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Identification of a UV-Induced *trans*-Acting Protein That Stimulates Polyomavirus DNA Replication

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Previous studies provided indirect evidence that the ability of a variety of DNA-damaging agents to induce asynchronous polyomavirus DNA replication in the H3 rat fibroblast cell line is mediated by a *trans*-acting factor. Using an erythrocyte insertion technique to introduce protein fractions from UV-irradiated cells into unirradiated H3 cells, we have now obtained evidence that this factor is a 60-kilodalton protein. These findings provide evidence that DNA damage in mammalian cells induces a factor that can alter the replication of a viral DNA.

Proteins that interact with specific DNA sequences are thought to contribute to the temporal and spatial restriction of DNA replication and transcription. Only a few of the proteins involved in the control of DNA replication have been well characterized in eucaryotic systems (2, 7–9, 20, 23). One approach to this problem is to study the factors responsible for the selective replication and amplification of specific DNA sequences, including viral DNAs (5, 10–14, 17, 18, 21, 24, 27, 29) or amplified cellular genes involved in drug resistance (26, 28). We have focused our studies on factors that induce the replication of polyomavirus DNA in response to various DNA-damaging agents; we have used the H3 cell line, a well-characterized rat fibroblast cell line transformed by a temperature-sensitive mutant of polyomavirus (ts-a) (10–12, 29). The exposure of these cells to chemical carcinogens (10–12) or UV irradiation (24) causes a striking and selective increase in polyomavirus DNA replication and the appearance of this DNA in the extrachromosomal fraction. The induced asynchronous polyomavirus DNA replication (APR) seen in this system may serve as a useful model for understanding the control of normal DNA synthesis as well as the phenomenon of DNA amplification that is often seen in tumor cells (1). Using cell fusion studies, we obtained evidence that the induction of polyomavirus DNA replication that results from DNA damage by chemical carcinogens or UV irradiation is mediated by a *trans*-acting factor (11, 24). Subsequent studies by others have also provided evidence that the induction of simian virus 40 DNA synthesis in hamster cells by DNA-damaging agents is also mediated by *trans*-acting factors (21, 29). In view of these results, it would be of considerable interest to identify this putative factor.

To identify this factor, we needed a method by which protein fractions obtained from nontransformed cells that had been treated with a DNA-damaging agent could be introduced into untreated H3 cells to assess whether specific proteins led to APR. Proteins and other macromolecules can be introduced into cultured mammalian cells by several methods, including microinjection, liposome fusion, osmotic lysis of pinocytic vesicles, and erythrocyte insertion (RCI) (for a review, see reference 19). We chose the latter method, since by modifying previously reported procedures, we were easily able to transfer proteins into a majority of the H3 cells on a tissue culture plate.

Proteins were introduced into cells by the RCI fusion technique as described by Ohara and Watanabe (22), with some modifications. Briefly, human erythrocytes (RBCs) (type O) were lysed in a hypotonic buffer and then mixed in a tube with the protein fraction for 45 min at room temperature by gentle rotation. The RBCs were resealed by increasing the osmotic concentration to 150 mM salt and then fused with the recipient cells in a ratio of 100:1, respectively, by using 25% polyethylene glycol 1000. The polyethylene glycol solution was added for 60 s to a pellet that contained both the RBCs and the recipient cells. This mixture was slowly diluted by adding Dulbecco modified Eagle medium (without serum) to a volume of 40 ml. The cells were centrifuged and plated in Dulbecco modified Eagle medium plus 10% calf serum, and 24 h later, the monolayer was washed extensively to remove residual free RBCs. To optimize the conditions for RCI, we loaded the RBCs with mouse immunoglobulins that were conjugated to rhodamine and then used a fluorescence cell sorter to quantitate both the total amount of protein that entered the cells and the fraction of cells that acquired the labeled protein. Thus, we could conveniently monitor the amount of protein at each stage of the RCI procedure. We found that about 52% of the H3 cells contained rhodamine-labeled proteins 24 h after they were fused to the preloaded RBCs. By microscopy, we could also detect a large number of RBCs at various stages of fusion with the rat fibroblasts (data not shown).

Having established the efficiency of the RCI method, we prepared nuclear and cytoplasmic protein fractions from either control (nontreated) or UV-C (254 nm; 2 J/m^2)-treated normal rat fibroblasts (that did not contain polyomavirus DNA sequences), loaded RBCs with these fractions, and then fused them to H3 cells that had not been exposed to UV. After 48 h, the Hirt supernatant fraction of DNA (extrachromosomal DNA) was extracted from the H3 cells (6), separated by gel electrophoresis, and blot hybridized by the Southern procedure to a ^{32}P-labeled polyomavirus DNA probe (24). In this and all subsequent studies, we analyzed the H3 cells 48 h later, since our previous studies indicated that optimal induction of polyomavirus DNA occurs within 48 to 72 h after cell fusion (10, 11, 24).

