

# Microinjection of partially purified protein factor restores DNA damage specifically in group A of xeroderma pigmentosum cells

(UV irradiation/DNA repair/human cells/factor A)

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Communicated by Frank H. Ruddle, October 9, 1985

**ABSTRACT** Microinjection of cell extracts prepared from both human placenta and HeLa cells into xeroderma pigmentosum (XP) cells of complementation group A restores unscheduled DNA synthesis (UDS) in these cells after UV irradiation [de Jonge, A., Vermeulen, W., Klein, B. & Hoeijmakers, J. (1983) *EMBO J.* 2, 637-641]. These cells also showed normal resistance to UV irradiation. The half-life of the factors in the cell extracts corresponding to the UDS activity (factor A) was 14 hr in XP cells of group A, and the maximal level of UDS was exerted 2 hr after microinjection. The factors were sensitive to protease treatment but not to RNase treatment and were found to be  $\approx 160$  and  $\approx 90$  kDa by gel filtration. These two fractions of the factor(s) acted specifically in XP cells of complementation group A among complementation groups A, B, C, D, F, G, and probably E and H.

Xeroderma pigmentosum (XP) is an autosomal recessive hereditary disease with a high incidence of skin cancer on exposure to sunlight. Cells from XP patients show higher sensitivity to UV irradiation than do wild-type cells, because they have a defective DNA repair system. From the results of somatic cell hybridization studies, XP cells are now divided into 10 complementation groups, including recently found group I (1). Complementation group A-I has defects in the excision repair system, whereas XP variants have normal excision repair but an impaired post-replication repair system (2). There have been many attempts to identify the factors involved in the UV light-damaged DNA repair system in eukaryotic cells, but very little is yet understood about the molecular basis of the complementation groups of this disease because no specific factor has been purified. As an alternative approach, some prokaryotic repair enzymes such as T4 endonuclease V (3) have been successfully used in eukaryotic cell systems to get information on the mechanism of DNA repair in eukaryotic cells. These studies imply that the excision step in the DNA repair reactions does not work in at least some of the complementation groups of XP cells.

In this work, we showed that after microinjection of normal human cell extracts, XP cells of complementation group A had a normal level of unscheduled DNA synthesis (UDS) and became resistant to UV irradiation. Then, as a first step to purify specific factors involved in DNA repair, we partially purified proteinaceous factors that are deficient in these XP cells and determined their molecular size by gel filtration.

## MATERIALS AND METHODS

**Cells.** XP 27OS (group A), XP 2OSSV (a transformant of XP 2OS by simian virus 40) and XP 2YO (group F) were gifts from H. Takebe. GM-3176, GM-0435, GM-2415, GM-3021A, and GM-3248 were purchased from NIGMS Human Genetic

Mutant Cell Repository (Camden, NJ). CRL-1199 was purchased from ATCC (Rockville, MD). Normal human skin fibroblasts were obtained from a volunteer in our laboratory. All cells described above were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 9% fetal calf serum. HeLa cells were cultured in suspension in Eagle's minimal essential medium containing 5% calf serum. Human placenta was used without freezing.

**Autoradiography.** For autoradiography, cells on coverslips were washed once with phosphate-buffered saline (PBS) and irradiated with UV light 30 J/m<sup>2</sup> and at a fluence rate of 0.74 J/m<sup>2</sup>/sec. These cells were then incubated for 2.5 hr at 37°C in DMEM containing 1% fetal calf serum and [*methyl* 1',2'-<sup>3</sup>H]thymidine (Amersham) at 50  $\mu$ Ci/ml (1 Ci = 37 GBq). They were fixed in a mixture of methanol and acetic acid (3:1), mounted on glass slides with Eukitt (O. Kindler, F.R.G.), coated with Sakura NR-M2 emulsion, and exposed for 7 days at 4°C. These slides were stained for 5 min with 3% Giemsa solution in 25 mM sodium phosphate buffer (pH 7.2).

