Proc. Nat. Acad. Sci. USA
Vol. 72, No. 10, pp. 4071–4075, October 1975
Genetics

Restoration of ultraviolet-induced unscheduled DNA synthesis of xeroderma pigmentosum cells by the concomitant treatment with bacteriophage T4 endonuclease V and HVJ (Sendai virus)

(excision repair/complementation group/incision)

KIYOJI TANAKA*, MUTSUO SEKIGUCHI†, AND YOSHIO OKADA*‡

*Department of Animal Virology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan; and †Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan

Communicated by James D. Ebert, July 25, 1975

ABSTRACT **Ultraviolet (UV)-induced unscheduled DNA synthesis of xeroderma pigmentosum cells, belonging to complementation groups A, B, C, D, and E, was restored to the normal level by concomitant treatment of the cells with T4 endonuclease V and UV-inactivated HVJ (Sendai virus). The present results suggest that (*1*) T4 endonuclease molecules were inserted effectively into the cells by the interaction of HVJ with the cell membranes, (*2*) the enzyme was functional on human chromosomal DNA which had been damaged by UV irradiation in the viable cells, (*3*) all the studied groups of xeroderma pigmentosum ("variant" was not tested) were defective in the first step (incision) of excision repair.**

Xeroderma pigmentosum (XP) is a rare hereditary disease caused by mutation of an autosomal recessive gene. It is characterized by development of pigmentation abnormalities and numerous malignancies on exposure of the skin to sunlight. Cleaver (1) first showed that cells from XP patients lack an ability to repair ultraviolet (UV)-induced damage of DNA. Inability of excision repair of UV-damaged DNA in XP cells could be measured autoradiographically, since levels of unscheduled DNA synthesis are lower than those in normal cells (1, 2). deWeerd-Kastelein *et al.* (3) reported that cells of classical XP patients and deSanctis-Cacchione patients were genetically complementary. Both types of cells showed low levels of unscheduled DNA synthesis, but when they were fused by HVJ (Sendai virus) the levels became normal. Thus, five complementation groups in XP cells have been recently identified by the cell fusion technique (4).

Setlow *et al.* (5) suggested that XP cells fail to start the excision process because they lack the required function of an UV-specific endonuclease. Paterson *et al.* (6) and Buhl and Regan (7) reported that an UV-specific endonuclease from *Micrococcus luteus* was effective for introducing single-strand breaks in UV-damaged DNA of XP cells (both classical and deSanctis-Cacchione). On the contrary, Cleaver (8) observed that after UV-irradiation strand breaks accumulated in DNA of XP cells (deSanctis-Cacchione). All these experiments (5–8) were carried out using an alkaline-sucrose gradient centrifugation technique *in vitro* to estimate the degree of strand breaks of UV-irradiated DNA.

We planned to identify autoradiographically what kind of cells in the five complementation groups was defective in the enzyme for strand breaks of UV-damaged DNA, by insertion of an UV-specific endonuclease into viable XP cells. HVJ is well known to have cell fusion activity (9). At an early stage of the cell fusion reaction, the structure of the cell membrane is partially damaged by the action of the virus and then the structure is restored again. Thus, it seemed possible that macromolecules could be inserted into viable cells from the outside during the interaction between the cell membrane and HVJ. As an enzyme, T4 endonuclease V was used, which was isolated from *Escherichia coli* infected with bacteriophage T4 (10, 11). This enzyme catalyzes the first step of excision repair in T4-infected cells (12). We tried to insert this enzyme into XP cells with help of HVJ, and this trial was successful.

Abbreviations: HVJ, hemagglutinating virus of Japan, synonym: Sendai virus; XP, xeroderma pigmentosum; UV, ultraviolet; PEME, phosphate–ethylene glycol–mercaptoethanol–EDTA buffer; ENase, endonuclease V.

‡ To whom all correspondence should be addressed.

MATERIALS AND METHODS

Cells. Human fibroblasts derived from biopsy specimens of skin of normal subjects and XP patients were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (Flow Lab.). Strain HNSF3 was derived from normal skin. Strains XP8OS, XP10OS, and XP6T0O were from Japanese XP patients and have been classified in complementation group A§. Strain CRL 1223, 1199, 1170, 1160, and 1259, belonging to the complementation groups A, B, C, D, and E, respectively, were obtained from the American Type Culture Collection.

