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Induction of transcription from the long terminal repeat of Moloney murine sarcoma provirus by UV-irradiation, x-irradiation, and phorbol ester

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ABSTRACT **The long terminal repeat (LTR) of Moloney murine sarcoma virus (Mo-MuSV) was used as a model system to study the stress response of mammalian cells to physical carcinogens. The chloramphenicol acetyltransferase (CAT) gene was inserted between two Mo-MuSV LTRs, and the LTR-CAT-LTR construct was used for virus production and was integrated into the genome of NIH 3T3 cells in the proviral form. This construct was used to assure that the integrated CAT gene was driven by the promoter of the LTR. Expression of the CAT gene was stimulated 4-fold by UV irradiation, and the peak of activity was observed at 18 hr. In contrast, stimulation of the CAT expression after x-irradiation was 2-fold and occurred at 6 hr. Phorbol myristate acetate also stimulated CAT activity 4-fold with a peak at 6 hr. Down-regulation of protein kinase C blocked totally the response to x-irradiation but only partially the response to UV. The protein kinase inhibitor H7 blocked the response to treatment by UV, x-ray, and phorbol ester.**

Prokaryotic cells respond to DNA damage by the induction of a series of enzymes. This is known as the SOS response (1). In mammalian cells, the "stress response" is in a general sense similar in that a series of genes are activated under various conditions. Herrlich *et al.* (2) demonstrated that γ-irradiation, UV-irradiation, other agents that damage DNA or block DNA replication, and phorbol ester increased the levels of a series of mRNAs and proteins.

The goals of this study were to establish a reporter system for stress response, to determine whether x-ray- and UV-irradiation could trigger that response, and to investigate some aspects of the internal signaling system for that response. A retroviral long terminal repeat (LTR) was chosen as the sensor for signals arising from DNA damage. Previous work by others had demonstrated the induction of avian tumor virus production by carcinogens (3), the induction of avian tumor virus transcription by pyrrolizidine alkaloids (4), and the induction of endogenous murine retroviruses by aflatoxin B_1 and 2-acetylaminofluorene (5). Also in transformed C3H/10T½ murine fibroblasts, transcription of Moloney murine sarcoma virus (Mo-MuSV) LTR sequences was observed that was absent or present at a very low level in untransformed cells (6). Thus, there was considerable evidence that an LTR might be transcriptionally activated due to a stress response from carcinogens.

For experiments reported here, the chloramphenicol acetyltransferase (CAT) gene was inserted into a Mo-MuSV vector and stably integrated into the genome of NIH 3T3 cells in the proviral form. Increased CAT expression was observed following UV-irradiation, x-irradiation, and phorbol ester addition. Differences in the kinetics of expression were observed. Down-regulation of protein kinase C affected the response of the CAT gene to both x-ray- and UV-irradiation. The response to both these challenges was blocked by the protein kinase inhibitor H7.

MATERIALS AND METHODS

Plasmids. The retroviral-derived vector poly-pMV was constructed by H. Short (Case Western Reserve University, Cleveland) by inserting a polylinker into the *Pst* I cloning site of pMV (7), which contains the two LTRs and the packaging signal from Mo-MuSV. Plasmid pPC732 containing the LTR-CAT-LTR construct was produced by ligating the 779-base-pair (bp) CAT-containing *Bam*HI fragment from a cartridge pCM-4 (8) (Pharmacia) to *Bgl* II-digested, alkaline phosphatase-treated poly-pMV. The orientation of the CAT gene relative to the two LTRs was verified by restriction enzyme mapping. For probing Northern blots, the human α-tubulin clone k-α-1 (9) was provided by D. Cleveland (The Johns Hopkins University, Baltimore), and the human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA clone (10) was provided by R. Wu and X.-H. Sun (Cornell University, Ithaca, NY). All enzymes used in nucleic acid analysis were from Boehringer Mannheim.

