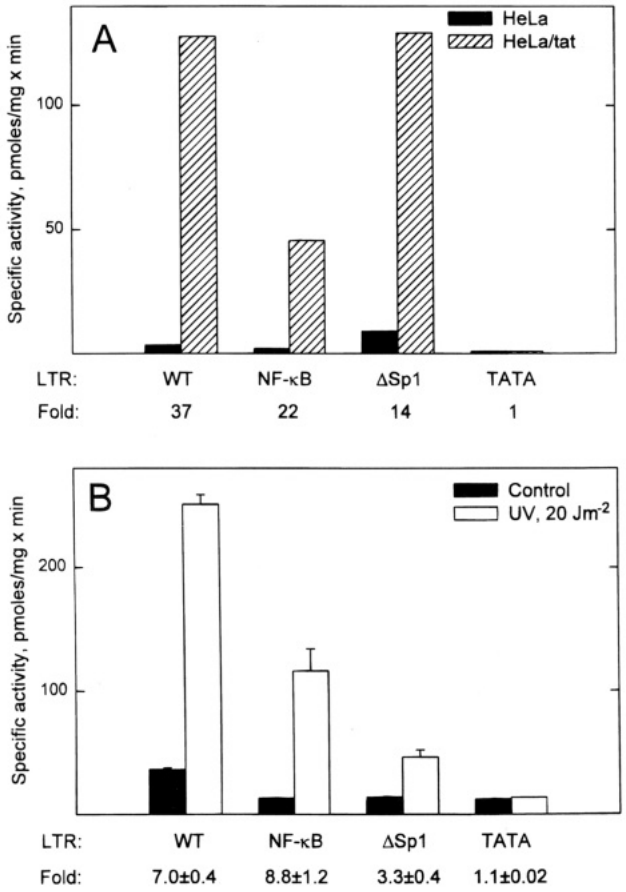

FIGURE 2: Effect of specific LTR point mutations on UV activation. HeLa cells stably transfected with WT, NF-κB, ΔSp1, and TATA HIV LTR plasmids were fused to HeLa or HeLa/tat cells (A) or UV irradiated (B) as described in the legend to Figure 1. No statistical difference was found between the UV activation levels of WT and NF-κB cells ($p = 0.2$), whereas the values obtained with WT and ΔSp1 ($p = 0.015$) and WT and TATA ($p = 0.0014$) cells were significantly different.

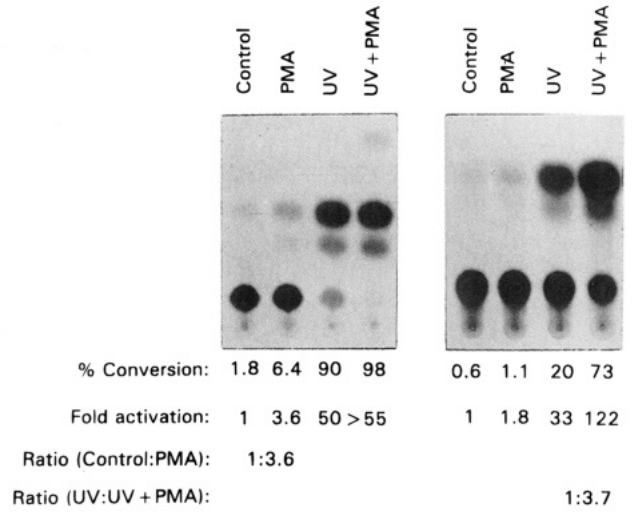

FIGURE 3: UV activation of HIV gene expression acts synergistically with phorbol ester stimulation. HIV cat/HeLa cells were treated with phorbol ester (PMA, 20 nM), UV (10 J/m^2), and the combination of the two. CAT assays were performed 20 h later for 15 min (left) and 10 min (right) using the same protein extracts.

experiments (Stein et al., 1989; Zider et al., 1993). Our results suggest that a minimal HIV promoter composed of the basal transcription elements is sufficient for efficient UV activation.