Enzymes. T4 endonuclease V was prepared from T4D-infected *E. coli* 1100 by a modification of the procedure described previously (10,¶). A crude extract was subjected to phase partition in dextran-500 and polyethylene glycol-6000 and the supernatant solution was dialyzed against PEME buffer (10 mM potassium phosphate, 10% ethylene glycol, 10 mM 2-merceptoethanol, and 2 mM EDTA, pH 6.5). The fraction was applied to a column of CM-Sephadex C-25 and eluted with a linear gradient of 0–0.5 M KCl in PEME buffer. The purity of the fraction eluted (Fraction III) was about 400-fold that of the crude extract, and after dialysis against PEME buffer, it was used in the present experiments. The enzyme preparation (19 units/ml) exhibits no detectable incorporation of [^{3}H]dTTP into acid-insoluble form under the standard assay conditions for DNA polymerase (13) and does not release pyrimidine dimers from T4 endonuclease V-treated, irradiated DNA under the conditions for the assay of dimer excision enzyme (5′ → 3′ exonuclease) (14), indicating that the preparation contains no or only little DNA polymerase and 5′ → 3′ exonuclease activities.

A preparation of bovine pancreatic deoxyribonuclease purified by chromatography was obtained from Sigma Chemical Co.

HVJ (Sendai Virus). HVJ, Z strain, propagated in embryonated eggs, was partially purified by differential cen-

§ H. Takebe and K. Tanaka, in preparation.

¶ S. Yasuda and M. Sekiguchi, in preparation.

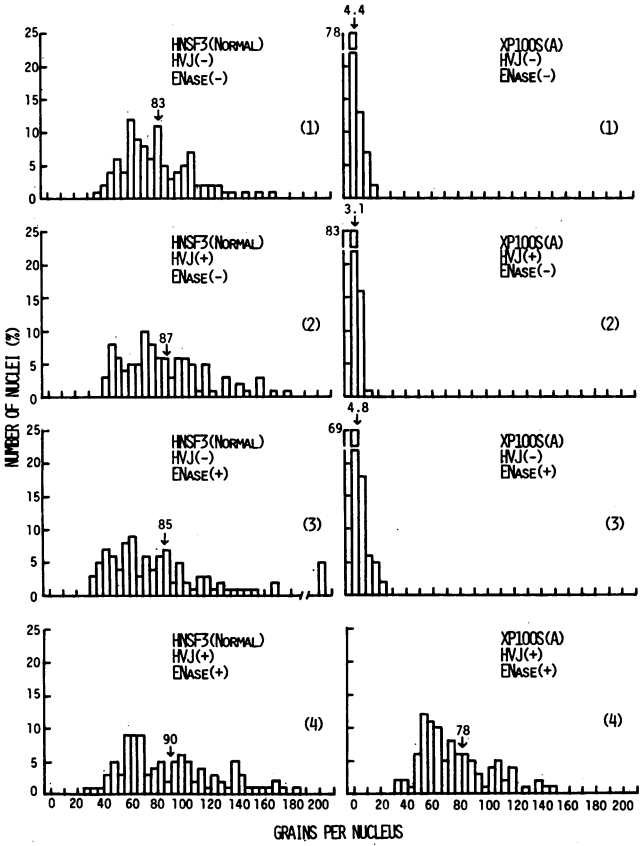

FIG. 1. Restoration of UV-induced unscheduled DNA synthesis of XP10OS cells by the concomitant treatment with T4 endonuclease V (ENase) and UV-inactivated HVJ. UV-induced unscheduled DNA synthesis of HNSF3 (normal, left column) and XP10OS (group A, right column) cells was estimated autoradiographically. Cells were UV irradiated (*1*), or then treated with HVJ (*2*) or with the enzyme (*3*) or with both the enzyme and HVJ (*4*). Ordinate: frequency of nuclei with the grain numbers shown on the abscissa. Arrows: mean grain numbers per nucleus calculated from numbers in 100 nuclei.

trifugation and suspended in a balanced salt solution (0.14 M NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 2.4 mM $CaCl_2$, buffered with 10 mM Tris·HCl at pH 8.0). The virus was inactivated by UV light before use (15).