Cell Culture. ψ-2 (11) and NIH 3T3 cell lines were grown in McCoy's 5A medium containing 10% (vol/vol) calf serum. Transfection was done by using a modification of the calcium phosphate procedure of Graham and van der Eb (12) in which no carrier DNA was added and the cultures were subjected to a 2-min exposure to 15% (vol/vol) glycerol at room temperature 4 hr after the DNA was added.

The cell line containing the LTR-CAT-LTR construct was derived as follows. pPC732 (150 μg) was transfected into 10^7 ψ-2 cells. Medium from the transfected population was collected 24 hr later, and cell debris was removed by centrifugation at 11,000 × *g* for 15 min. Viral particles in clarified medium were pelleted by centrifugation at 55,000 × *g* for 60 min in a SW 27 rotor. The viral pellet was suspended in 0.5 ml of 100 mM NaCl/10 mM Tris·HCl, pH 7/1 mM EDTA, sonicated at 4°C for 2 min, diluted with 3 ml of 37°C culture medium, and used to infect 10^5 NIH 3T3 cells in the presence of 8 μg of Polybrene per ml for 3 days. The resulting cells (NC2) were assayed for CAT activity either as a population or as colonies derived from single cells. Sublines containing the CAT gene were identified by the CAT assay.

UV-irradiation was performed on cells covered with 4 ml of phosphate-buffered saline. X-irradiation was performed on cells in the absence of medium. Cycloheximide, phorbol 12-myristate 13-acetate (PMA, Sigma), and 1-(5-isoquinolinyl sulfonyl)-2-methylpiperazine (designated H7; Seikagaku Kogya, Tokyo) were added directly to the culture medium in

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Abbreviations: LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate; GAPDH, glyceraldehyde phosphate dehydrogenase; Cm, chloramphenicol.

the presence of serum at final concentrations of 10 μg/ml, 100 nM, and 50 μM, respectively.

RNA (Northern) Blot Hybridization. Total cellular RNA was purified by the guanidine thiocyanate/CsCl procedure (13). Northern analysis was carried out as described (14). Probes were labeled by nick-translation with [α-^{32}P]dCTP to a specific activity of 2 × 10^8 cpm/μg.

S1 Nuclease Mapping. The procedure for S1 nuclease mapping was modified from that of Berk and Sharp (15) with an RNA·DNA hybridization temperature of 42°C. To prepare the probe, plasmid pPC732 was digested with *Eco*RI, treated with alkaline phosphatase, and labeled with [γ-^{32}P]ATP at the 5′ end by using T4 polynucleotide kinase.

CAT Assay. CAT activity was assayed by the method of Gorman *et al.* (16). For each assay, 200 μg of total protein, determined by rose Bengal assay (17), was used. Reactions were carried out with 0.1 μCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol (Cm; 50–60 Ci/mol, New England Nuclear), 150 mM Tris·HCl (pH 8), and 4 mM acetyl CoA (Calbiochem) at 37°C overnight. After the reaction, Cm and acetyl-Cm were extracted with 1 ml of ethyl acetate. Samples were dried in a Speed Vac for 1 hr, dissolved in 30 μl of ethyl acetate, spotted on silica gel TLC plates (J. T. Baker), and developed in the ascending solvent chloroform/methanol, 95:5 (vol/vol). For quantitative analysis, areas of the silica corresponding to Cm and acetyl-Cm spots were scraped off the plate and counted in a liquid scintillation cocktail consisting of toluene/triton X-100, 2:1 (vol/vol), containing 4 g of 2,5-diphenyloxazole per liter and 0.5 g of 1,4-bis[5-phenyl(oxazolyl)]benzene (POPOP) per liter. CAT activity was expressed either as percent conversion of Cm into acetyl-Cm or as activity relative to the basal level in untreated cells. All experimental points were done at least in triplicate, and the standard error of the mean (SEM) was calculated.

