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Use of in vivo and in vitro assays for the characterization of mammalian excision repair and isolation of repair proteins

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Summary

Elucidation of the molecular mechanism of mammalian nucleotide excision repair requires the availability of purified proteins, DNA substrates with defined lesions and suitable repair assays. Repair assays introduced in recent years vary from testing individual steps and successions of steps in vitro to systems that closely reflect the entire process in vivo. In the first part of this review, an in vivo microinjection system is discussed. The second part of the article reviews an in vitro system for study of repair synthesis promoted by cell extracts. Both systems can be utilized as assays during the purification of protein factors that complement repair-defective xeroderma pigmentosum cells. The effect of purified repair proteins from other organisms on mammalian repair is also considered.

Excision repair is responsible for removing most ultraviolet radiation damage to DNA, including damage caused by exposure to the UV in sunlight. Excision repair also acts on damage caused by a broad class of chemical mutagens, including many that occur in every diet as components of natural and cooked foods. Many DNA-damaging agents are also used for cancer chemotherapy.

Much of the research on DNA repair in humans has been carried out with cells derived from patients with xeroderma pigmentosum (XP). Studies of XP have emphasized the particular importance of DNA repair in counteracting carcinogenesis. Individuals with this autosomal, recessively inherited syndrome are subject to a high incidence of sunlight-induced skin disorders, including cancers. Cells from XP patients exhibit reduced levels of DNA-repair synthesis in response to ultraviolet light and many chemical mutagens. The disease is genetically heterogeneous, and includes 8 complementation groups. The groups designated XP-A through XP-G have a defect in incision of damaged DNA, and the XP-Variant group is thought to have a defect in 'post-replication repair'. These groups may represent separate proteins which act together in a complex to repair damaged DNA. The precise molecular nature of the particular defects in different XP groups is unknown.

Elucidation of the molecular mechanism of mammalian nucleotide excision repair requires the

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availability of purified proteins, DNA substrates with defined lesions and suitable repair assays. The detailed understanding of the uvrABC(D) excision-repair pathway in *Escherichia coli*, gained in recent years (for reviews see Grossman et al., 1988; Sancar and Sancar, 1988) provides a good example of the power of this combination of approaches. Repair assays vary from testing individual steps and successions of steps in vitro to systems that closely reflect the entire process in vivo.

The latter category involves the use of living mammalian cells as 'test tubes' and methods to introduce exogenous material which do not disturb the endogenous repair process. Several such systems have been developed or can be envisaged for the analysis of mammalian repair processes. These include:

(a) systems based on transiently permeabilized cells using hypotonic shock (Castellot et al., 1978), electroporation (Winegar et al., 1989; Neumann et al., 1982), lysolecithin (Miller and Ruddle, 1978), or inactivated Sendai virus (Tanaka et al., 1975).

(b) fusion of cells with enveloped carriers such as erythrocyte ghosts (Furusawa et al., 1974), liposomes (Tyrell et al., 1976) or isolated cytoplasts of mammalian cells (Giannelli et al., 1982; Keijzer et al., 1982). The application of lipofectin (Felgner et al., 1987) for these purposes may prove a promising new extension of this type of approach.

(c) microneedle injection into living cells. Compared to the other methods mentioned above this technique is either more direct and efficient and/or less toxic to the cells.

Several in vitro or semi-in vitro approaches for the study of mammalian DNA excision repair have been used in recent years. These include permeabilized cell systems (Dresler, 1984; Kaufmann and Briley, 1987) and studies of isolated nuclei (Nishida et al., 1988). Several studies have also been carried out to analyze excision repair in cell-free extracts (Mortelmans et al., 1976; Kano and Fujiwara, 1983; Wood et al., 1988b; Sibghat-Ullah et al., 1989).

In the first part of this review, an in vivo microinjection system will be discussed. The second part of the review will discuss an in vitro system for study of repair synthesis promoted by cell extracts.

Use of the in vivo microinjection assay

Since this in vivo system is most extensively used for repair studies it is the main focus of the first part of this chapter. The basic principle of the microinjection assay, outlined in Fig. 1, utilizes human (wild-type or repair-deficient) fibroblasts as living test tubes for the introduction of various compounds with the aid of a glass microneedle. UV-induced unscheduled DNA synthesis (UDS) or monoclonal antibodies against specific lesions are used in a single cell assay to assess the effect of the injected substance on the endogenous excision repair process. Notwithstanding its obvious limitations and its conceptual simplicity this system has a surprisingly wide variety of possibilities and applications to the analysis of mammalian excision repair (Hoeijmakers, 1988). Here we discuss some of the results obtained with this method.

Main characteristics and critical parameters of the micro-injection repair assay

Since the microinjection assay is performed in living cells, nucleotide excision is examined in its

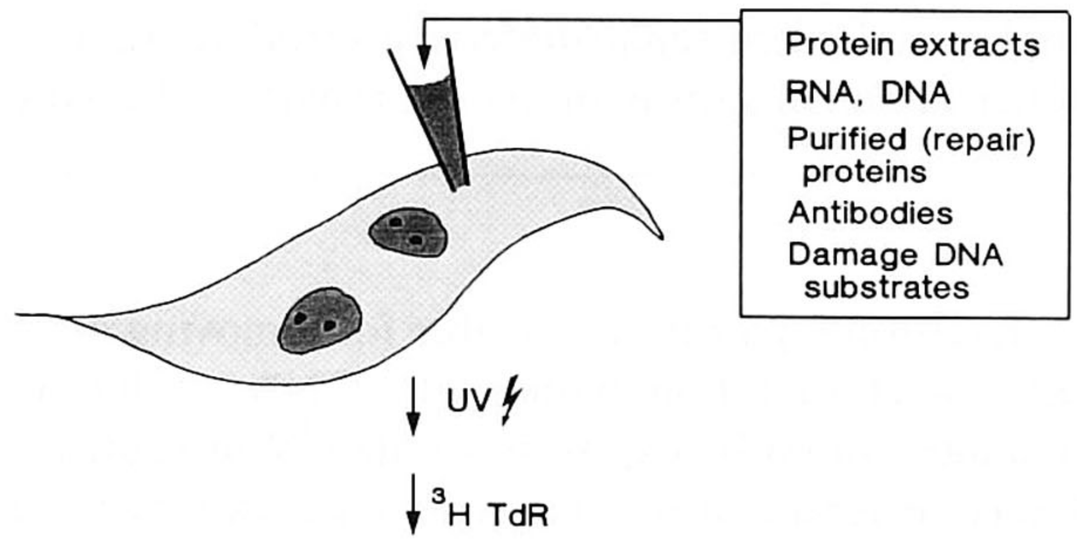


Fig. 1. Schematic outline of the microinjection assay for mammalian excision repair. Cells (repair-deficient or normal human homopolykaryons, generated by cell fusion) are injected with the indicated components: proteins and RNA into the cytoplasm, DNA into the nucleus. In the case of RNA and DNA injection cells are incubated for 8–24 h to permit gene expression. To determine the effect of the injected substance on the repair capability of the injected cells, the cell population is exposed to UV-light and incubated in the presence of [^{3}H]thymidine, then fixed and processed for autoradiography to detect newly synthesized DNA-repair patches (UDS assay, see Fig. 3a, b). Alternatively, dimer removal can be directly visualized by quantitative immunofluorescence using a monoclonal antibody against TT–cyclobutane dimers (see Fig. 5a, b). For some of the applications not mentioned in this article see Hoeijmakers (1988).