Estimation of Unscheduled DNA Synthesis. Fibroblasts were grown as monolayers in petri dishes (35 mm in diameter) containing two coverslips (15 mm in diameter). The dishes were washed once with phosphate-buffered saline and irradiated with UV light using a low pressure mercury discharge tube (0.54 J/m^2 per sec). Immediately after UV irradiation (30 J/m^2), one drop of UV-inactivated HVJ (2000 hemeagglutinating units) and one drop of T4 endonuclease V (19 units/ml) were put on each coverslip. The coverslips were then incubated for 15 min on ice and then for 15 min at 37°. Then the cells were cultured with minimum essential medium–10% fetal calf serum medium containing 10 μCi/ml of [^{3}H]thymidine for 3 hr at 37°. The coverslips were then washed and fixed with methanol. After acid-soluble material had been removed with 5% trichloroacetic acid at 4°, the coverslips were dipped in Kodak NTB3 nuclear emulsion. They were developed after 7 days exposure and then stained with Giemsa. In autoradiographic preparations silver grains on "lightly labeled" nuclei of both fused and nonfused cells were counted at random. The average number of grains per nucleus was calculated from the number in 100 nuclei and a histogram was made of the distribution of numbers of grains per nucleus.

RESULTS

Concomitant treatment of XP cells with T4 endonuclease V and HVJ

HVJ has frequently been used for insertion of macromolecules into cells, since Enders *et al.* (16) first used it for infection of nonsusceptible cells with poliovirus. The mechanism of insertion of macromolecules is not yet clear, but it may be related to damage of cell membranes by the virus at an early stage of fusion of cell membranes with viral envelopes and then the repair of the membrane damage (17, 18). It seemed likely that T4 endonuclease V could be inserted into XP cells by HVJ because this enzyme has a small molecular weight (about 20,000).

In the first experiment, XP10OS (group A) was used to determine whether the T4 endonuclease could be inserted effectively into cells by HVJ and whether it could function on UV-damaged human chromosomal DNA. Since UV-induced unscheduled DNA synthesis is very small in group A, a clear-cut result should be obtained. XP10OS cells were irradiated with UV light and then exposed to the enzyme and UV-inactivated HVJ for 15 min at 0° and then for 15 min at 37°. As shown in Figs. 1 and 2, it was found that unscheduled DNA synthesis in the XP10OS cells increased markedly, reaching the same level as that in normal cells. When the XP cells were exposed to either the enzyme or HVJ alone, no increase in the level of unscheduled DNA synthesis was observed. The effect of the enzyme was specific for UV-damaged DNA, because no unscheduled DNA synthesis was seen in non-irradiated cells on exposure to both the enzyme and HVJ. The level of unscheduled DNA synthesis of irradiated normal cells was not affected by exposure to the enzyme and HVJ. These results suggest that (*1*) T4 endonuclease V molecules were inserted effectively into XP cells by the action of HVJ, (2) the enzyme was functional on human chromosomal DNA which had been damaged by UV irradiation, and (3) the lack of UV-induced unscheduled DNA synthesis in XP10OS cells was due to a defect in the first step of excision repair, that is, incision near pyrimidine dimers.

Cleaver (8) reported that cells from a patient with de-Sanctis-Cacchione syndrome (belonging to group A) accumulated strand breaks in DNA after irradiation for various periods. He suggested that either the cells may possess UV-specific endonucleolytic activity but be defective in a later stage of repair or the breaks accumulated during cell death due to the action of nucleases which differed from repair enzymes. Our results obtained with viable cells containing inserted T4 endonuclease V implied that XP10OS cells lacked the first step of excision repair. To confirm this, the effect of the enzyme was tested in three strains belonging to group A. As shown in Fig. 3, treatment of all these cells with both the enzyme and HVJ was effective, and after treatment essentially similar histograms of the distributions of nuclei synthesizing DNA were obtained with normal and XP cells. The average numbers of silver grains per nucleus were also similar, being 76 in normal cells, XP8OS and XP10OS cells, and 75 in XP6TO cells.