RESULTS

Cells That Permit Expression of the CAT Gene. The steps by which NIH 3T3 sublines containing the LTR-CAT-LTR construct were derived are described in *Materials and Methods*. This procedure ensured that the CAT reporter gene was downstream from the LTR promoter. The resulting population (NC2) was a mixture of infected and wild-type cells. As a group, these cells expressed a CAT activity of 17% conversion from Cm to acetyl-Cm per 200 μg of total soluble protein. These cells were cloned, and one of the clones, NC2-11, was chosen for further study. NC2-11 had a CAT activity similar to that of the original uncloned population. Southern analysis indicated that NC2-11 contained a single integrated provirus (data not shown) with a restriction map consistent with normal proviral integration.

Effect of UV-Irradiation. NC2-11 cells were irradiated with UV, and the CAT activity was measured 20 hr later. The CAT activity as a function of the dose of UV-irradiation is shown in Fig. 1*A*. The relative CAT activity increased with increasing UV up to a dose of 9.5 J/m^2. This dose resulted in the killing of 40% of the cells. All subsequent UV-irradiation was done with a dose of 10 J/m^2, which was lethal for 45% of the cells.

The kinetics of the response of CAT activity to UV-irradiation is shown in Fig. 1*B* (solid line). Eighteen hours after the NC2-11 cells were irradiated with 10 J/m^2 of UV, CAT activity increased 4-fold over the basal level and then gradually decreased to the basal level by 72 hr. There was a small reproducible decrease in activity 6 hr after irradiation.

To determine whether the response to UV-irradiation was unique to the NC2-11 clone, an identical experiment was performed with another clone, NC2-17. NC2-17 had a slightly lower basal CAT activity and a slower growth rate in the culture. As indicated in Fig. 1*B* (dotted line), the response to

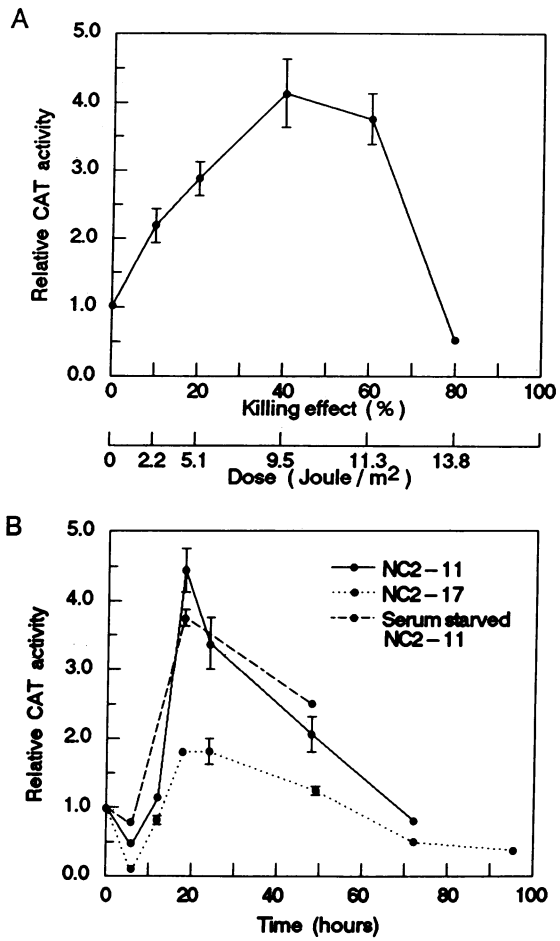

FIG. 1. Effect of UV-irradiation on CAT activity. (*A*) Dose–response effect of CAT activity. NC2-11 cells were irradiated with UV, and the CAT activity was measured 20 hr later. The dosage is indicated both as the percent killing effect and as J/m^2. (*B*) Response of CAT activity as a function of time measured after 10 J/m^2 of UV-irradiation. The NC2-11 serum-starved cells were grown in 0.5% calf serum for 3 days. Fluorescence-activated cell sorting analysis confirmed that, after 3 days of starvation, 94% of the cells were arrested in G_0/G_1 phase, and no S-phase cells were present. SEMs (vertical bars) are shown only when values are >5% of the mean.