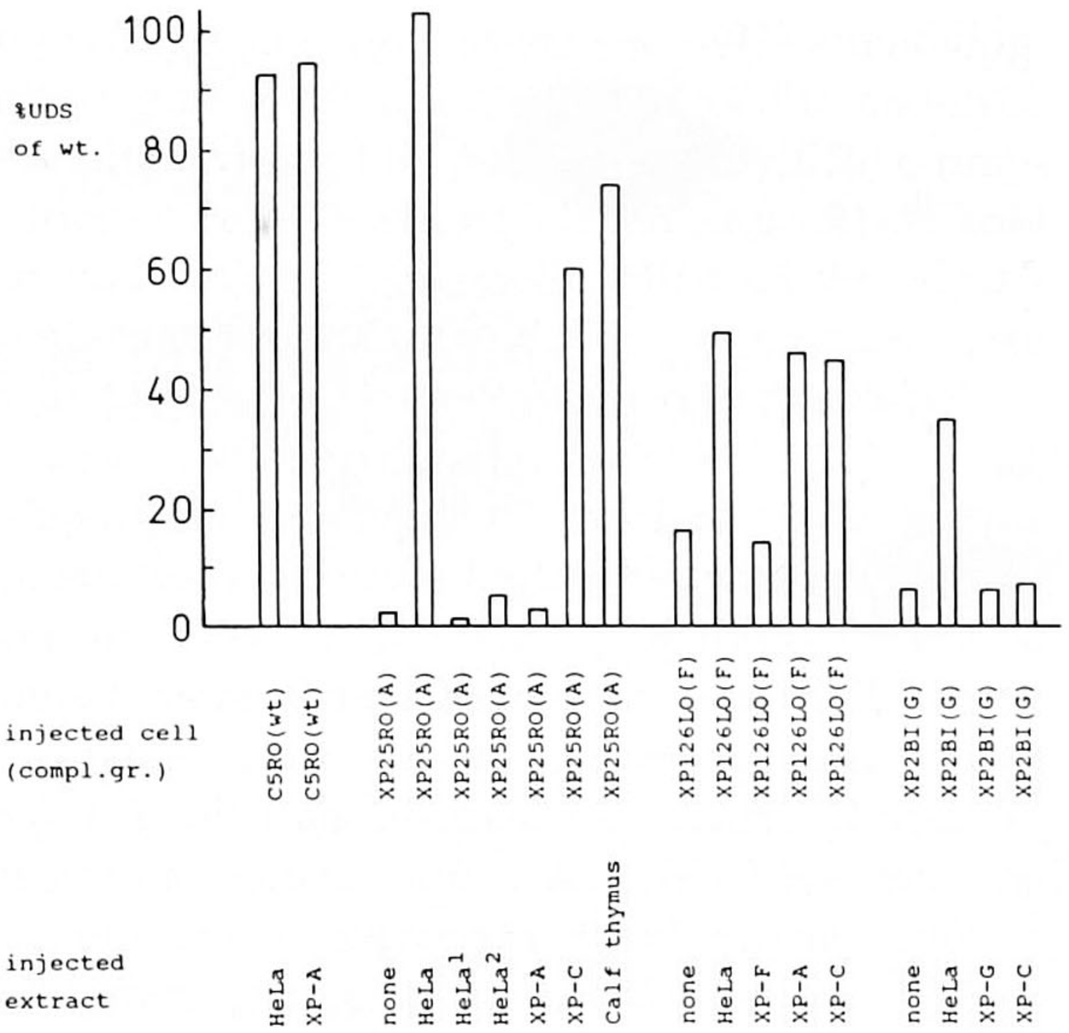


Fig. 2. Effect of microinjected cell extracts and purified repair proteins on UV-induced UDS of XP-fibroblasts. After injection, cells were exposed to UV-radiation (15 J/m^2) and incubated for 2 h in the presence of [^{3}H]thymidine, fixed and processed for autoradiography (Vermeulen et al., 1986). For quantitating the level of UDS, the average number of grains from counting at least 25 nuclei was determined. UDS in injected cells is expressed as % of UDS in a repair competent human fibroblast line (C5RO) used as the internal standard in every experiment. [1] No UV-irradiation; [2] HeLa extract treated with proteinase-K beads prior to injection.

natural context. This includes native chromatin configuration, and important aspects such as preferential repair of specific lesions and of transcriptionally active regions in the genome. Furthermore, optimal controls are automatically included in each experiment by the presence of neighbouring, non-injected cells, which have — apart from the injection — undergone exactly the same treatment as injected cells. A prerequisite is that the injection itself has no significant effect on the repair system. This holds for injection into the cytoplasm (Fig. 2) but not for nuclear injection. We have noted that injection of (physiological) buffer alone into the nucleus of primary fibroblasts causes a significant reduction of UDS in the injected nucleus that lasts for a period of several hours (Hoeijmakers, 1988). Apparently, excision repair is sensitive to mechanical or physiological disturbance of the nucleus. Another critical parameter of the microinjection assay is the physiological composition of the sample to be injected: although cells can tolerate surprisingly unphysiological solutions (e.g. 1 M NaCl, 1 M urea), they are also very sensitive to some compounds (e.g. traces of detergents). Furthermore, the sample should be free of particles to avoid clogging of the needle, and the needle must be of high quality. Homopolykaryons generated by cell fusion are selected for injection. These cells show hardly any S-phase nuclei after 3 days of cultivation avoiding confusion of UDS with labeling due to beginning or ending S-phase DNA replication. Furthermore, these large, multinuclear cells can be more easily injected and reidentified later on. As shown by a dilution experiment of a highly active XP-A correcting calf-thymus preparation injection into XP-A fibroblasts (Table 1), it is possible to quantitate UDS in a reproducible fashion. This allows different experiments to be compared directly when internal standards are included. The data indicate a linear relationship, ranging from 0 to approx. 75% of wild-type UDS, between the amount of partially purified XP-A factor and the UDS found in the microinjection assay. Table 1 also indicates that after injection of a suitable amount (3-fold dilution) of XP-A factor correc-

TABLE 1

EFFECT OF DILUTION ON THE XP-A CORRECTING ACTIVITY OF PARTIALLY PURIFIED CALF-THYMUS EXTRACTS MEASURED BY THE MICRO-INJECTION ASSAY

A Dilution *x*-fold	B UDS in XP-A cells grains/nucleus	A×B
60	13	780
30	24	720
15	50	750
10	74	740
5	95	475
3	108	324
2	103	206
1	83	83

UDS is corrected for the residual UDS found in non-injected cells. The UDS of wild-type C5RO cells was 111 grains/nucleus. The linear relationship between the amount of microinjected XP-A factor and UDS is demonstrated by multiplying the dilution factor and UDS yielding a constant value (3rd column) for the more diluted samples.

tion up to wild-type level occurs. At higher concentrations there is a clear divergence from linearity, stressing the need to use appropriately diluted samples for microinjection.

Finally, the assay has been modified in such a way that UDS results can be obtained after overnight exposure of the injected cells to autoradiographic emulsion i.e. within 24 h when necessary. Immunofluorescence assays using monoclonal antibodies against DNA damage can be done in an even shorter time. Details on the procedure used can be found elsewhere (Hoeijmakers, 1988).

Purification and characterization of XP-A correcting factor

Microinjection of crude cell extracts of repair proficient cells (HeLa, K562) was shown to cause a UV-dependent, transient increase of the level of UDS in XP-fibroblasts from all excision-deficient complementation groups (de Jonge et al., 1983; Vermeulen et al., 1986; see also Fig. 2). An example is shown in Fig. 3a. Phenotypic correction was also achieved using extracts from complementing XP cells (except in the case of XP-C extracts injected into XP-G fibroblasts) but never with homologous extracts (Vermeulen et al., 1986; see also Fig. 2). This finding provides evidence that in each case a separate complementation group specific factor is responsible for the observed effect and that the microinjection assay is a 'bona fide' complementation test. The level of correction varied between individual groups depending in part on the concentration of each correcting factor in the extract and at least in part on the rate of complementation that is known to differ between complementation groups (Giannelli et al., 1982; Keijzer et al., 1982). The specific correction provides a means to purify and characterize the factor involved. The XP-A correction is most prominent, and a low level of correction can be easily determined due to the low residual UDS in XP-A cells (Fig. 2). Therefore, studies have concentrated mainly on this factor and some of the recent findings are summarized here.