**Microinjection.** A cell extract was injected into cultured cells by a method described elsewhere (4) with some modifications. For the present experiments, we developed a new type of hydraulic micromanipulator in collaboration with Narishige Scientific Instrument Laboratory (Tokyo). With this micromanipulator, a capillary can be moved in four directions by four independent knobs, and rapid and precise microinjection is possible. For experiments to determine the survival of XP 2OSSV cells after microinjection of cell extracts, we used special coverslips with fine grids (5) to identify individual cells. We usually microinjected 50-100 cells for one point in each experiment.

**Preparation of Cell Extract.** Cell extracts were prepared as described by Manley *et al.* (6) with some modifications. All preparation procedures were carried out at 4°C. About 10 ml of cell pellet was induced to swell by adding 40 ml of buffer A (1 mM EDTA/5 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/10 mM Tris-HCl, pH 7.9) for 20 min. Then it was homogenized, and 40 ml of buffer B [10 mM MgCl<sub>2</sub>/50% (vol/vol) glycerol/2 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/50 mM Tris-HCl, pH 7.9] was added. In some cases, the nuclear fraction was separated from the cytoplasmic fraction by centrifugation at this step. Then 10 ml of saturated ammonium sulfate was added drop-by-drop, and the mixture was stirred for 20 min and then centrifuged for 3 hr at 50,000 rpm in a Hitachi 65 RP rotor. The supernatant was treated further with ammonium sulfate and material precipitated at 20-50% saturation was collected by centrifugation for 20 min at 16,000 rpm in the same rotor, redissolved in 7.5 ml of buffer C (3 mM MgCl<sub>2</sub>/20 mM KCl/0.1 mM EDTA/1 mM dithiothreitol/250 mM sucrose/25 mM Tris-HCl, pH 7.9) and dialyzed against the same buffer. Such preparations are called crude cell extracts in this

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Abbreviations: XP, xeroderma pigmentosum; UDS, unscheduled DNA synthesis; EF-2, elongation factor 2.

paper. The dialyzed crude cell extracts were applied to a DE-52 column. Factor A was mainly eluted with buffer consisting of 70 mM KCl/3 mM MgCl<sub>2</sub>/0.2 mM EDTA/1 mM dithiothreitol/250 mM sucrose/50 mM Tris·HCl, pH 7.9. The eluted fractions were concentrated by precipitation at 70% saturation of ammonium sulfate, redissolved in ≈1.5 ml of buffer D (6 mM MgCl<sub>2</sub>/140 mM KCl/0.2 mM EDTA/1 mM dithiothreitol/250 mM sucrose/50 mM Tris·HCl, pH 7.9) and applied to a Sephadex G-200 column equilibrated with the same buffer. Fractions of 0.6 ml were collected and their factor A activity was examined by microinjection.

**Chemicals.** Trypsin, soybean trypsin inhibitor, and RNase A were purchased from Sigma. Human placenta RNase inhibitor was prepared according to Blackburn *et al.* (7) by T. Arioka in this laboratory. DE-52 and Sephadex G-200 were purchased from Whatman and Pharmacia, respectively.

## RESULTS

**UDS and Survival of Microinjected XP Cells After UV Irradiation.** Many attempts to develop an *in vitro* assay system for the specific factors involved in UV light-damaged DNA repair reactions that are defective in XP cells have been unsuccessful. Therefore, as an alternative approach, we used a living cell system combined with a capillary microinjection technique. When crude cell extracts prepared either from HeLa cells or from human placenta were microinjected into XP cells of complementation group A, they showed a normal level of UDS on autoradiography, as shown in Fig. 1. Such UDS was not seen even after microinjection of the same extract when UV irradiation was omitted. Furthermore, no UDS was detected after microinjection of a crude cell extract prepared from XP cells of complementation group A. Thus, crude cell extract of normal human cells contains some factor(s) with a defect in XP cells of complementation group A. We call such factor "factor A" according to Giannelli *et al.* (8).

To see the effect of concentration of cell extracts on the amount of UDS, a series of diluted extracts was microinjected into XP cells of group A, and the percentage of cells showing positive UDS was determined (Fig. 2). While almost 100% of the microinjected cells showed positive UDS up to a 1:8 dilution, the grain number per nucleus at this dilution rate was roughly one-third that of the original extract. Upon further dilution, the percentage declined rapidly with a decrease in the grain number per nucleus. From such a dose-response curve of UDS, we can estimate the titer of the A activity of a given cell extract.