Transcriptional Activation by UV and Phorbol Ester Involves Independent Mechanisms. Previous work by others has demonstrated that the tumor promoter phorbol ester (PMA) activates HIV gene expression in transient transfection experiments through the enhancer region (−104/−81) (Kaufman et al., 1987; Nabel & Baltimore, 1987; Tong-Starksen et al., 1987) and more specifically is associated with binding of the NF-κB factor to the enhancer element (Nabel & Baltimore, 1987). Furthermore, a previous report suggested that UV and PMA act through similar pathways (Stein et al., 1989). To see whether PMA would stimulate HIV gene expression in stably transfected HIVcat/HeLa cells, and whether UV would influence this expression, we incubated the cells with PMA with or without concomitant UV treatment. PMA alone gave an ~4-fold increase in CAT activity, while UV alone produced a 33-fold activation (Figure 3). The combined treatment with UV and PMA gave a 122-fold increase in CAT activity, which is ~4-fold greater than that with UV alone. The result of this experiment shows that PMA and UV act independently in stably transfected HeLa cells, supporting the idea that these two agents activate HIVcat gene expression by separate mechanisms. Similar synergistic effects between UV and PMA were also observed in two other independently isolated HIVcat HeLa clones (clones B3 and A4; Valerie et al., 1988), and in several monkey CV-1 clones (Cheng and Valerie, unpublished observations), suggesting that this result is independent of cell line, chromosomal integration site, and species.

The synergy observed between UV and PMA was also seen at the steady-state mRNA level. An RNA protection experiment was carried out on total cytoplasmic RNA isolated from HIVcat/HeLa cells after treatment with PMA, UV, and the combination of the two (Figure 4). Whereas 10 and 20 J/m^2 of UV produced an ~9- and 15-fold increase in steady-state HIVcat mRNA, respectively, the PMA treatment resulted in an ~2-fold increase and the combination of PMA and 10 J/m^2 of UV produced a 16-fold increase (Figure 4A). In clear contrast to the increased HIVcat mRNA levels, SVneo steady-state mRNA varied only a few fold with the same treatments (data not shown). Again, this result supports the idea that UV and PMA stimulate HIVcat transcription independently in stably integrated cells. The increases in steady-state *cat* mRNA after these various treatments reflect the level of CAT activity (Figure 4B) obtained in parallel, suggesting that the primary effect of UV is by transcriptional stimulation.

These results demonstrate that UV increases HIVcat gene expression primarily at the transcriptional level. The treatment with PMA treatment also increases steady-state HIVcat mRNA levels and acts in a synergistic fashion with UV, suggesting that these two agents increase HIVcat transcription by independent mechanisms in stably integrated cells.

UV and Phorbol Ester Activation Can Be Separated at the Level of the LTR. Because we observed a synergistic effect of UV and PMA on HIVcat expression with the HIVcat/HeLa cells (Figures 3 and 4), we wanted to investigate if these two effects were also separable at the LTR level. We again used the two cell populations differing only in that one (−119/+80) has the enhancer region (−104/−81), while the other (−69/+80) does not, and tested for activation by PMA and UV (Figure 5). We found that, as expected, the (−119/+80) cells responded to PMA and UV in a fashion similar to that of the (−485/+80) cells. In