Purification of XP-A correcting activity. After verification that bovine cells have the capability to complement the XP-A defect by interspecies cell fusion, several bovine tissues were screened for

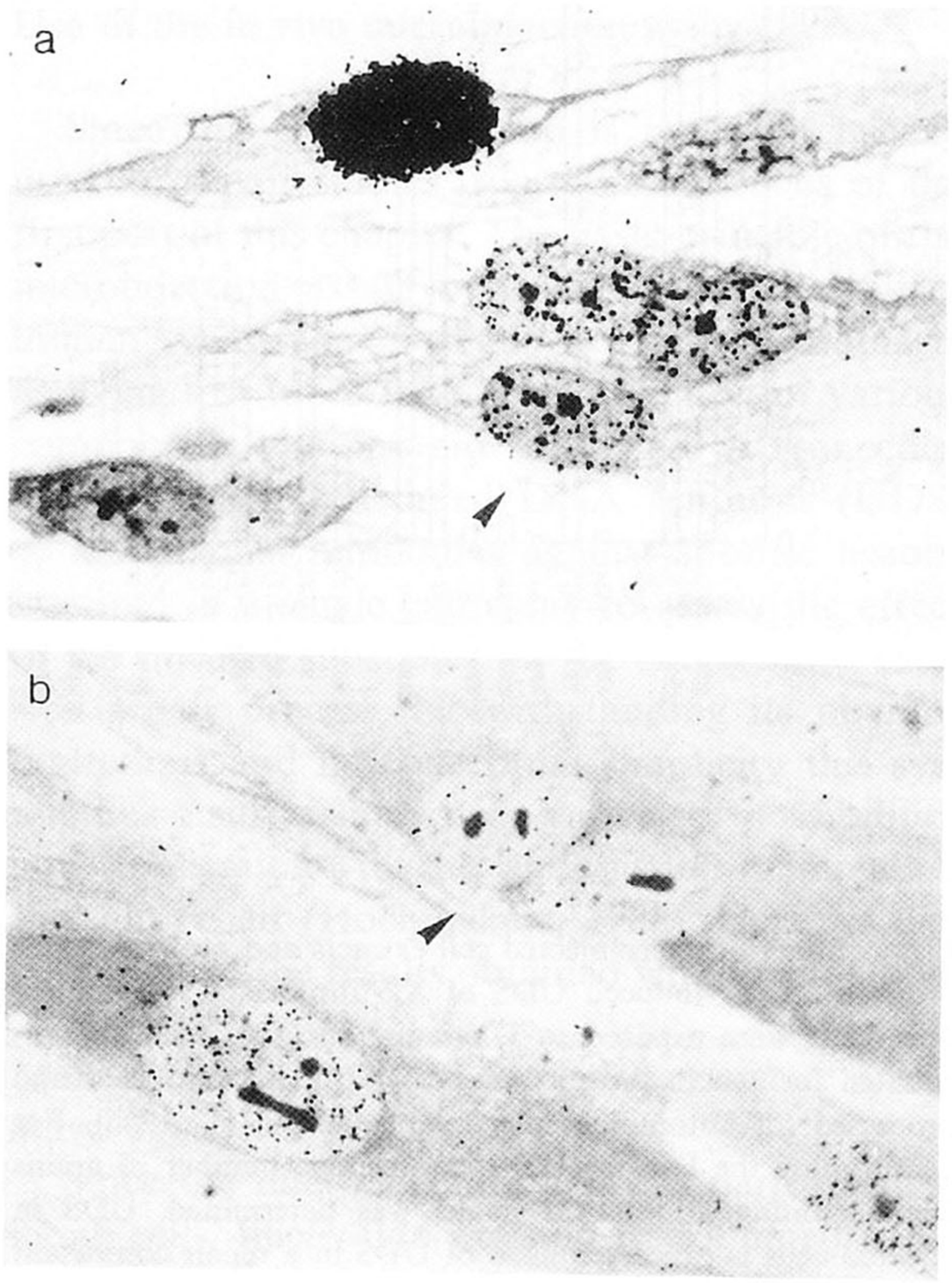


Fig. 3. (a) Micrograph of a homopolykaryon of XP complementation group A (arrow head) containing 3 nuclei, injected with a HeLa crude cell extract, exposed to UV (15 J/m^2) and assayed for UDS. The 3 neighbouring monokaryons (of which one is in S-phase) were not injected. The UDS (number of grains) above the nuclei of the injected cell is corrected close to the UDS level of repair-proficient fibroblasts assayed in parallel. (b) Micrograph of a repair-competent human C5RO homopolykaryon (arrow head containing 2 nuclei), injected with purified yeast PRE, UV-irradiated (10 J/m^2), illuminated with photoreactivating light and assayed for UDS (2 h). The 2 monokaryons did not receive any injection. The injected cell shows a clearly reduced level of UDS.

XP-A correcting activity. Clear differences between tissues were observed. Calf thymus was chosen as starting material for large scale XP-A correcting protein (XPAC) purification although bovine-brain tissue also contained a high XP-A correcting activity. HeLa cells served as a human source in the experiments described below. Cell extracts were prepared from calf thymus with an ultraturrax disperser or from HeLa cells using an ultrasonic disintegrator, followed by centrifugation to remove cell debris. Both extracts were purified by column chromatography on porous

silica beads and blue-sepharose, 60% ammonium sulfate precipitation and dialysis of the dissolved precipitate. With this protocol highly active preparations were obtained which are 30–75-fold purified with 50–70% yield. Most of the experiments presented in the following sections were done with these partially purified preparations.

Specificity. Partially purified calf-thymus preparations showed a high activity on microinjection in XP-A cells: most preparations can be diluted more than 100-fold to yield still clearly detectable correction. No correction was found with the calf-thymus extract in other XP complementation groups except for XP-C where some correction was observed. However, we estimate the correcting activity for XP-C is approx. 50-fold lower than for XP-A, in accordance with results obtained with crude extracts from calf thymus, which gave detectable correction in XP-A only. In contrast, partially purified HeLa extracts showed, besides very high activity in XP-A cells, also a high activity in XP-C, D, E, F and G cells, whereas no significant correction was found in XP-B. Crude HeLa extracts showed correction in all XP complementation groups (Vermeulen et al., 1986). The absence of correction in several complementation groups found with the calf-thymus preparation rules out the possibility of a general by-passing activity as was found for T4-endonuclease or micrococcal UV-endonuclease (see below). The calf-thymus factor induced the same amount of correction in XP-A cells with low (XP25RO, XP2CA) and high (XP8LO) residual UDS giving additional evidence for the presence of the same biochemical defect in these cell lines irrespective of the large difference in residual UDS.

DNA binding. The XPAC polypeptide binds to both unirradiated single-stranded DNA-agarose and UV-DNA-cellulose, suggesting this factor is a DNA-binding protein. The elution curves indicate only a slight preference for UV-DNA over single-stranded DNA, leaving it uncertain whether the XPAC protein binds preferentially to UV-lesions in DNA.