Next, we tested whether these UDS-positive microinjected cells became resistant to UV irradiation. In this experiment, XP 20SSV cells, which are transformants of simian virus 40, were used instead of primary culture cells because it was easy to determine the viability of these cells from their rapid growth rate. As shown in Fig. 2, XP cells microinjected with the original undiluted extract showed almost the same resistance to UV irradiation as wild-type cells. The resistance, however, declined more rapidly than the UDS activity and no detectable activity remained after a 1:7 dilution.

**Stability of Factor A in XP Cells.** To determine the stability of factor A in XP cells of complementation group A, a crude cell extract was diluted in such a way that ≈90% of the microinjected cells still showed positive UDS but on any further dilution, only some of them became UDS positive under the conditions of titration of factor A shown in Fig. 1. Under these conditions, the stability of factor A is reflected in the percentage of UDS-positive cells. After microinjection of the diluted cell extract, the cells were cultured for various times at 37°C. Then these cells were exposed to UV light and labeled with [<sup>3</sup>H]thymidine for 2.5 hr to see the UDS-positive cells. As shown in Fig. 3, the number of UDS-positive cells remained constant for the first 2 hr. The grain number per nucleus,

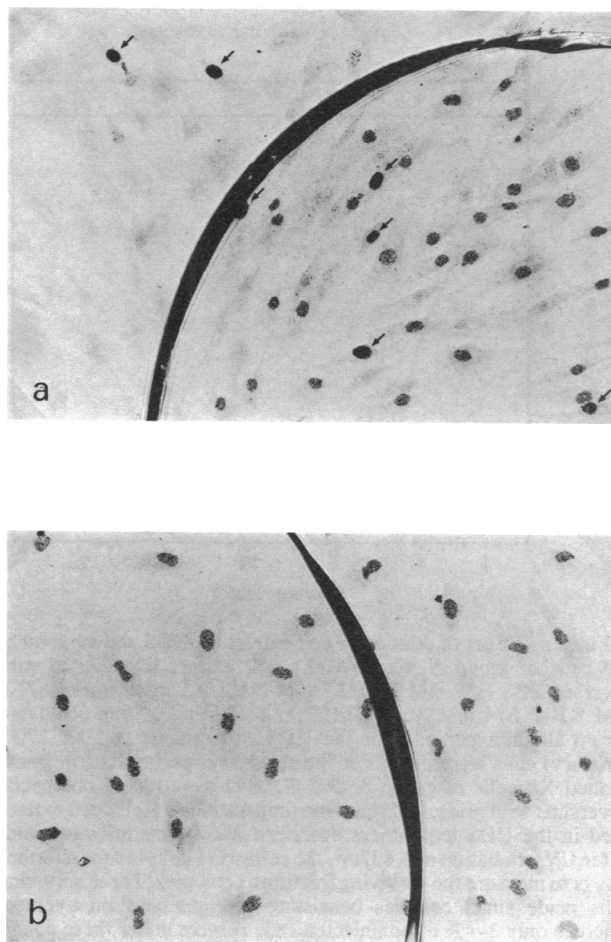


FIG. 1. Autoradiography of UDS after microinjection of a cell extract. Cells on coverslips with small circles engraved with a diamond knife were cultured for 3 days. Those cells inside the small circle (part of which is seen in these pictures) had been injected with a HeLa cell crude extract. (a) XP 27OS (group A); (b) untreated normal human skin fibroblasts (normal cells). Arrows show heavily labeled S-phase cells. Note the grain number of UDS on XP cells is equal to that on wild-type cells. Almost the same level of UDS was observed after microinjection of partially purified and concentrated factor A into XP 27OS cells (see footnote to Table 1).

however, reached maximal 2 hr after microinjection. After that, the percentage declined slowly with a half-life of ≈14 hr.