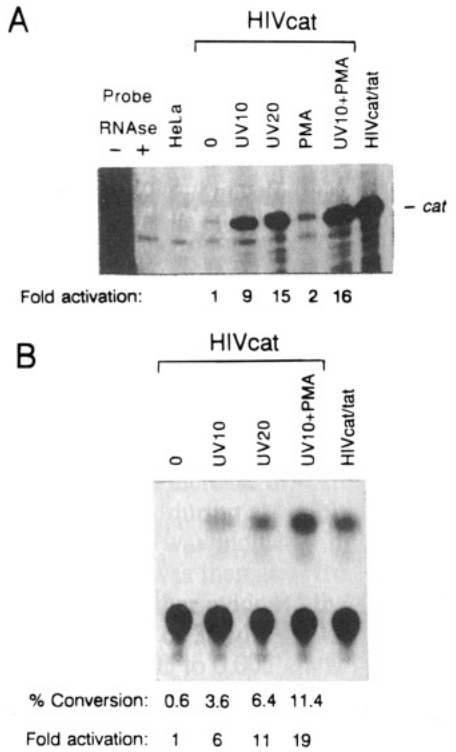

FIGURE 4: Transcriptional activation of HIV gene expression after UV. HIVcat/HeLa cells were treated with UV (10 or 20 J/m^2), PMA (20 nM), and UV in combination with PMA. Steady-state *cat* mRNA (A) and CAT activities (B) were determined 10 h after treatment. As control, we used HIVcat/tat cells (constitutively producing CAT through *tat* activation). Fold activation was calculated by densitometric scanning of the protected RNA bands. The *cat* value obtained from untreated (0) cells was taken as 1, and all other values were normalized against this value. An RNA sample isolated from cells with no HIVcat plasmid (HeLa) was used as negative control for the *cat* transcript. RNase (±) is riboprobe treated or not treated with RNases.

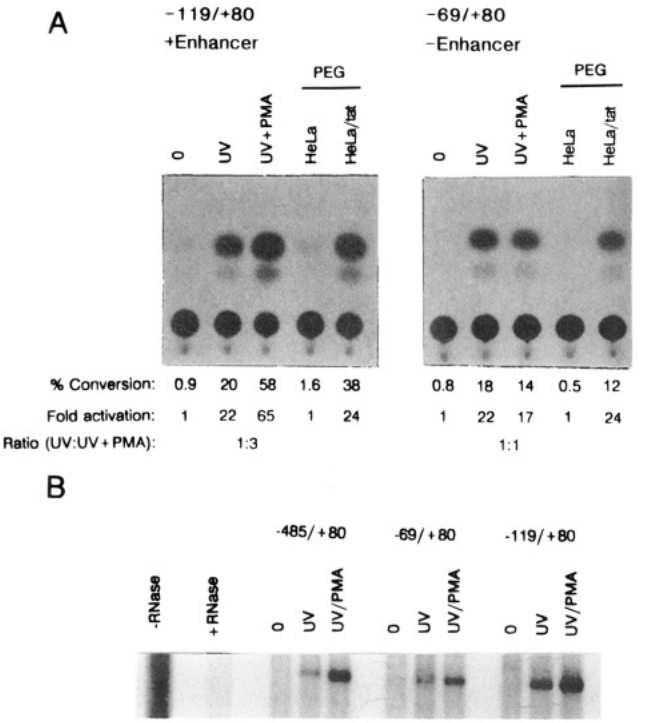

FIGURE 5: Phorbol ester response maps to the enhancer, while UV response does not. Stably transfected enhancer$^+$ (−119/+80) and enhancer$^-$ (−69/+80) HeLa cells were treated with UV (10 J/m^2) and UV and PMA (20 nM). As control, they were fused to HeLa and HeLa/tat cells. (A) CAT assays were performed and fold activations calculated as described in the legend to Figure 1. (B) Total RNA was isolated from wild-type (−485/+80) HIVcat/HeLa, enhancer$^+$ (−119/+80), and enhancer$^-$ (−69/+80) cells 5 h after treatment with UV (30 J/m^2) or UV and PMA (20 nM) or without any treatment.

contrast, the cells having the −69/+80 LTR showed the same level of CAT activity irrespective of PMA treatment, suggesting the lack of a requirement for the −119 to −69 region for the UV response. The fact that PMA had no effect on the UV response with the (−69/+80) cells indicates that PMA requires the −119 to −69 region and that the PMA and UV responses act independently.

To investigate CAT effects at the level of steady-state HIVcat mRNA, we performed an RNA protection experiment with RNA isolated from wild-type (−485/+80) HIVcat/HeLa, (−119/+80), and (−69/+80) cells after UV or the combination of UV and PMA. The result presented in Figure 5B shows that the (−485/+80) HIVcat/HeLa and (−119/+80) cells both exhibited synergistic increases in HIVcat mRNA after the combined UV and PMA treatment compared to the results with UV alone (3.9- and 2.4-fold, respectively), whereas the (−69/+80) cells showed little to no synergistic response (1.4-fold). All together, these results demonstrate that UV does not act through the enhancer region (−104/−81) located within the −119 to −69 region and works independently of the PMA response. Both responses are primarily transcriptional effects on HIV gene expression.