Stability. The XP-A factor appears to be extremely stable as it withstands for instance heating

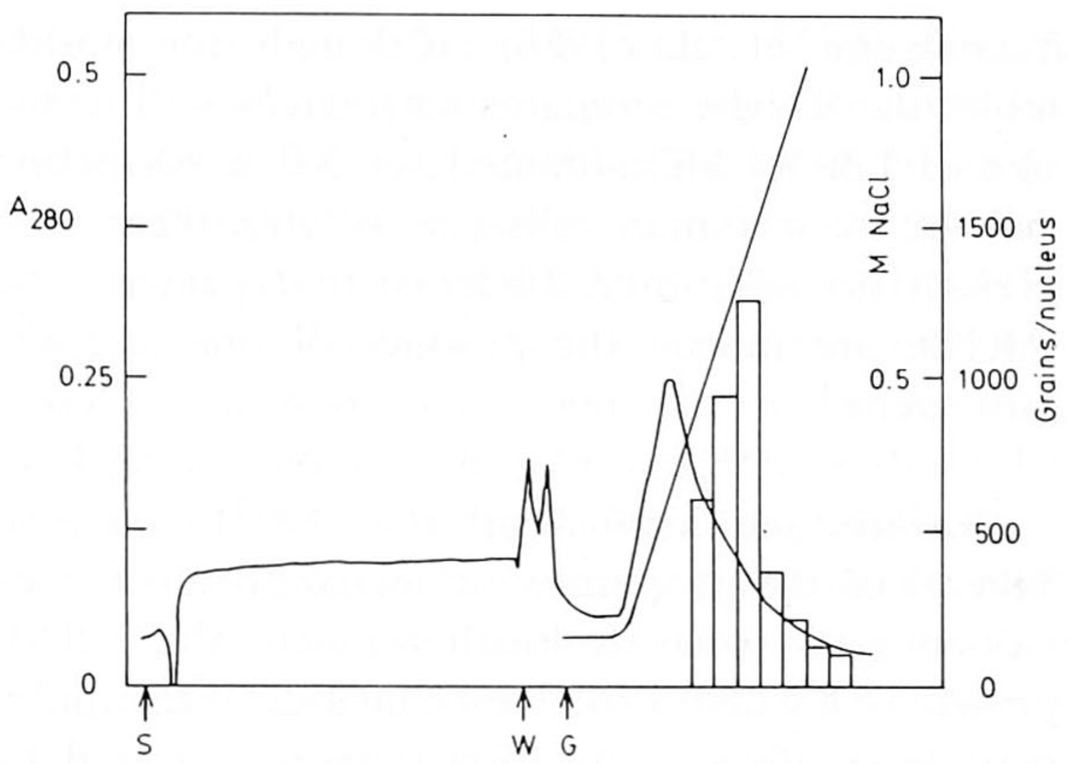


Fig. 4. Chromatography of calf-thymus XP-A correcting factor on a hydroxylapatite column. The column (0.66 × 6.3 cm) was loaded with a partially purified calf-thymus sample (S). After washing (W) the column was eluted with a 30-ml linear 0.08–1.08 M NaCl gradient (G). Fractions were analyzed with the micro-injection assay (bars). The peak fraction was approx. 12-fold purified with respect to the loaded sample.

for 5 min at 95 °C in the presence of 0.1% SDS and 280 mM 2-mercaptoethanol. Heating without SDS results in extensive precipitation, but activity can be partially recovered by solubilization of the precipitate in SDS-containing buffer. Furthermore, HeLa cell extracts showed hardly any loss of XP-A correcting activity upon storage for > 6 weeks at 4 °C. Incubation with proteinase K destroys the XP-A correcting activity, indicating the proteinaceous nature of the XP-A factor (Fig. 2).

Molecular weight. The resistance of the XP-A factor towards SDS offers the possibility to determine the molecular weight by SDS PAGE. After electrophoresis the gel was cut into pieces, crushed and extracted. The resulting supernatant was dialyzed to remove excess SDS which seriously interferes with the microinjection assay by disruption of internal cell structures. In this way it was possible to detect XP-A correcting activity in the 45 kD region for HeLa and calf-thymus factor. Approximately the same molecular weight was found by gel filtration on Sephacryl-200 in the absence of SDS. Results of both gel filtration and SDS electrophoresis indicated the occasional occurrence of biologically active high-molecular weight aggregates. This may provide an explanation for the higher MW values reported by

Yamaizumi et al. (1986). Although the 45-kD molecular weight compares reasonably well to the size of 1.3–1.4 kb estimated for XP-A correcting mRNA from human cells (see below), there is at present no additional evidence that protein and mRNA are indeed the product of one and the same gene.

Further purification of the XPAC protein. Several of the properties summarized above provide obvious tools to further purify the XPAC protein. An example of the behaviour of this polypeptide in other purification steps is provided by the single peak of activity eluting upon hydroxylapatite chromatography, as presented in Fig. 4. However, since the protein is present in small amounts purification to homogeneity has not yet been achieved.