UV was similar in NC2-17 except that the increase was only 2-fold at 18 hr.

LTR expression has been shown to occur in the G_1 phase of the cell cycle (18). It was possible that the UV treatment preferentially killed cells in S phase, and the increase in CAT activity described above was due to the death of the subpopulation of cells that did not express CAT. To obtain a total population of cells in G_0/G_1, NC2-11 cells were incubated in 0.5% serum for 3 days. Analysis by the fluorescence-activated cell sorter showed that only the G_0/G_1 population was present. Fig. 1*B* shows that there was no significant difference between the experiments done with serum-starved or serum-fed cells (dashed line vs. solid line). This suggests that the UV-irradiation effect was not due to an artifact of cell killing.

CAT Protein and mRNA Analysis. The stability of CAT protein was measured in control and UV-irradiated NC2-11 cells. Incorporation of [^{35}S]methionine into protein was essentially absent following the addition of cycloheximide at 10 μg/ml. There was no significant decrease in CAT activity during 8 hr of incubation with cycloheximide in cells with or without UV-irradiation (data not shown). These results suggest that the CAT protein is stable in control cells and that UV does not alter this long-term stability.

The effect of UV-irradiation on RNAs in NC2-11 cells was studied. Fig. 2*A* shows the Northern blot analysis of CAT RNA. By 18 hr after irradiation, CAT RNA had increased about 4-fold relative to RNA from untreated cells. The level of the increase was similar to the increase in CAT enzyme activity (compare with Fig. 1*B*) in parallel experiments. However, by 48 hr after irradiation, the accumulated level of CAT RNA had dropped to the basal level, while the enzyme activity was still 2 times the basal level. By 6 hr after irradiation, there was a reproducible decrease in CAT enzyme activity, while CAT mRNA remained at the normal level.

To determine whether the effect of UV was specific for the CAT gene only, Northern analysis of other cellular mRNAs was performed. Fig. 2 *B* and *C* suggest that the level of accumulated tubulin RNA also responded to UV-irradiation, while glyceraldehyde phosphate dehydrogenase (GAPDH) RNA did not.

Effect of X-Irradiation. A dose–response curve of CAT activity in NC2-11 cells measured 6 hr after x-irradiation is shown in Fig. 3*A*. The maximum 2-fold increase of CAT activity after x-irradiation occurred at a dose of 90 rads (1 rad = 0.01 Gy). This resulted in the death of 20–30% of the cells. An x-ray dose that killed 50% of the cells did not stimulate CAT activity measured after either 6 or 72 hr.

The kinetics of the CAT response following 90 rads of x-irradiation is shown in Fig. 3*B*. CAT activity started to increase 2 hr after NC2-11 cells were irradiated. The effect was maximal 6 hr after irradiation and gradually declined to the basal level by 48 hr. Thus, CAT activity in NC2-11 cells responded to x-irradiation more rapidly but to a lesser degree than to UV-irradiation.

To ensure that the effects observed were due to transcripts originating in the LTR of the provirus in NC2-11, S1 nuclease analysis was performed. A single S1 nuclease-protected fragment of approximately 790 bp was detected by autoradiography and was quantitated by densitometry (Fig. 3*C*). The length of the protected fragment is that expected for a CAT RNA transcript initiated from the Mo-MuSV LTR. The abundance of CAT RNA after the x-irradiation increased approximately 2-fold over the basal level and thus was comparable to the increase in enzyme activity. Furthermore, x-irradiation did not induce a new transcription initiation site, since the protected fragments were of the same size before and after treatment.