Transcription Complexes Are Present on the LTR Prior to UV Activation. Previous reports have demonstrated that short 50−60 nt transcripts are continuously produced by the HIV LTR, and in the presence of Tat, increased rates of transcription elongation occur from transcription complexes assembled on the LTR (Jones, 1989; Kao et al., 1987; Laspia et al., 1991). Such preassembled transcription complexes, if elongated in response to UV, could account for the effects of UV on HIVcat gene expression. To see if our stably transfected cells produce these short transcripts, we analyzed RNA isolated from untreated and UV-treated wild-type (−485/+80) HIVcat/HeLa, (−119/+80), and (−69/+80) cells and specifically looked for the presence of short HIVcat-specific mRNA molecules. RNA protection experiments revealed that all three cell populations produced the short 50−60 nt RNA species in the absence of UV (Figure 6, top, lanes 2, 4, 7, and 9), except that the level obtained with the RNA from (−69/+80) cells was reduced (~2-fold) in this particular experiment. However, in a subsequent experiment, we found no difference in the level of the short RNA species between the (−119/+80) and (−69/+80) cells (Figure 6, bottom, compare lanes 4−6 with lanes 7 and 8). RNA isolated from control HeLa cells did not produce any such short protected RNAs (Figure 6, top, lane 1, and bottom, lanes 2 and 3), suggesting that they are synthesized by the HIVcat transcription units. After UV, these short RNA molecules disappeared and the longer HIVcat-specific mRNAs appeared in all three cell populations (Figure 6, top, compare lanes 2, 7, and 9 with lanes 3, 8, and 10). The reduction in the level of the short transcript and the appearance of the longer mRNAs was time-dependent (Figure 6, top, lanes 4−6 and 3).

This result demonstrates that integrated HIVcat transcription units produce short mRNAs in the absence of any stimulus, suggesting that transcription complexes are as-