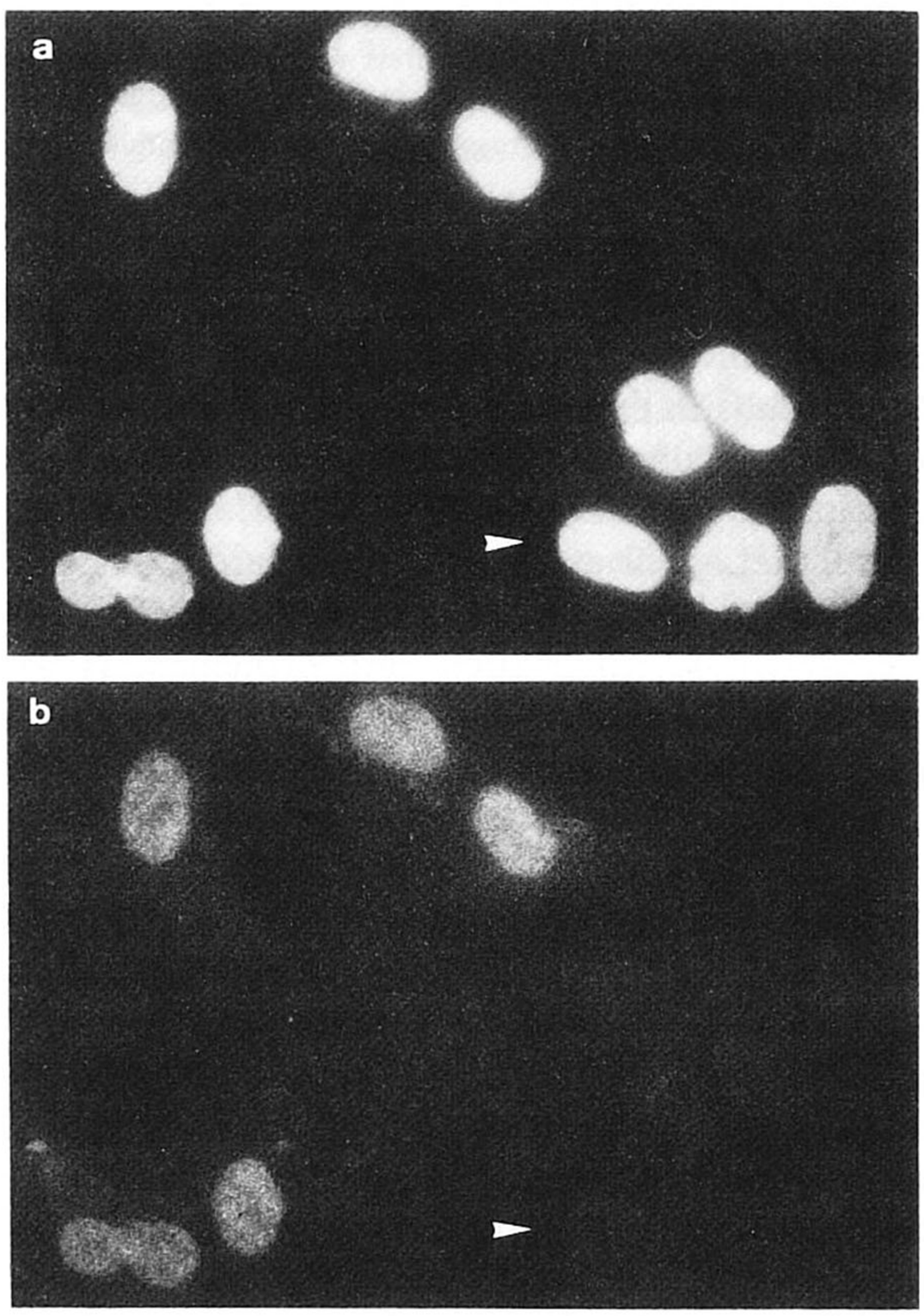


Fig. 5. Immunofluorescence of a repair-proficient, human, homo-polykaryon (arrow head), containing 5 nuclei, injected with purified yeast PRE, UV-irradiated (10 J/m^2), illuminated with photoreactivating light and processed for immunofluorescence, using a monoclonal antibody directed against TT–cyclobutane dimers. (a) The nuclei of all cells in the area of injection as visualized by staining with propidium iodide. (b) TT-dimers as visualized by immunofluorescence staining. Only the homopolykaryon with 5 nuclei was injected.

Introduction of repair enzymes of lower species into mammalian cells

UV-endonucleases of Micrococcus luteus and bacteriophage T4. Introduction of the dimer specific UV-endonucleases of *M. luteus* and T4 into XP fibroblasts either by cell permeabilization using Sendai virus or by microinjection results in a significant induction of UDS above residual activity in all excision-deficient XP complementation groups (Tanaka et al., 1975, 1977; Hayakawa et al., 1981; de Jonge et al., 1985; Yamaizumi et al., 1989). Under conditions where the enzyme is not limiting a complete restoration to wild-type repair synthesis can be reached (Yamaizumi et al., 1989). In fact, a stimulation above wild-type levels was found for repair-competent mouse cells and (in early periods following UV-irradiation) for repair proficient human fibroblasts (Yamaizumi et al., 1989). The T4 *denV* enzyme also increased UV-resistance (Tanaka et al., 1977). These results are confirmed and extended by experiments in which the gene for this protein is introduced by DNA-transfection into various mammalian excision defective mutants (Valerie et al., 1985, 1987; Arrand et al., 1987; Ley et al., 1989). Apparently, the reaction catalyzed by the prokaryotic enzymes, a dimer-specific DNA-glycosylase followed by an AP-endonuclease step, provides an aspecific bypass of the normal mammalian damage-incision process and results in a product that can be effectively processed by endogenous repair systems.

UvrABC(D) nuclease. Microinjection has been used to deliver the well characterized *E. coli* UvrABC(D) proteins in the form of purified polypeptides into the cytoplasm and nucleus of normal and XP-A and -C fibroblasts, to probe the ability of the bacterial enzymes to remove DNA damage from human chromatin in situ. Although a T4 endonuclease preparation with the same in vitro activity did induce UDS, no notable effect could be registered with the *E. coli* excision complex

(Zwetsloot et al., 1986a). Obviously, trivial explanations for this negative result — such as rapid degradation of (one of) the components — cannot be ruled out completely, notwithstanding the fact that the proteins were delivered directly into the nucleus and that the UDS assay was conducted immediately after microinjection. Transfection experiments have shown that at least the UvrA and B proteins appear to be quite stable in mammalian cells (Zwetsloot et al., 1986a; Dickstein et al., 1988); on the other hand the UvrC protein seems to be very unstable at least in vitro (Yeung, 1986; Sancar et al., 1988). In view of fundamental differences in chromatin conformation between prokaryotes and eukaryotes it is also not unlikely that the lack of detectable induction of repair synthesis is due to the inability of the *E. coli* repair complex to gain access to lesions in native eukaryotic chromatin.

Photoreactivating enzyme. In contrast to the UvrABC(D) complex, photoreactivating enzyme (PRE) purified from *S. cerevisiae, E. coli* and *Anacystis nidulans* was able to effectively remove dimers in human and other mammalian cells in situ, in a photoreactivating light dependent way (Hoeijmakers, 1988; Roza et al., 1990). Photoreactivation of dimers was determined by quantitative immunofluorescence using a monoclonal antibody against TT-cyclobutane dimers (Fig. 5). In normal cells it was found to be rapid and efficient: virtually no dimers induced by a UV dose up to 40 J/m^2 (254 nm) could be detected after administration of 15 min of photoreactivating light. The indirect effect of the sudden disappearance of dimers by photoreactivation on UDS revealed an unexpected complexity since it was found to depend on the time lapse between UV-irradiation and photoreactivation. When dimers were photoreversed immediately after UV-irradiation UDS remained initially unaffected for a period of approx. 30 min after which a rapid drop set in to a plateau UDS value of 20% of that of neighbouring, non-injected cells (see Fig. 3b). However, the retarded response of UDS upon dimer monomerization was absent when photoreactivation was postponed: illumination with photoreactivating light 2 h after UV exposure led to an immediate reduction in UDS to 20%, the same level as reached (after a lag phase of 30–45 min) after immediate photoreactivation (Roza et al., 1990; Zwetsloot et al., 1985). One explanation for the difference in response of UDS to photoreactivation of dimers at different times is that UDS early after UV-irradiation is predominantly derived from non-cyclobutane dimer lesions (e.g. 6–4 photoproducts). In addition, it is possible that the loss of contribution of dimers to UDS can be compensated for by accelerated repair of other lesions. In contrast, at later times the bulk of repair synthesis is caused by excision of dimers. At this time the loss of the contribution of dimers to UDS apparently cannot be replaced by accelerated repair of other UV-photoproducts. This interpretation is consistent with data obtained by others, demonstrating a more rapid repair of 6–4 photoproducts and slower repair of cyclobutane dimers in human cells (reviewed in Mitchell and Nairn, 1989).

The effect of photoreactivation was also examined in XP-fibroblasts of various complementation groups. The immunofluorescence dimer assay showed a very rapid and efficient dimer removal in PRE injected fibroblasts of all XP complementation groups, comparable to that in normal cells. However, the decrease of residual UDS due to photoreactivation was absent in XP-D and much delayed in XP-A and E compared to normal cells (Roza et al., 1990). This supports the idea that in these XP-cells preferential repair of non-cyclobutane dimer lesions does occur, but at a much lower rate. The considerable residual UDS in XP-D may not be derived from dimer removal. In XP-C a more rapid decrease in residual UDS was observed than in XP-A and E (Roza et al., 1990; Zwetsloot et al., 1986b). This suggests that the residual repair-functioning in XP-C is of a more normal type. This interpretation is in agreement with the idea that XP-C fibroblasts perform preferential repair of dimers in transcribed regions, similar to normal cells but are deficient in the overall repair of the genome (Mayne et al., 1988).

Total cell extracts from heterologous organisms. To investigate whether some of the factors lacking in XP are functionally conserved between distantly related species, total cell extracts prepared from *Xenopus laevis* (oocytes), Drosophila (cul-

tured cells) and the yeast *S. cerevisiae* were injected into XP-fibroblasts. A clear correction of the UDS in XP-A fibroblasts was exerted by extracts of Xenopus oocytes. No UDS-inducing effect was seen with Drosophila and yeast material for XP-A and several other XP-complementation groups. These data, together with the results obtained with the calf tissues, indicate that the XP-A factor is sufficiently conserved at least among vertebrates to yield functional complementation.