We found that both the nuclear and cytoplasmic fractions from the UV-treated cells caused a marked induction of APR

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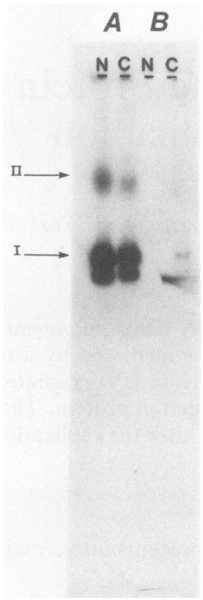

FIG. 1. Effects of nuclear or cytoplasmic proteins obtained from UV-treated normal rat 6 fibroblasts on polyomavirus DNA synthesis in H3 cells following RCI. Cytoplasmic and nuclear protein extracts were prepared as previously described (15, 16) from normal rat 6 fibroblasts (10) at 24 h after UV-C irradiation of these cells (254 nm; 2 J/m^2). These extracts or control fractions obtained from unirradiated rat 6 cells, were loaded into RBCs at a concentration of 0.6 mg/ml and then fused with the unirradiated polyomavirus-transformed rat fibroblast cells (H3) grown at 39°C. The cultures were then shifted to 33°C, the permissive temperature for polyomavirus DNA replication in H3 cells (10), and 48 h later, the extrachromosomal DNA fraction (Hirt supernatant) was isolated, separated by gel electrophoresis, and hybridized to a ^{32}P-labeled polyomavirus DNA probe by the Southern method, as previously described (10, 24). N and C, Samples obtained from H3 cells that received nuclear or cytoplasmic extracts, respectively, from UV-irradiated (A) or unirradiated (B) rat 6 fibroblasts; I and II, two major species of free viral DNA found in this cell line (10).

in the H3 cells, whereas the corresponding fractions from unirradiated cells lacked this activity (Fig. 1). To quantitate this response, we fused H3 cells with RBCs that contained increasing concentrations of cell extracts obtained from UV-irradiated normal rat fibroblasts. We found that the induction of APR was dose dependent and was saturated when the RBCs were loaded with approximately 0.6 mg of cell protein per ml (Fig. 2A). Using the latter protein concentration, we studied the time course for the production of the relevant factor(s). Whole-cell extracts were prepared from UV-irradiated and control, normal rat fibroblasts at 2, 6, 12, 24, 48, and 72 h after UV exposure. Each fraction was then introduced into the H3 cells by the RCI method, and the extent of polyomavirus DNA replication was determined. We observed inducing activity in the cell extract prepared as early as 6 h after UV exposure. This activity reached a peak at 24 h and then declined to the base-line level by 48 h after UV exposure (Fig. 2B).

Since the experiments described above were performed with crude cell extracts, it was of interest to purify the active fraction. A whole-cell extract was prepared from normal rat fibroblasts 24 h after UV exposure, and this material was fractionated by fast-protein liquid chromatography by using a Mono Q (Pharmacia) anion-exchange column. The eluate was monitored at 280 nm, and each peak was pooled. These pools were then loaded into RBCs, and these were then assayed for induction of polyomavirus DNA replication in H3 cells. A single major peak of activity was detected (Fig. 3A) and was then further fractionated by employing a Superose 6 (Pharmacia) gel filtration column. This procedure yielded a well-resolved peak which demonstrated a 12-fold induction of APR when it was introduced into H3 cells by the RCI technique (Fig. 3B). We estimate that the molecular mass of the active fraction is about 60 kilodaltons, based on protein markers applied to the Superose 6 gel column.

The present studies provide direct evidence that UV irradiation of rat fibroblasts leads to the accumulation of a protein with an apparent molecular weight of 60,000 that can induce APR. Although the precise mechanism by which this factor is formed or becomes activated is not known, it is very likely that DNA damage plays a critical role, since the major known effect on cells of the UV source used in the present