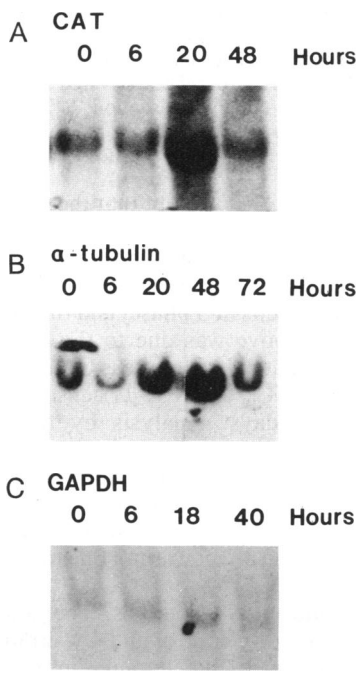

FIG. 2. Northern hybridizations of RNA from NC2-11 cells after UV-irradiation. Total RNA (25 μg) was loaded in each lane. The numbers above each lane represent the number of hours after UV-irradiation (10 J/m^2). The Northern blots were hybridized with the ^{32}P-labeled CAT DNA pCM-4 (*A*), the α-tubulin cDNA (*B*), and the human GAPDH cDNA (*C*).

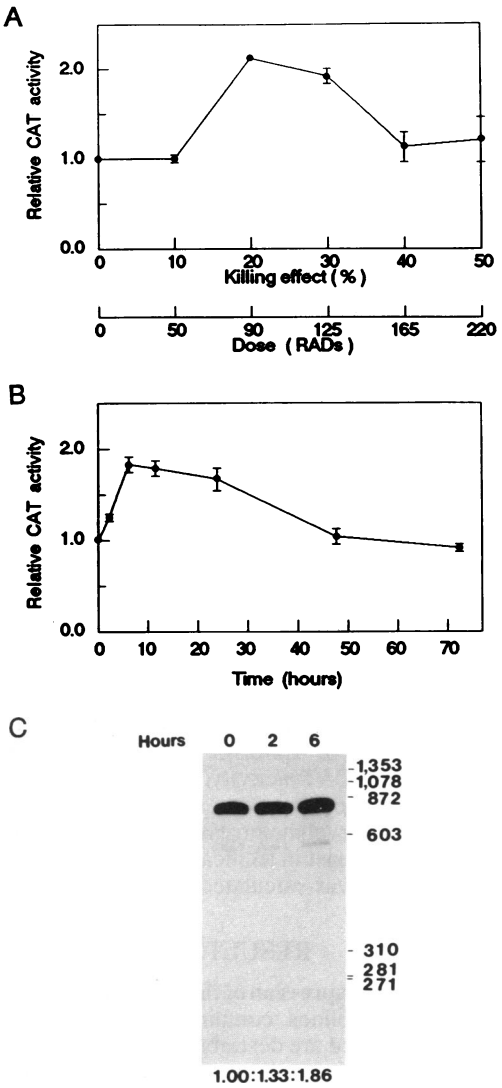

FIG. 3. Effect of x-irradiation on CAT activity in NC2-11 cells. (*A*) Dose–response of CAT activity. Cells were irradiated with x-ray, and the CAT activity was measured 6 hr later. The dosage is indicated both as the percent of cell killing and as rads. (*B*) Response of CAT activity as a function of time measured after 90 rads of x-irradiation. (*C*) S-1 nuclease analysis of CAT RNA. Total RNA from control and x-irradiated cells (90 rads) was analyzed with the probe pPC732 which had been labeled with ^{32}P at the 5′ end of an *Eco*RI site. The number above each lane represents the number of hours after irradiation. The number below each lane is the ratio of signal intensity based on results of the densitometer tracing. SEMs (vertical bars) are shown only when the value was >5% of the mean.

Effect of Phorbol Ester. When NC2-11 cells were exposed to phorbol 12-myristate 13-acetate (PMA) at a final concentration of 100 nM, the kinetics of the CAT activity response was similar to that following x-irradiation. The activity started to increase 2 hr after treatment, peaked at 6 hr, and then gradually declined to the basal level after 48 hr (data not shown). The extent of the increase in CAT activity was almost 4-fold with this treatment.