Introduction of nucleic acids into XP-fibroblasts

RNA. As first demonstrated by Legerski and coworkers, cytoplasmic injection of total poly(A)$^+$ RNA of repair competent HeLa cells induced a transient restoration of UDS in XP-A and G fibroblasts (Legerski et al., 1984). Size-fractionation of the mRNA prior to injection indicated a size of 0.6 kb for the correcting XP-A mRNA and 0.7 kb for that of XP-G. We (A.E. and J.H.) have confirmed the temporary induction of UDS by total poly(A)$^+$ RNA in XP-A; however, the XP-A correcting mRNA is found to reside principally in a RNA fraction with a size of 1.3 to 1.4 kb. This size is just sufficient to accommodate the open reading frame for the approx. 45-kD XP-A correcting protein described above. No significant correction of XP-G could be achieved after injection of total or size-fractionated poly(A)$^+$ RNA of HeLa or K562 cells that was positive for XP-A (A.E. and J.H., unpublished results).

cDNA. To be expressed DNA should be injected directly into the nucleus. In this way cDNA's provided with proper mammalian expression signals can be tested for their ability to genetically complement the repair deficiency of mammalian mutants. We have injected the cDNA of the human repair gene *ERCC-1* into fibroblasts of those XP complementation groups for which no immortalized cell lines suitable for DNA transfection are available (van Duin et al., 1989). However, no complementing effect could be observed, although the coinjected SV40 early region (included as positive control) induced T antigen expression in a substantial fraction of the injected cells. This suggests that the *ERCC-1* cDNA does not correct any of the known XP groups, indicating that it probably is not any of the XP-genes (Van Duin et al., 1989). However, we have recently obtained evidence that another *ERCC* gene is able to restore specifically and very efficiently the UDS of one of the XP complementation groups (G. Weeda et al., 1990), demonstrating the value and validity of the microneedle injection assay for analysis of mammalian excision repair.

Use of the in vitro DNA-repair synthesis assay

The second part of this article is devoted to a recently developed cell-free system for the study of the biochemistry of DNA-excision repair in human cells (Wood et al., 1988b). In this system, repair is carried out by enzymes in gently prepared soluble extracts from human cell lines. The assay is based on monitoring the introduction of short patches of nucleotides into damaged circular plasmid DNA. The scheme is outlined in Fig. 6.

Bacterial plasmid DNA is prepared from *E. coli* hosts. The DNA is isolated by cesium chloride centrifugation in the presence of ethidium bromide; closed circular molecules are further purified on one or two neutral sucrose gradients. Sufficient purification of the DNA circles is essential for two reasons. Elimination of nicked circular molecules reduced the background incorporation in nondamaged DNA caused by priming of DNA synthesis at the nicked sites. In addition, the gradients remove contaminating degraded DNA from the plasmid preparations that can interfere with the reactions.

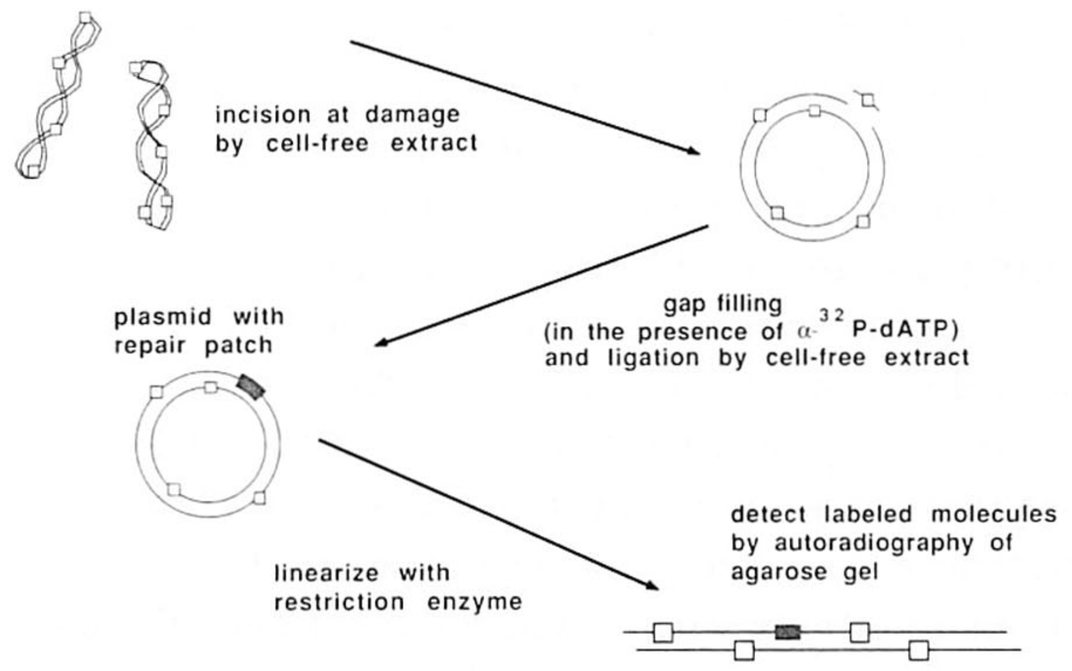


Fig. 6. Scheme for detection of repair synthesis mediated by cell-free extracts. Sites of DNA damage in plasmid DNA are indicated by open-square symbols (□).

Whole-cell extracts are prepared from one-liter suspension cultures ($6–8 \times 10^5$ cells/ml) essentially by the method of Manley et al. (1980), as described in Wood et al. (1988b). Protease inhibitors are present during cell lysis in hypotonic buffer (phenylmethylsulfonylfluoride, pepstatin, leupeptin, chymostatin and aprotinin). Active extracts have been prepared from lymphoid cell lines derived from normal individuals, from HeLa cells, and from 293 cells (a human embryo kidney cell line).

In vitro repair synthesis

To observe repair replication of damaged plasmid DNA in vitro, the DNA is incubated with whole cell extracts in a reaction mixture which includes the 4 deoxynucleoside triphosphates and α-[^{32}P]dATP. During incubation at 30 °C, enzymes in the extract incise the damaged plasmid DNA, and repair patches are formed. Damage-dependent repair synthesis occurs during the course of several hours incubation, and requires the presence of ATP and an ATP regenerating system in the reaction buffer (Wood et al., 1988; Wood and Robins, 1989). The plasmid DNA is then extracted from the reaction mixture, isolated by gel electrophoresis, and analyzed for the presence of radioactively labeled DNA-repair patches by autoradiography. The data can be quantified by scanning densitometry of the autoradiograph, and normalized for DNA recovery by scanning the photographic negative of the ethidium bromide-stained gel. Bands can also be excised from the gel and analyzed by scintillation counting in order to calibrate the densitometry results with reference to incorporation of radioactive material.

DNA-repair synthesis reactions with a mixture of UV-irradiated plasmid and nonirradiated plasmid are shown in Fig. 7. Repair synthesis is equally efficient on DNA that is initially either supercoiled, or relaxed by eukaryotic topoisomerase I. This result is not unexpected, since topoisomerases in the human extract eliminate supercoiling within a few minutes incubation at 30 °C. Thus plasmid circles are probably incised while in a relaxed state. Plasmid circles containing nicks introduced by pancreatic DNAase I (Fig. 7A, lane 4) also undergo damage-dependent repair (Fig. 7B). The background incorporation in non-damaged DNA increases with such a nicked DNA substrate (Fig. 7B, lane 5), indicating the occurrence of limited exonucleolytic digestion before ligation. DNA ligase activity in the extracts can seal the nicks produced by DNAase I to produce closed circular molecules, as shown in Fig. 7B, lanes 8–14 (Wood and Robins, 1989).