The preparation of T4 endonuclease V used (Fraction III, see *Materials and Methods*) contained no detectable DNA polymerase or 5′ → 3′ exonuclease activity. The specific role of T4 endonuclease V in the reaction was further demonstrated by the following observations. (*1*) This enzyme is

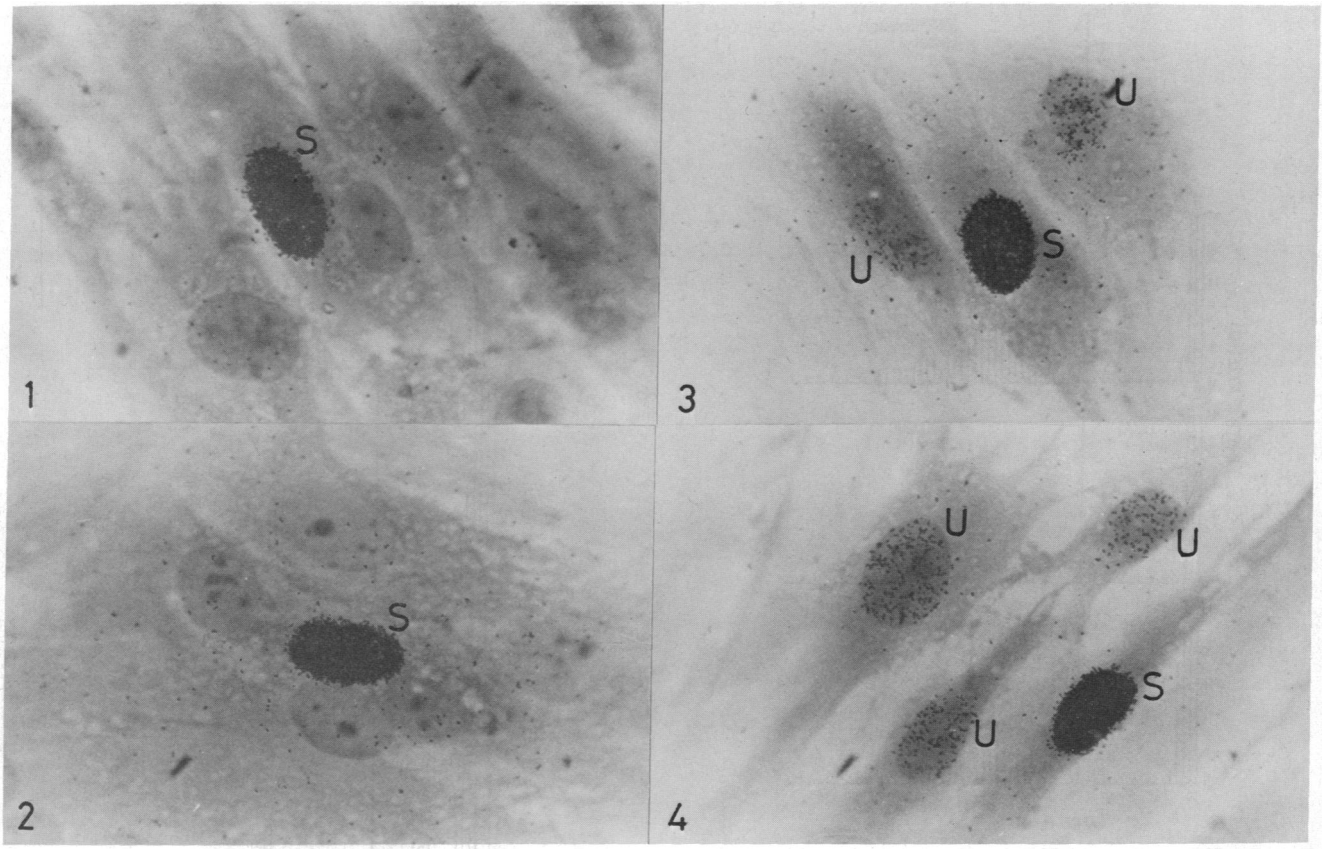

FIG. 2. Autoradiograms of normal cells and XP cells. XP10OS cells were UV irradiated and then treated with the enzyme alone (*1*), or with HVJ alone (*2*), or with both the enzyme and HVJ (*3*), and incubated with [^{3}H]thymidine for 3 hr. HNSF3 cells were UV irradiated and treated with HVJ alone and then incubated with [^{3}H]thymidine for 3 hr (*4*). S is "heavily labeled" cells in S-phase. U is "lightly labeled" cells showing unscheduled DNA synthesis.