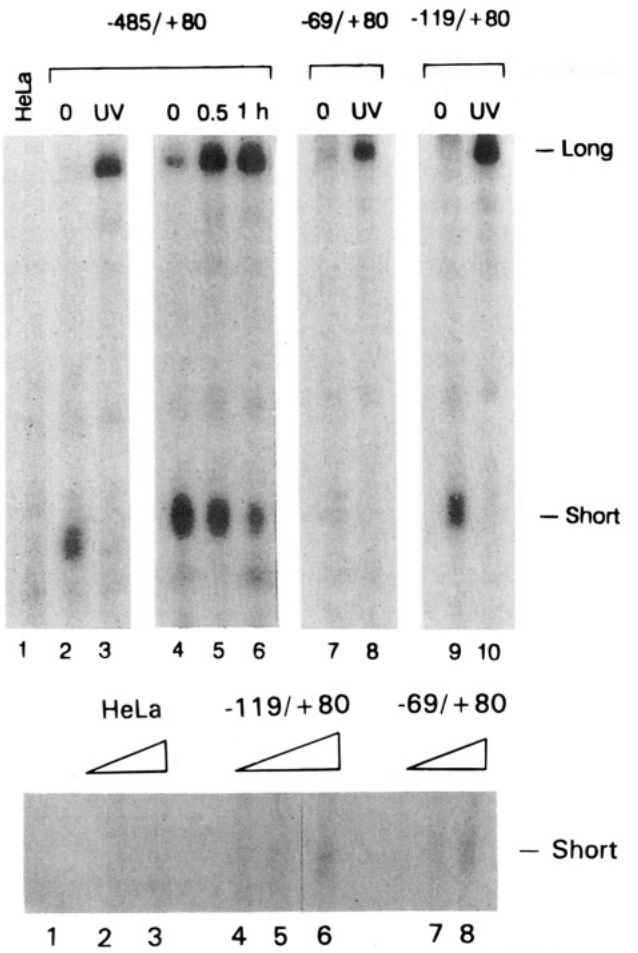

FIGURE 6: Short transcripts are produced from the HIV LTR prior to UV activation in stably transfected cells. (Top) Total RNA was isolated from untreated or UV-irradiated (30 J/m^2) HIVcat/HeLa (−485/+80), enhancer$^+$ (−119/+80), and enhancer$^-$ (−69/+80) cells. Protected RNA was separated on a 5% urea polyacrylamide gel in order to identify the shorter HIVcat transcripts. HeLa RNA was included as control: lane 1, HeLa RNA; lanes 2 and 4, untreated (−485/+80) cells; lane 3, 30 J/m^2 for 5 h; lane 5, 30 J/m^2 for 0.5 h; lane 6, 30 J/m^2 for 1 h; lane 7, untreated (−69/+80) cells; lane 8, 30 J/m^2 for 5 h; lane 9, untreated (−119/+80) cells; lane 10, 30 J/m^2 for 5 h. Long is 330 nt riboprobe-protected HIVcat transcript; short is 50−60 nt transcripts. The autoradiogram was scanned with a densitometer to quantitate the relative level of the 50−60 nt RNA species. The normalized level for (−69/+80) cells (lane 7) was ~45% of (−119/+80) cells (lane 9). (Bottom) Total RNA from HeLa, (−119/+80), and (−69/+80) cells was isolated from untreated cultures and hybridized to the HIVcat riboprobe as described above. Protected RNA species were separated on an 8% urea polyacrylamide gel: lane 1, no RNA; lane 2, 10 μg of HeLa; lane 3, 30 μg of HeLa; lane 4, 10 μg of (−119/+80); lane 5, 20 μg of (−119/+80); lane 6, 30 μg of (−119/+80); lane 7, 10 μg of (−69/+80); lane 8, 30 μg of (−69/+80). The level of the 50−60 nt RNA species in the (−119/+80), lanes 4−6, and (−69/+80), lanes 7 and 8, cells were almost identical.

sembled and, to some extent, elongate on the HIV promoter prior to UV activation. These complexes appear to be associated with the basal promoter in as much as the short transcripts were also detected in the (−69/+80) cell population. After UV, these shorter RNA molecules disappear and the production of longer HIVcat transcripts occurs in a time-dependent fashion.

DISCUSSION

UV Activation of HIV Gene Expression in Stably Transfected Cells Does Not Involve the Enhancer and Can Be Separated from Phorbol Ester Activation. The main finding in this study is that, when the HIVcat transcription unit is stably integrated in the genome, UV activation of HIV gene expression does not require any specific upstream LTR transcription element and can be functionally separated from the enhancer region and, consequently, NF-κB influence. Several of our results support this contention. First, promoter-mapping experiments revealed no single upstream LTR transcription element associated with UV activation, which instead appears to be linked to the overall basal promoter strength. A mutation in the TATA element correspondingly eliminated basal expression and UV activation, and a specific deletion of the three Sp1 elements resulted in a 50−60% reduction. Second, whereas the PMA response, as expected, mapped to the −119 to −69 region spanning the enhancer (−104/−81), the UV response was not diminished by removal of this region or by specific mutation of the NF-κB binding sites. Third, UV and PMA activated HIV *cat* transcription in a synergistic fashion, suggesting that these two agents activate by independent mechanisms.

UV Produces Different Results Depending on Whether the Transcription Unit Is Integrated or Episomal. Our finding that UV activation does not map to a single upstream LTR element, including the enhancer region, in stably transfected cells contrasts with two studies which employed a transient transfection assay to map the UV response to the HIV enhancer (Stein et al., 1989; Zider et al., 1993). However, Zider et al. (1993) also demonstrated in the same study that various mutant HIV LTR-βgal constructs stably integrated into cells of transgenic mice showed no specific involvement of the NF-κB elements in UV activation, which is in agreement with what we report here. Therefore, UV may activate HIV gene expression differently when the transcription unit is stably integrated in the genome compared to when it is episomal.