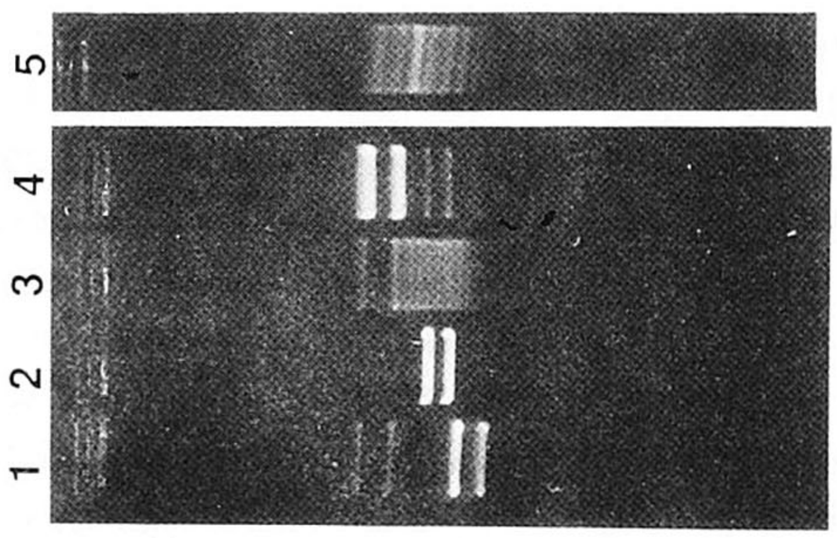
In contrast, DNA that has been linearized before introduction into cell-free extracts is a poor substrate for repair synthesis reactions (Fig. 7B). The extent of repair synthesis with linearized irradiated substrates is 15–40% of that found with identically treated circular substrates. The number of DNA termini present in reaction mixtures with linear DNA (0.6 μg total linear plasmid DNA contributes 0.43 pmoles of 5′ ends) is insufficient to cause this inhibition; up to 3.6 pmoles of 5′ restriction fragment ends do not inhibit reactions containing circular DNA (not shown). The reason for the preference for circular DNA is unknown, but it has been observed in other systems. A reduced efficiency of incision of linear DNA as compared to circular DNA has previously been observed for the *E. coli* UvrABC enzyme (Van Houten et al., 1988). Reduced DNA synthesis has been noted in linear as compared to circular UV-damaged plasmids after microinjection into Xenopus oocytes (Legerski et al., 1987).

Repair synthesis stimulated by different types of DNA damage

UV photoproducts. The extent of repair replication is dependent on the UV fluence given to the plasmid DNA. Fig. 8A shows results for repair mediated by an extract from the normal lymphoid cell line GM1953. Repair incorporation is approximately linear up to 200 J/m^2, with a gradually decreasing slope above this fluence.

The major lesions produced by UV irradiation of DNA are cyclobutane pyrimidine dimers and (6–4) pyrimidine dimers. Experiments have been performed to selectively remove cyclobutane dimers from UV-irradiated DNA with purified *E. coli* DNA photolyase. Photoreactivation of irradiated DNA using DNA photolyase removed more than 95% of the cyclobutane dimers from the DNA and reduced the observed repair synthesis by 20–40% (Wood, 1989). The greater part of the

7A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4
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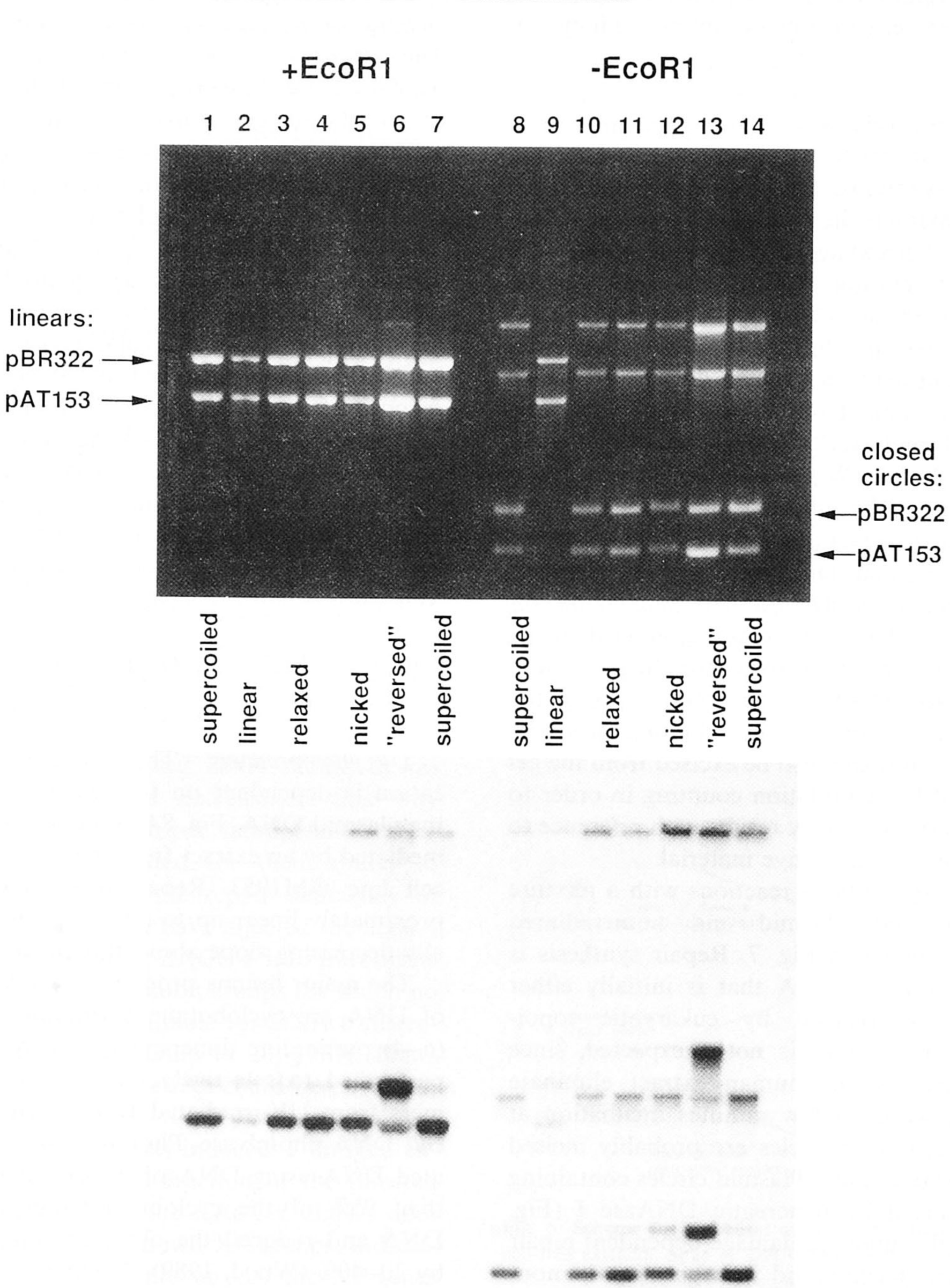
7B