**Molecular Weight of Factor A.** Crude cell extracts from either HeLa cells or human placenta were fractionated by ammonium sulfate and DE-52 column chromatography. Fractions containing factor A were applied to a Sephadex G-200 column. The activity of factor A in each fraction was examined by microinjection of an aliquot into XP 27OS cells. As shown in Fig. 4, we detected UDS activity in two positions in the eluate. Since the positions of these materials corresponded to ≈160 and ≈90 kDa, we named these materials 160K and 90K factor, respectively. To confirm their molecular sizes, we determined the amount of elongation factor 2 (EF-2) in each fraction obtained by this gel filtration. Eukaryotic EF-2, an essential factor for protein synthesis, is a highly conserved protein of ≈100 kDa. The maximal amount of EF-2 was found in fraction 31 (shown by an arrow in Fig. 4). This position is just in front of the second peak of factor A. A similar gel filtration pattern with two peaks of factor A at the same positions was obtained from a crude HeLa cell extract, which was prepared by precipitation with 50% saturated ammonium sulfate only without DE-52 fractionation. We could not detect any activity of factor A by the

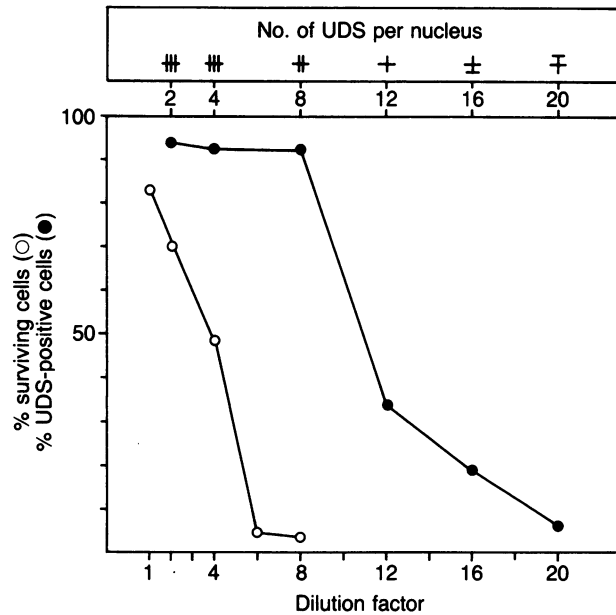


FIG. 2. Effect of dilution of cell extract on UDS and survival of XP cells of group A. Crude HeLa cell extract was diluted with reversed PBS (137 mM KCl/2.7 mM NaCl/8.1 mM  $\text{Na}_2\text{HPO}_4$ /1.5 mM  $\text{KH}_2\text{PO}_4$ /4 mM  $\text{MgCl}_2$ , pH 7.4) containing 0.5 mg of bovine serum albumin per ml. For the UDS experiments (●), XP 27OS (primary) cells were used. For the survival experiments (○), transformed XP cells of group A (XP 20SSV) were plated on special coverslips with grids, and the same preparation of HeLa cell extract used in the UDS experiment described above was microinjected. After UV irradiation at  $3.4 \text{ J/m}^2$ , the cells were cultured for a further 3 days to measure the surviving fraction as colonies. These surviving cells made small colonies consisting of eight cells on average, whereas only 3–4% of noninjected cells survive under these conditions. For the amount of UDS per nucleus, five grades (̄ through +++ were set in this experiment. UDS shown by +++ is a level of that of normal cells, whereas UDS shown by ̄ is just above the surrounding noninjected XP cells.

same method in a crude cell extract of XP 20SSV of complementation group A.

**Activity of the Two Fractions of Factor A in Other Complementation Groups.** Next, the two fractions of factor A separated by Sephadex G-200 were each concentrated and injected into XP cells of different complementation groups. After microinjection of these concentrated preparations, all the XP 27OS cells (group A) tested showed positive UDS and the grain number over their nuclei became almost comparable to that of wild-type cells. As shown in Table 1, the 160K and 90K factors had specific effects on cells of complementation group A.

**Some Characteristics of Factor A.** Factor A in crude extracts was quite stable and could be stored for 1 month at  $4^\circ\text{C}$  without detectable loss of activity. It was also resistant to repeated freeze-thawing. Unfortunately, during or after DE-52 chromatography and gel filtration, the stability of these factors decreased rapidly. The nature of these factors was examined by treating crude cell extracts from HeLa cells ( $5.6 A_{280}$ ) with either trypsin (final concentration, 0.2 mg/ml) or RNase A (0.02 mg/ml) for 30 min at  $37^\circ\text{C}$  and then adding excess trypsin inhibitor or RNase inhibitor and continuing incubation for a further 20 min at  $37^\circ\text{C}$ . Subsequent measurement of factor A activity showed that trypsin caused complete inactivation, whereas RNase had no effect.