Table 1. Effects of the protein kinase inhibitor H7 on the response to UV, x-ray, and PMA

H7 preincubation, hr	Treatment		CAT expression, fold increase*
	Agent	Time, hr	
—	—	—	1.0
0.5	—	—	1.0
—	UV	20	3.9
0.5	UV	20	1.0
—	X-ray	6	1.9
0.5	X-ray	6	1.1
—	PMA	6	3.9
0.5	PMA	6	1.1

*Compared with the basal level of CAT activity.

Effect of the Protein Kinase Inhibitor H7 on the Response to UV, X-Ray, and Phorbol Ester. The protein kinase inhibitor H7 (19) at a concentration of 50 μM had no effect on the basal level of the CAT activity in NC2-11 cells. Preincubation of NC2-11 cells with 50 μM H7 for 30 min before UV-irradiation, x-irradiation, or PMA addition abolished the stimulating effect normally observed with these agents (Table 1).

Effect of Down-Regulation of Protein Kinase C on the Response to X- and UV-Irradiation. Exposure of cells to PMA for several hours will block subsequent signaling in some systems initially responsive to PMA (20, 21). This phenomenon is presumably due to down-regulation of protein kinase C in these exhaustively treated cells. NC2-11 cells show a similar down-regulation phenotype after prolonged treatment with PMA (Table 2, experiment 1). The 3.9-fold increase in CAT activity observed 6 hr after addition of PMA was eliminated by prior incubation with PMA for 46 hr. Next, the response of the CAT activity to x-irradiation was abolished by prior treatment of the NC2-11 cells for 46 hr with PMA (Table 2, experiment 2). Finally, the response of the CAT activity to UV-irradiation was only partially inhibited by down-regulation of protein kinase C (Table 2, experiment 3). After 48 hr of PMA preincubation, addition of fresh PMA increased the CAT level after a 6-hr incubation from 1.4-fold to 1.6-fold. Thus, the CAT activity response to the fresh addition was only 5% of the original response. With prior exposure to PMA for 48 hr followed by UV-irradiation and incubation for 20 hr, the CAT increase was 2.5-fold while the increase was 3.9-fold without PMA preincubation. In this case, with the down-regulation of protein kinase C, the CAT response to UV-irradiation (minus the residual PMA response) was 59% of the response without down-regulation.

DISCUSSION

NC2-11 Cells and the Expression of the CAT Gene. Southern analysis of the NC2-11 subline indicated that one copy of this construct was integrated in the genome. S1 nuclease analysis confirmed that CAT RNA was initiated from the upstream LTR. Therefore, a system was established to study the response, presumably via transcription, of a specific proviral LTR under a variety of experimental conditions.

Effects of UV-Irradiation, X-Irradiation, and PMA Treatments. UV-irradiation, x-irradiation, and PMA treatments stimulate CAT activity. A number of points should be made regarding the response. First, this response to UV-irradiation was not due to the preferential killing of nonexpressing cells as explained in *Results*. Second, the effect of UV-irradiation was not restricted to a single clone of infected cells but was demonstrated in the original NC2 cell population (data not shown) and two independently derived colonies, suggesting that the effects were not an artifact of a particular proviral location. Third, the CAT protein was very stable over an 8-hr

Table 2. Effect of down-regulation of protein kinase C on the response of CAT activity to x-ray and UV treatment

Exp.	PMA preincubation, hr	Treatment		CAT expression, fold increase*
		Agent	Time, hr	
1	0	PMA	6	3.9
	46	—	—	1.6
	46	PMA	6	1.6
2	0	X-ray	6	1.9
	46	—	—	1.3
	46	X-ray	6	1.2
3	6	—	—	3.8
	48	—	—	1.4
	68	—	—	1.2
	48	PMA	6	1.6
	0	UV	20	3.9
	48	UV	20	2.5

*Compared with the basal level of CAT activity.

period with or without exposure to UV, thus making irradiation effects on the protein an unlikely factor although not one that can be eliminated completely. Fourth, the S1 nuclease analysis indicated that there was no alteration in the transcriptional start site of the CAT mRNA after x-irradiation.