heat-labile and it lost more than 90% of its activity when heated at 45° for 10 min in PEME buffer. It was found that only slight unscheduled DNA synthesis was induced when XP 10OS cells were treated with this heated preparation and HVJ (Fig. 4). (2) It has been shown that T4 enzyme induces a break on the 5′-side of a pyrimidine dimer, whereas pancreatic deoxyribonuclease induces strand breaks in a rather random fashion, although both enzymes produce terminal 3′-hydroxyl and 5′-phosphoryl groups (10–12, 19). Treatment of XP10OS cells with the pancreatic enzyme (1 or 100 μg/ml) instead of T4 endonuclease V caused no stimulation of unscheduled DNA synthesis. This enzyme at a concentration of 1 μg/ml caused roughly the same number of strand breaks *in vitro* as T4 endonuclease V used here. However, it was difficult to determine whether this enzyme could be inserted enough into the cells under the present conditions.

Effect of T4 endonuclease V on 5 groups of XP cells

Studies were made on the effects of the T4 endonuclease V on different kinds of XP cells. For this purpose its effects were compared in normal cells and the following standard strains from the American Type Culture Collection: CRL 1223 (group A), CRL 1199 (group B), CRL 1170 (group C), CRL 1160 (group D), and CRL 1259 (group E). These cells were irradiated and then treated with both T4 endonuclease V and HVJ or with HVJ alone. The levels of unscheduled DNA synthesis after treatment with HVJ alone were 5% of the level in normal cells in group A, 7% in B, 13% in C, 20% in D, and 67% in E. These levels were in agreement with the standard levels in these cells estimated without treatment with HVJ. As shown in Figs. 5 and 6, the unscheduled DNA synthesis of all these XP cells was restored to almost the normal level on treatment with both the enzyme and HVJ. That is, the level increased to 95% of normal level in A, 91% in B, 99% in C, 101% in D, and 105% in E.

These results suggest that the first step of excision repair in human cells is catalyzed by an enzyme which has a similar mode of action to T4 endonuclease V and that all the XP cells studied ("variant" was not tested) are defective in this step.

DISCUSSION

The existence of six genetically distinct groups in XP patients has been established by the complementation test after fusion of two kinds of cells by HVJ. One of the phenotypic characteristics of these groups is a difference in their levels of UV-induced unscheduled DNA synthesis; the levels increased in the order A < B < C < D < E < variant = normal (1–5). XP variant is thought to have an abnormality in post-replication repair (20–22). Groups A to E are wholly or partially defective in excision repair activity, and the differences between them appear to be genetic.

The mechanism of excision repair has been studied extensively in T4-infected *E. coli*, and it has been suggested that it consists of the following reactions (12); (*1*) formation of a single-stranded break on the 5′-side of a pyrimidine dimer by T4 endonuclease V, (*2*) excision of a nucleotide fragment containing the dimer by 5′ → 3′ exonuclease, (*3*) repair rep-