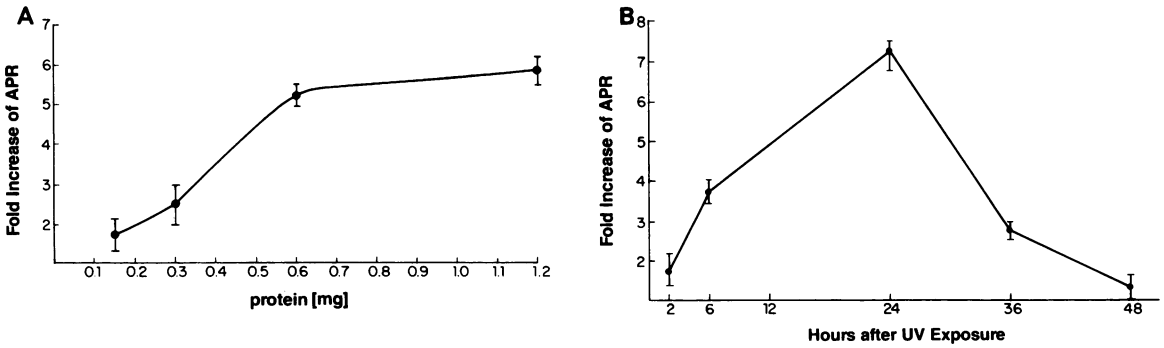

FIG. 2. Concentration and time course studies. (A) A whole-cell extract was prepared from UV-treated rat 6 cells, and increasing concentrations of this extract (0.3 to 1.2 mg/ml) were loaded into RBCs, which were then fused to unirradiated H3 cells. After 48 h, the extrachromosomal DNA was extracted from the H3 cells, following the methods described in the legend to Fig. 1. The abundance of polyomavirus DNA was determined by dot blot hybridization (24), by using a ^{32}P-labeled polyomavirus DNA probe. The blots were quantitated by densitometry of the radioautographs, and the data are expressed as fold increase of APR relative to the results obtained with DNA samples from H3 cells that were fused with RCBs loaded with an extract from unirradiated rat 6 cells. (B) Whole-cell extracts were prepared from UV-irradiated and unirradiated rat 6 cells at the indicated times following irradiation. Each of these extracts was loaded into RBCs, which were then fused with unirradiated H3 cells, and 48 h later, the Hirt DNA fraction was isolated from H3 cells and analyzed as described for panel A.

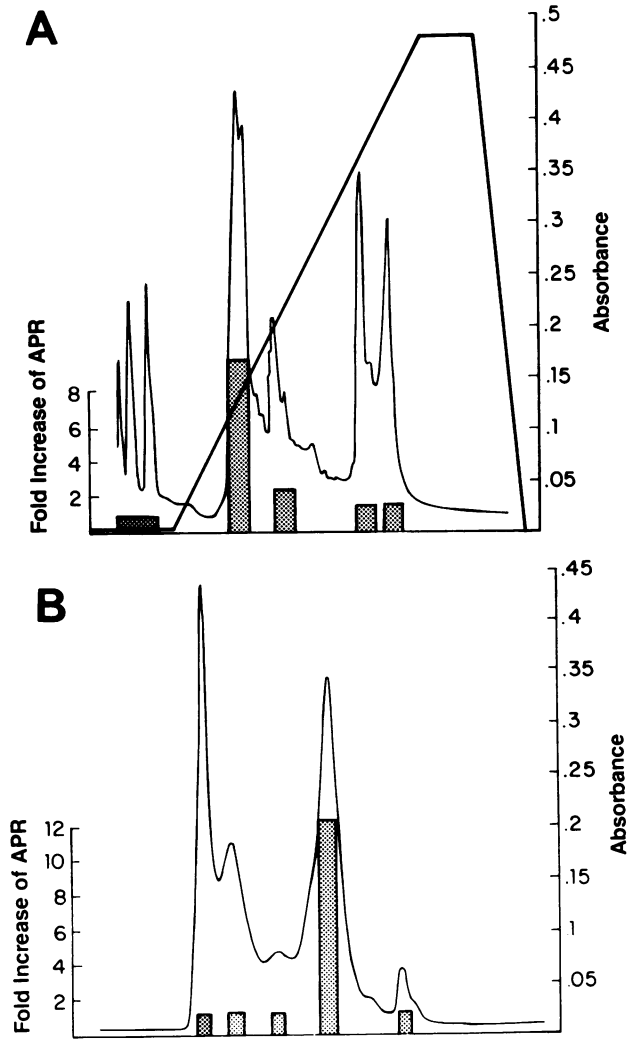

FIG. 3. Separation of the *trans*-acting factor on anion-exchange and gel filtration fast-protein liquid chromatography columns. (A) Whole-cell extract (5 mg) obtained from rat 6 cells at 24 h after UV irradiation (Fig. 2) was fractionated on a Mono Q column by using a gradient of 0.02 to 1 M NaCl in 0.02 M Tris (pH 8). The eluate was tested for its ability to induce polyomavirus DNA replication when 200 μg of each pooled peak was introduced into unirradiated H3 cells by the RCI procedure (▭) as described in the legends to Fig. 1 and 2. (B) The separation described for panel A was repeated, and 0.5 mg of the active peak obtained from the Mono Q column was then loaded onto a Superose 6 column and eluted with 0.05 M potassium phosphate buffer (pH 7.6) that contained 0.15 M NaCl. The major peaks (50 μg from each) were then assayed for induction of polyomavirus DNA synthesis in unirradiated H3 cells by the RCI technique.

studies (254 nm) is the formation of pyrimidine dimers in DNA (4, 25, 30). Furthermore, a number of previous studies have indicated that a variety of DNA-damaging agents or drugs can induce the asynchronous replication of polyomavirus (10–12, 17, 24) or simian virus 40 (5, 13, 27, 29) DNA or can result in the amplification of cellular genes (3, 14, 26, 28), presumably by causing transient arrest of DNA replication. The *trans*-acting factor that we have identified is clearly not the polyomavirus large T antigen nor any other protein encoded by the virus, since the formation of this factor can be induced in rat fibroblasts lacking polyomavirus DNA. We hope that the further purification and characterization of this protein will provide insights into its mechanism of action. Since it appears to be encoded by cellular DNA, we assume that it might play a more general role in the response of cells to DNA damage. Thus, the present findings add to the accumulating evidence that DNA damage in mammalian cells can not only cause targeted mutations but can also lead to the induction of a variety of functions that can have complex effects on the genome and the phenotype of the target cell.

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