In general, the level of accumulated CAT RNA paralleled the level of CAT activity. However, there were discrepancies after UV-irradiation. First, there was a transient decrease in CAT activity 6 hr after UV-irradiation. Northern analysis indicated that the amount of RNA at that time was about the same as in untreated cells. The cause of this initial decrease in CAT activity is not clear. Second, by 48 hr after UV-irradiation, CAT RNA levels returned to the basal level but the CAT activity remained elevated, probably because of the stability of the CAT protein. We favor a hypothesis in which UV-irradiated cells (and possibly x-irradiated and PMA-treated cells as well) respond with only a short period of CAT gene expression. The gradual decline in enzyme activity after the peak value may reflect dilution of the protein as the cells grow and divide. An unlikely alternative is that UV is affecting the translation of CAT RNA (22).

Cis Elements Related to the Response. The response to UV-irradiation was observed with both α-tubulin and CAT RNA but not with GAPDH RNA. Thus, UV-irradiation does not affect the synthesis or stability of all RNAs. PMA treatment and UV-irradiation are known to induce expression of the human metallothionein and collagenase genes (23, 24). In the 5′ flanking region of these genes, there is a PMA-responsive element containing the sequence TGA$^{G}_{C}$-TCA with the ability to bind the AP-1 protein (25, 26). The metallothionein gene 5′ flanking sequence also contains an AP-2 binding site responsive to phorbol ester (27). Sequences closely related to these known PMA-responsive cis regulatory elements are not present in the Mo-MuSV LTR (28) or CAT gene.

Signaling Pathway. The difference in the kinetics of the response of CAT activity to UV-irradiation as opposed to x-ray may reflect differences in DNA repair mechanisms. In eukaryotic cells, DNA repair synthesis after x-ray damage involves rapid replacement of short patches of around four nucleotides (29). In contrast, DNA repair after UV damage involves long patches of 30–100 nucleotides (29). In UV-irradiated human fibroblasts, the time for half-maximal repair synthesis of DNA measured by nucleotide incorporation was 4.5 hr, while the time for half-maximal loss of thymine dimers ranged from 12 to 22 hr (30). In experiments reported here, the peak of CAT activity after UV-irradiation was at 18 hr, while the peak after x-irradiation and PMA treatment was at

6 hr. The possibility exists that the signal for CAT activation after UV is related to the removal of thymine dimers from a long-lived, single-stranded oligonucleotide, while the signal after x-ray arises from a short-lived stretch of single-stranded DNA. This signaling might be analogous to the binding of RecA protein to single-stranded DNA in the prokaryotic SOS system (1).

Evidence is presented here to support the idea that signaling, after x-ray exposure, involves protein kinase C. Down-regulation of protein kinase C with PMA (20, 21) eliminated the subsequent response of the CAT gene to x-irradiation. The UV response was partially blocked by PMA treatment, while H7, an inhibitor of protein kinase C as well as a weaker inhibitor of the cAMP and cGMP protein kinases (19), also totally blocked the response of the CAT gene to both UV- and x-irradiation.

As this project was nearing completion, a number of reports appeared confirming the ability of agents that induce the stress response (2) to activate transcription from an LTR. The human immunodeficiency virus (HIV) LTR has been shown to contain a tandem repeat sequence that is responsible for a 2- to 20-fold increase in CAT expression following PMA addition (31). However, this sequence is not present in the Mo-MuSV LTR. UV or mitomycin C, both agents that induce the stress response (2), can increase the HIV LTR CAT expression as well as viral growth and development (32). It is also of interest that the peak of the LTR CAT response to UV occurred at 20 hr (32). Finally, the level of endogenous rat leukemia virus RNA increased 7 hr after UV-irradiation (33).

The data presented in this paper emphasize the fact that UV- and x-irradiation can act not only by mutational mechanisms that affect DNA directly but also through a signaling system that is usually associated with the nonmutagenic process defined as tumor promotion.

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