The result of our PMA-mapping experiment using stably transfected cells agrees with previous results generated from transient transfection experiments which mapped the PMA response to the enhancer (Kaufman et al., 1987; Nabel & Baltimore, 1987; Tong-Starksen et al., 1987). The finding that activation of HIV transcription by okadaic acid (a protein phosphatase inhibitor) also maps to the enhancer region using these same cell populations (Valerie et al., unpublished observations) further substantiates the proposal that the cells respond in a way which is very similar, if not identical, to the responses observed in transient transfections (Thévenin et al., 1990). In contrast, UV activation relies on a mechanism distinct from the PMA and okadaic acid activation pathways in stably transfected cells since the UV response was not affected by removal of the −119 to −69 region encompassing the enhancer (−104/−81), or by specific mutation of the NF-κB binding sites. These findings demonstrate that the UV effect is unique among these three activating agents in that different results are obtained depending on whether transient transfections or stably transfected cells are being used. Most importantly, a previous report suggested that UV and PMA stimulate HIV gene expression through similar pathways (Stein et al., 1989), whereas we clearly show here that in a stably transfected system these two responses operate via separate pathways.

Alterations in Chromatin Structure as a Possible Mechanism for UV Activation of Stably Integrated Transcription Units, a DNA Repair Side Effect. One possibility which may

account for the observed differences between the stable and transient systems may involve changes in chromatin structure, which are important for transcriptional activation (Croston et al., 1991; Felsenfeld, 1992; Wolfe, 1994; Workman & Buchman, 1993). Integrated plasmid DNA is part of higher order chromatin structure and is replicated and repaired along with other cellular genes. Therefore, the integrated plasmid is likely to behave more like a provirus or any other cellular gene, and the cell system is thus more physiologically relevant than a system based on transient transfection. In line with this idea is the finding that global chromatin decondensation is observed after UV, but not ionizing radiation (Valerie et al., 1995), perhaps as a prerequisite for, or as a result of, the different types of DNA repair induced by these two types of radiation. Interestingly, only UV, not ionizing radiation, activates HIVcat expression (Valerie & Rosenberg, 1990; Valerie et al., 1995). Furthermore, both UV and ionizing radiation activate NF-κB post-translationally to a similar extent and with similar kinetics, as measured by gel shift assays (Valerie et al., 1995), suggesting that with stably integrated cell constructs the binding of NF-κB to the HIV enhancer measured by *in vitro* gel shift assays is a poor indicator for the involvement of NF-κB in these responses. However, we cannot rule out that NF-κB plays a smaller and more indirect role in these responses.

Other possibilities that explain our results may involve changes in DNA topology or the induction of Tat-like host proteins after UV damage (Carrier et al., 1994). However, because we do not observe any consistent effect with different inhibitors of topisomerase I and II on HIVcat expression (Valerie et al., unpublished results), we believe that changes in DNA topology are less likely to play any major role in UV activation of HIVcat transcription. If Tat-like cellular proteins are involved, they must act independently of TAR since a TAR deletion did not significantly affect UV activation in our study. Increased mRNA stability may also contribute to the increased HIVcat steady state after UV.

Our results suggest that the stably integrated HIVcat plasmids have transcription complexes associated with the LTR because short 50−60 nt transcripts form in the absence of any stimulus. This finding is in agreement with earlier studies (Jones, 1989; Kao et al., 1987; Laspia et al., 1991). We find that after UV the short HIV-specific transcripts disappear and full-length mRNAs appear in a time-dependent fashion. This was also observed with the (−69/+80) cells, suggesting that UV activates HIV transcription at least in part by allowing basal transcription complexes on the LTR to overcome a transcriptional block to elongation of the short nascent transcripts. The nucleotide excision repair process itself may play a direct or indirect role in this transcriptional activation since recently it has become clear that proteins involved in repair of UV damage are also basal transcription factors (Drapkin et al., 1994).

These results support a mechanism whereby UV produces changes in chromatin structure, perhaps due to the DNA repair process itself, which may allow the basal HIV promoter to become transcriptionally activated without the initial involvement of NF-κB. Because it is believed that the role of upstream transcription factors is to stimulate basal promoter factors to initiate transcription, it is possible that UV-induced changes in chromatin structure could substitute for the action of these factors during the initial steps of gene activation.