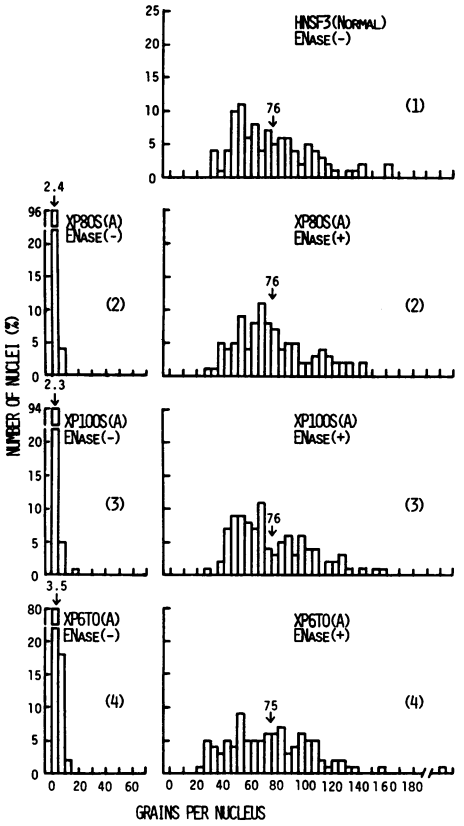

FIG. 3. Restoration of UV-induced unscheduled DNA synthesis in three strains of XP group A by T4 endonuclease V. XP8OS (*2*), XP10OS (*3*), and XP6TO (*4*) cells were UV irradiated and then treated with HVJ alone (left column) or with both the enzyme and HVJ (right column). HNSF (normal) cells were UV irradiated and treated with HVJ alone (*1*). Ordinate: frequency of nuclei with the grain numbers shown on the abscissa. Arrows: mean grain numbers per nucleus calculated from numbers in 100 nuclei.

lication by DNA polymerase, using the undamaged strand as template, and (*4*) rejoining of the repaired segment to the parental DNA strand by DNA ligase. According to this model, the decreased level of unscheduled DNA synthesis might be caused by a defect in any one of the three reactions, (*1*), (*2*), or (*3*). These types of defects may be present in patients with XP or other hereditary diseases. However, if the enzyme is involved in a process which is essential for cell proliferation, it is not possible to isolate such cells under ordinary conditions.

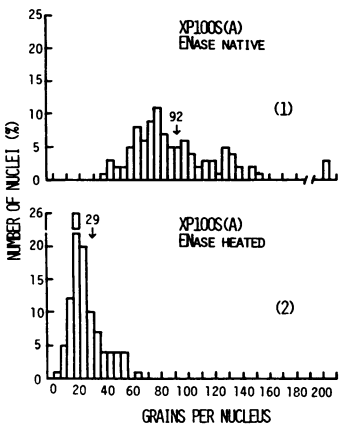

FIG. 4. Loss of effect of T4 endonuclease V on heat inactivation. XP10OS (group A) cells were UV irradiated and treated with native enzyme and HVJ (*1*) or enzyme which had been heated at 45° for 10 min and HVJ (*2*). Ordinate: frequency of nuclei with the grain numbers shown on the abscissa. Arrows: mean grain numbers per nucleus calculated from numbers in 100 nuclei.

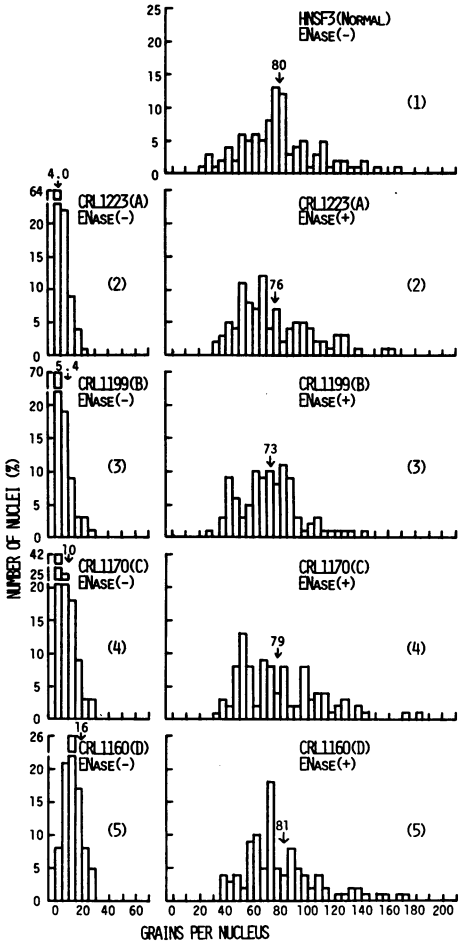

FIG. 5. Restoration of UV-induced unscheduled DNA synthesis of XP cells of groups A to D. CRL 1223 (group A), CRL 1199 (group B), CRL 1170 (group C), CRL 1160 (group D) cells were UV irradiated and then treated with HVJ alone (left column) or with both the enzyme and HVJ (right column). HNSF3 (normal) cells were UV irradiated and then treated with HVJ alone. Ordinate: frequency of nuclei with the grain numbers shown on the abscissa. Arrows: mean grain numbers per nucleus calculated from numbers in 100 nuclei.