## DISCUSSION

We partially purified proteinaceous factor A of  $\approx 160$  and  $\approx 90$  kDa by gel filtration. These factors restored UDS in XP cells

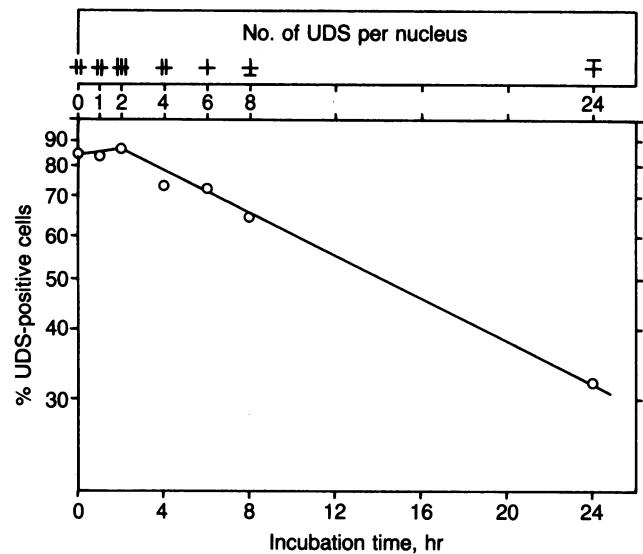


FIG. 3. Stability of factor A in XP cells of group A. HeLa cell extract with a titer of factor A that is equivalent to a 1:8 dilution in Fig. 2 were microinjected into XP 27OS cells. After incubation for different periods of time at  $37^\circ\text{C}$ , these cells were exposed to UV light ( $30 \text{ J/m}^2$ ) and then labeled for 2.5 hr with  $^3\text{H}$ thymidine under the conditions described in Fig. 2 to find UDS-positive cells. The amount of UDS per nucleus was determined in five grades (̄ through +++ as described in Fig. 2.

only of complementation group A and made these cells resistant to UV irradiation. There are three possibilities concerning the relationship between these two factors. One is that the larger factor is a dimer of the smaller one. The second possibility is that the 160K factor is a complex of the 90K factor and an unknown factor of  $\approx 70$  kDa. We first

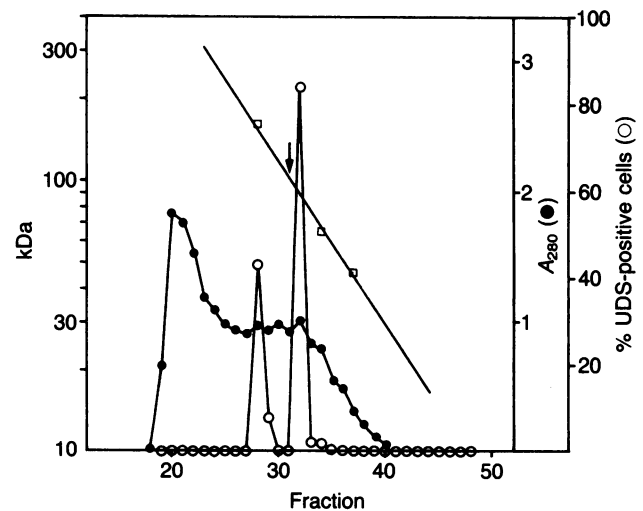


FIG. 4. Gel filtration of factor A. HeLa cell extracts were partially purified. After treatment with Sephadex G-200, each fraction was tested for activity of factor A as described in Fig. 1. Under the conditions in this experiment, the grain number of UDS on XP 27OS cells was less than that of normal cells, and the activity of factor A was expressed as the percentage of cells showing positive UDS among those injected. Rabbit IgG (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa) were used as molecular size markers (□). For more accurate determination of the size of the second peak at  $\approx 100$  kDa, we measured the amount of EF-2 in each fraction tested for factor A activity as described elsewhere (9). Because eukaryotic EF-2, including that of humans, has a molecular mass of 100 kDa, this protein is a good internal marker. The position of the maximal EF-2 activity is shown by an arrow.