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REFERENCES

Beer, J. Z., Olvey, K. M., Lee, W., & Zmudzka, B. Z. (1994) *Photochem. Photobiol. 59*, 643−647.
Berkhout, B., & Jeang, K.-T. (1992) *J. Virol. 66*, 139−149.
Brach, M. A., Hass, R., Sherman, M. L., Gunji, H., Weichselbaum, R., & Kufe, D. (1991) *J. Clin. Invest. 88*, 691−695.
Carrier, F., Gatignol, A., Hollander, M. C., Jeang, K. T., & Fornace, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1554−1558.
Cavard, C., Zider, A., Vernet, M., Bennoun, M., Saragosti, S., Grimber, G., & Briand, P. (1990) *J. Clin. Invest. 86*, 1369−1374.
Chen, E. J., & Seeburg, P. H. (1985) *DNA 4*, 165−170.
Croston, G. E., Kerrigan, L. A., Lira, L. R., Marshak, D. R., & Kadonaga, J. T. (1991) *Science 251*, 643−649.
Devary, Y., Rosette, C., DiDonato, J. A., & Karin, M. (1993) *Science 261*, 1442−1445.
Drapkin, R., Sancar, A., & Reinberg, D. (1994) *Cell 77*, 9−12.
Favoloro, J., Treisman, R., & Kamen, R. (1980) *Methods Enzymol. 65*, 718−749.
Felsenfeld, G. (1992) *Nature (London) 355*, 219−224.
Friedberg, E. E. (1985) in *DNA repair*, W. H. Freeman & Co., San Francisco.
Frucht, D. M., Lamperth, L., Vicenzi, E., Belcher, J. H., & Martin, M. A. (1991) *AIDS Res. Hum. Retroviruses 7*, 729−733.
Gaynor, R. (1992) *AIDS 6*, 347−363.
Gorman, C. M., Moffat, L. F., & Howard, B. H. (1982) *Mol. Cell. Biol. 2*, 1044−1051.
Herrlich, P. E., Angel, P., Rahmsdorf, H. J., Mallick, U., Potting, A., Hieber, L., Lücke-Huhle, C., & Schorpp, M. (1986) *Adv. Enzyme Regul. 25*, 485−504.
Jones, K. A. (1989) *New Biol. 1*, 127−135.
Kao, S.-Y., Calman, A. F., Luciw, P. A., & Peterlin, B. M. (1987) *Nature (London) 330*, 489−493.
Kaufman, J. D., Valandra, G., Roderiquez, G., Bushar, G., Giri, C., & Norcross, M. A. (1987) *Mol. Cell. Biol. 7*, 3759−3766.
Laspia, M., Rice, A., & Matthews, M. (1991) *Cell 59*, 283−292.
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.
Mulligan, R., & Berg, P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2072−2076.
Nabel, G., & Baltimore, D. E. (1987) *Nature (London) 326*, 711−713.
Nabel, G. J., Rice, S. A., Knipe, D. M., & Baltimore, D. (1988) *Science 239*, 1299−1302.
Rosen, C. A., Sodroski, J. G., & Haseltine, W. A. (1985) *Cell 41*, 813−823.
Sadaie, M. R., Tschachler, E., Valerie, K., Rosenberg, M., Felber, B. K., Pavlakis, G. N., Klotman, M. E., & Wong-Staal, F. (1990) *New Biol. 3*, 479−486.
Sambrook, J., Fritsch, W. F., & Maniatis, T. (1989) in *Molecular cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Smerdon, M. J. (1991) *Curr. Opin. Cell Biol. 3*, 422−428.
Southern, P. J., & Berg, P. (1982) *J. Mol. Appl. Genet. 1*, 327−341. 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.
Thévenin, C., Kim, S.-J., Rieckmann, P., Fujiki, H., Norcross, M. A., Sporn, M. B., Fauci, A. S., & Kehrl, J. H. (1990) *New Biol. 2*, 793−800.
Tong-Starksen, S. E., Luciw, P. A., & Peterlin, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6845−6849.

Valerie, K., & Rosenberg, M. (1989) in *RNA Tumor Viruses*, p 67, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
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., Laster, W. S., Kirkham, J. C., & Kuemmerle, N. B. (1995) *Biochemistry 34*, 15768−15776.
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.
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.
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.

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