The present experiments show that the impaired ability of XP cells to perform unscheduled DNA synthesis is restored to the level of normal cells by treatment with T4 endonuclease V. It is likely that the enzyme was artificially inserted into cultured XP cells by the action of HVJ and restored unscheduled DNA synthesis to normal, since the enzyme had no effect when HVJ was omitted from the culture.

Since the purified preparation of T4 endonuclease V used here contained no detectable DNA polymerase or 5′ → 3′ exonuclease, it seems likely that T4 endonuclease V itself acted as the enzyme which is missing in XP. Moreover, it was found that T4 endonuclease was effective in all XP cells tested belonging to complementation groups A–E, suggesting that at least five genes are involved in controlling the incision step in human cells. It has been shown that the incision step in *E. coli* is controlled by four genes, *uvrA–D*, whereas only one gene, the *v* gene, is involved in bacterio-

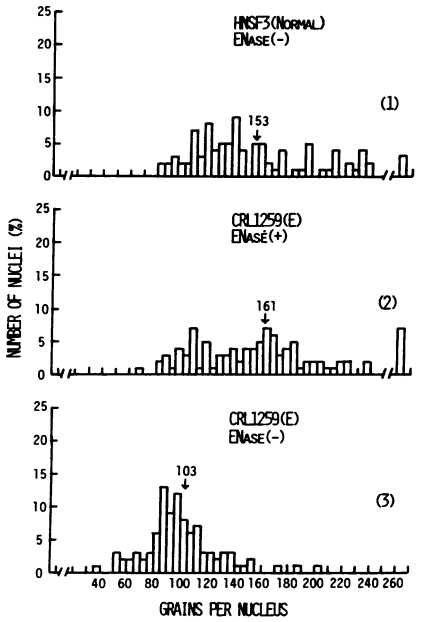

FIG. 6. Restoration of UV-induced unscheduled DNA synthesis of XP cells of group E. CRL 1259 (group E) cells were UV irradiated and then treated with HVJ alone (*3*) or with both the enzyme and HVJ (*2*). HNSF3 (normal) cells were treated with HVJ alone (*1*). Ordinate: frequency of nuclei with the grain numbers shown on the abscissa. Arrows: mean grain numbers per nucleus calculated from numbers in 100 nuclei.

phage T4 (23). Thus the processes of excision repair in human cells and *E. coli* are more complex than in T4-infected cells, although they involve similar enzymes.

There are several possible explanations of the functions of these five genes: (*1*) human cells may possess more than one species of enzyme catalyzing the incision step; (*2*) the enzyme(s) may be composed of subunits, and their formations may be controlled by different genes; (*3*) some other protein factor(s) besides an enzyme(s) may be required for the reaction *in vivo;* and (*4*) there may be a regulator gene, which controls the formation of the enzyme and/or factor. These mechanisms are nonexclusive and the observed phenomena may be due to a combination of several mechanisms. For example, gene *A* may be the regulator gene while gene *B* and *D* are structural genes for one enzyme and its factor and gene *C* and *E* for another. More studies are clearly necessary to identify the functions of these genes.

An endonuclease activity that preferentially incises UV-irradiated DNA has been found in extracts of human fibroblasts and HeLa cells (24, 25). It was shown, however, that the activity is present to a similar degree in extracts of XP and normal cells (26). This was also the case in *E. coli*, in which the same level of endonuclease activity specific for irradiated DNA was detected in crude extracts of wild type and excision-defective mutants (27). This paradox was recently resolved by demonstration of two endonucleases, one of which was absent in *uvrA* and *uvrB* mutants (28). It seems that the "true" repair endonuclease was masked by another endonuclease in crude extracts. In *in vitro* assay systems using crude extracts of cells as an enzyme material and human chromosomal DNA, probably sheared, as the substrate, a possibility must exist that both specific and nonspecific reactions are involved. This is unlikely in the present assay system. To exclude the possibility completely, however, the assay with an UV-specific endonuclease(s) derived from human cells is a necessity.

The present results suggest that it may be possible to relieve the symptoms of XP patients by treatment with this kind of enzyme.