Table 1. Effect of factor A on other complementation groups

Cell	Complementation group	UDS	
		160K	90K
XP27OS	A	+	+
CRL1199	B	-	-
GM3176	C	-	-
GM0435	D	-	-
GM2415	E	(-)	(-)
XP2YO	F	-	-
GM3021A	G	-	-
GM3248	H	(-)	(-)

The 160K and 90K factors shown in Fig. 4 were concentrated  $\approx 5$ -fold by precipitation with 70% saturated ammonium sulfate and redissolved in buffer D. The grain numbers of UDS on XP 27OS cells after microinjection of these concentrated factors were almost the same as those on control normal cells. These concentrated factors were injected into other complementation groups of XP cells. UDS was determined as described in Fig. 1. The symbol (-) indicates no increase in grain number over that of surrounding untreated cells. Because group E cells, and more especially H cells, have high residual values of UDS, it was very difficult to see a change in grain number in these two complementation groups. The final answer to these two groups must await further purification of factor A with a high titer.

suspected that this unknown factor might be a defective factor in some other complementation group and expected positive results when the 160K factor was injected into XP cells of other complementation groups. Although we found that these factors were effective only in group A, it seems possible that the 160K factor may be effective in the recently found group I (1) or in some as yet unidentified complementation group. The third possibility is that the 90K factor is a degradation product of the 160K factor. In connection with these possibilities, the data of Giannelli *et al.* (8) are very suggestive. They speculate that factor A might be a polymer from the kinetics of XP cells of group A in heterokaryons between group A and wild-type cells. Recently, Legerski *et al.* (10) reported that the molecular size of factor A mRNA is 11S (10), which would encode a protein of  $\approx 31$  kDa. Based on this size, our 90K factor could be either a homopolymer of three factor A polypeptides or a heteropolymer associated with some unidentified factor(s). NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis of the partially purified fractions failed to identify such proteins because many bands are detected in these active fractions (data not shown). We have previously demonstrated that endonuclease V derived from T4 phage restores UV light-induced UDS in XP cells of group A-E (3). While it is an interesting question whether the partially purified factor A has an endonuclease activity specific to UV-induced DNA damage or not, we could not get a definite answer because our active fractions still contain strong nonspecific endonuclease activity measured as described (11). Further purification of these factors is required to answer these questions.

Similar amounts of factor A were obtained from the cytoplasm and nuclei of wild-type cells (data not shown). In our standard procedure, the final volume of the crude cell extract was almost the same as the initial volume of the cell pellet. When the average volume injected per cell was 5–10% of the volume of a single cell (4), the grain number of UDS in cells microinjected with undiluted cell extract was equal to that of wild-type cells. Thus one cell seems to have at least 10 times the necessary amount of factor A. For detection of the activity of factor A, we injected cell extracts into the cytoplasm of XP cells and immediately processed these cells for autoradiography. From the stability experiments shown in Fig. 3, the maximal amount of UDS by factor A in nucleus was detected 2 hr after microinjection. Therefore, these factors must migrate rapidly from the cytoplasm into the nucleus.

Although XP cells microinjected with a crude extract showed a normal level of UDS up to a 1:4 dilution, a normal level of resistance to UV irradiation was observed only when undiluted extract was microinjected (Fig. 2). This discrepancy can be explained as follows. While the reaction of excision repair in human cells lasts a long time— $>1$  day (12)—we checked the UDS activity in microinjected cells by labeling only for the initial 2.5 hr after microinjection and then UV irradiation. Therefore, even if enough of factor A is microinjected to get a normal level of UDS under our conditions, it will not be enough to repair all DNA damage.

We believe that the present *in vivo* assay system with the microinjection technique should be useful for purification and isolation of other biologically active materials for which an ordinary *in vitro* assay system is not available.

This work was in part supported by Grants-in-Aid 56222007 and 60127010 from the Ministry of Education, Science and Culture of Japan.

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