We wish to thank Dr. H. Takebe (Osaka University Medical School) for supplying XP cells and for valuable discussion, Dr. S. Yasuda (Kyushu University Faculty of Science) for providing the preparation of T4 endonuclease V, and Dr. J. Furuyama (Hyogo Medical College) for providing facilities. This work was supported by Cancer Research Grants from the Ministry of Education of Japan and a Grant aided by the Ministry of Welfare of Japan for the Research on Handicapped Children.

1. Cleaver, J. E. (1968) *Nature* **218,** 652–656.
2. Epstein, J. H., Fukuyama, K., Reed, W. B. & Epstein, W. L. (1970) *Science* **168,** 1477–1478.
3. deWeerd-Kastelein, E. A., Keijzer, W. & Bootsma, D. (1972) *Nature New Biol.* **238,** 80–83.
4. Kraemer, K. H., Coon, H. G., Petinga, R. A., Barret, S. R., Rahe, A. E. & Robbins, J. H. (1975) *Proc. Nat. Acad. Sci. USA* **72,** 59–63.
5. Setlow, R. B., Regan, J. D., German, J. & Carrier, W. L. (1969) *Proc. Nat. Acad. Sci. USA* **64,** 1035–1041.
6. Paterson, M. C., Lohman, P. H. M. & Sluyter, M. L. (1973) *Mutat. Res.* **19,** 245–256.
7. Buhl, S. N. & Regan, J. D. (1973) *Nature* **246,** 484.
8. Cleaver, J. E. (1974) *Radiat. Res.* **57,** 207–227.
9. Okada, Y. & Tadokoro, J. (1962) *Exp. Cell Res.* **26,** 108–118.
10. Yasuda, S. & Sekiguchi, M. (1970) *Proc. Nat. Acad. Sci. USA* **67,** 1839–1845.
11. Friedberg, E. C. & King, J. J. (1971) *J. Bacteriol.* **106,** 500–507.
12. Sekiguchi, M., Shimizu, K., Sato, K., Yasuda, S. & Ohshima, S. (1975) in *Molecular Mechanism for the Repair of DNA,* eds. Hanawalt, P. C. & Setlow, R. B. (Plenum Press, New York), pp. 135–141.
13. Goulian, M., Lucas, Z. J. & Kornberg, A. (1968) *J. Biol. Chem.* **243,** 627–638.
14. Ohshima, S. & Sekiguchi, M. (1972) *Biochem. Biophys. Res. Commun.* **47,** 1126–1132.
15. Okada, Y. & Murayama, F. (1966) *Exp. Cell Res.* **44,** 527–551.
16. Enders, J. F., Holoway, A. & Grogan, E. A. (1967) *Proc. Nat. Acad. Sci. USA* **57,** 637–644.
17. Okada, Y. (1972) in *Membrane Research,* ed. Fox, C. F. (Academic Press, New York and London).
18. Okada, Y., Koseki, I., Kim, J., Maeda, Y., Hashimoto, T., Kanno, Y. & Matsui, Y. (1975) *Exp. Cell Res.,* in press.
19. Laskowsky, M. (1961) in *The Enzymes,* eds. Boyer, P. D., Lardy, H. & Myrback, K. (Academic Press, New York), Vol. V, p. 123.
20. Cleaver, J. E. (1972) *J. Invest. Dermatol.* **58,** 124–128.
21. Lehman, A. R., Kirk-Bell, S., Arlett, C. F., Paterson, M. C., Lohman, P. H. M., deWeerd-Kastelein, E. A. & Bootsma, D. (1975) *Proc. Nat. Acad. Sci. USA* **72,** 219–223.
22. Day, R. S. (1975) *Nature* **253,** 748–749.
23. Taketo, A., Yasuda, S. & Sekiguchi, M. (1972) *J. Mol. Biol.* **70,** 1–14.
24. Brent, T. P. (1972) *Nature New Biol.* **239,** 172–173.
25. Bacchetti, S., van der Plas, A. & Veldhuisen, G. (1972) *Biochem. Biophys. Res. Commun.* **48,** 662–669.
26. Ducker, N. J. & Teebor, G. W. (1975) *Nature* **255,** 82–84.
27. Takagi, Y., Sekiguchi, M., Okubo, S., Nakayama, H., Shimada, K., Yasuda, S., Nishimoto, T. & Yoshihara, H. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33,** 219.
28. Braun, A. & Grossman, L. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 1838–1842.