+EcoR1
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1 2 3 4 5 6 7 8 9 10 11 12 13 14
linears:
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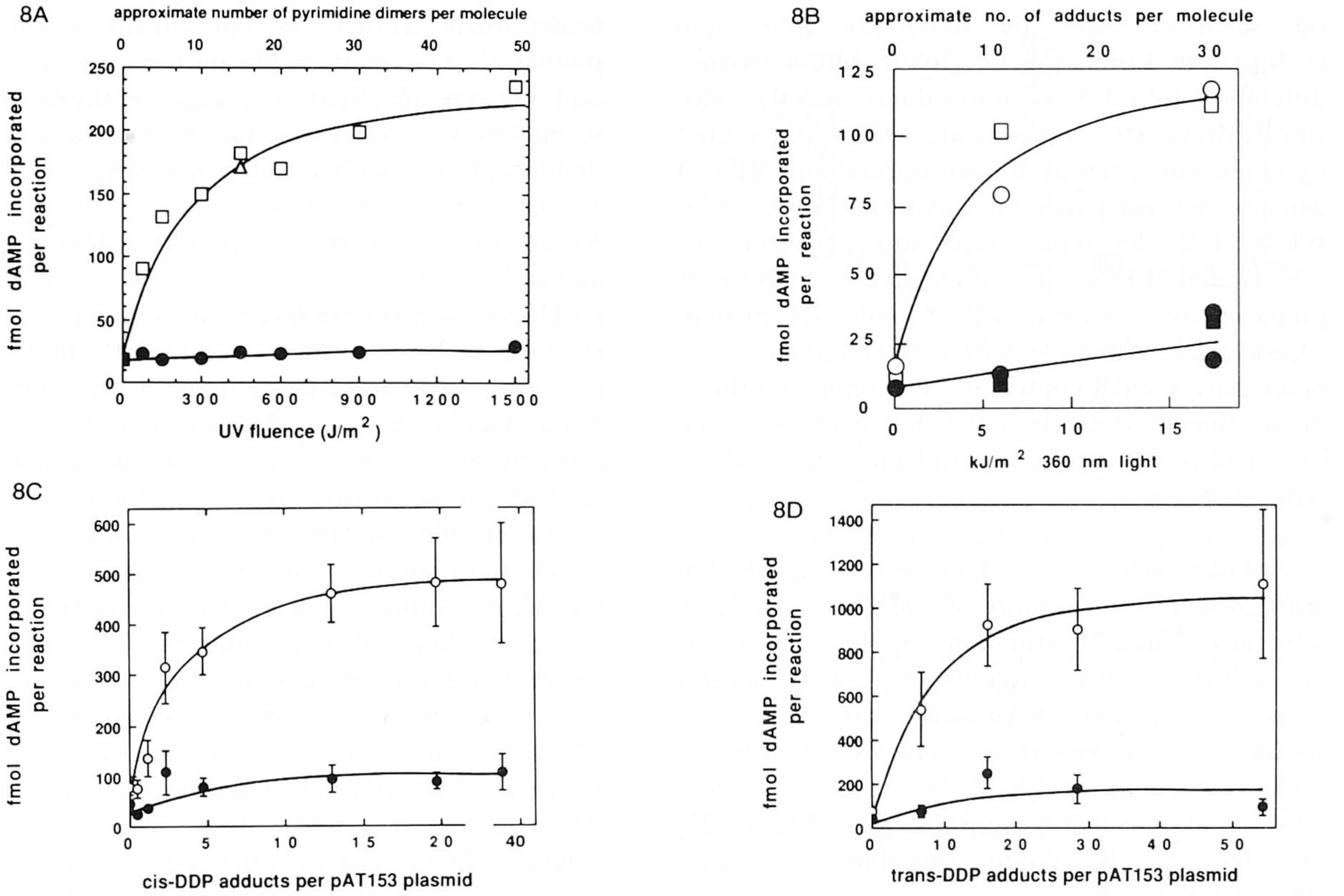

Fig. 8. Dose–responses for repair synthesis stimulated by (A) UV light-induced dimers (100 μg GM1953 extract protein per reaction); (B) 8-methoxypsoralen adducts (80 μg GM1953 extract protein per reaction); (C) *cis*-DDP adducts, and (D) *trans*-DDP adducts (150 μg GM1953 extract protein per reaction). Open symbols: Repair synthesis in damaged pAT153 DNA. Closed symbols: Repair synthesis in nondamaged pBR322 DNA coincubated in the same reactions with the damaged pAT153 DNA. Data are taken from: (A) Wood (1989); (B) Wood et al. (1988b); (C) and (D) Hansson and Wood (1989). Incorporation of dAMP in a particular experiment depends on a number of factors, including the amount of extract protein used, the nature of the DNA lesion, and on the preparation of α-[^{32}P]dATP used in the reactions. Thus it is not possible to strictly compare the absolute values for fmoles dAMP incorporated between all 4 panels.

repair synthesis is highly likely to be caused by (6–4) pyrimidine dimer photoproducts. This class of lesions is rapidly repaired by mammalian cells and their removal is known to be important for cell survival after ultraviolet irradiation. The data suggest that (6–4) photoproducts are 4–8-fold bet-

Fig. 7. Repair synthesis in UV-irradiated plasmid DNA. (A) Plasmid DNA mixtures. Lane 1, mixture consisting of 300 ng each of UV-irradiated (450 J/m^2) pAT153 and unirradiated pBR322 (supercoiled forms); Lane 2, mixture treated with EcoRI restriction endonuclease to linearize both plasmids; Lanes 3 and 5, mixture treated for 60 min at 37°C with calf-thymus DNA topoisomerase I (BRL), 10 U (lane 3) or 50 U (lane 5) to produce relaxed molecules; Lane 4, mixture treated for 15 min at 25°C with 10 ng/ml DNAase I (Sigma) to produce an average of 3 nicks per molecule. Samples were electrophoresed on a 1% agarose gel, and subsequently stained with ethidium bromide. (B) UV-stimulated DNA-repair synthesis. DNA mixtures as shown in part A were used in repair reactions under standard conditions, in a volume of 100 μl with (except for lanes 6 and 13) 600 ng each of UV irradiated pAT153, 600 ng pBR322, and 400 μg extract protein from GM1953 cells. After incubation and purification of the DNA, half of the sample was digested with EcoRI before electrophoresis (lanes 1–7), and half was electrophoresed without restriction cleavage (lanes 8–14). At the start of the reaction, the DNA had the following topological forms: Lanes 1, 7, 8, 14: supercoiled; lanes 2, 9: EcoRI-linearized; lanes 3, 10: topoisomerase I-relaxed as in Fig. 7A lane 3; lanes 4, 11: topoisomerase I-relaxed as in Fig. 7A lane 5; lanes 5, 12: DNAase I-nicked; lanes 6, 13: 'reversed' mixture of supercoiled unirradiated pAT153, and UV irradiated (450 J/m^2) pBR322.

ter substrates for the repair reaction than cyclobutane dimers (Wood, 1989). Minor pyrimidine hydration UV photoproducts, mostly cytosine hydrates (Boorstein et al., 1989) are repaired by an enzyme present in both normal and XP cell extracts, termed pyrimidine hydrate–DNA glycosylase. For the repair replication experiments, UV-irradiated DNA free of pyrimidine hydrates is prepared by treatment with *E. coli* Nth protein (Wood et al., 1988a). However, these minor lesions make only a small contribution to repair synthesis in the fluence range 0–450 J/m^2, because of the low yield of these products and their short DNA-repair patch size.

Psoralen adducts. Adducts produced by 360 nm light-activated binding of psoralen derivatives were also found to stimulate repair synthesis in the cell-free extract system (Fig. 8B). Several psoralen derivatives have been investigated, including 8-methoxypsoralen (Wood et al., 1988b), 4′-hydroxymethyl-4,5′,8′-trimethylpsoralen (HMT) and 5-methylisopsoralen (5-MIP) (D. Coverley and R. Wood, unpublished results; Sibghat-Ullah, 1989).

Platinum adducts. The cell-free system is also being used to study repair synthesis in DNA treated with the cancer chemotherapeutic agent *cis*-diamminedichloroplatinum(II) (CDDP), and its inactive *trans* isomer (TDDP). Plasmid DNA treated with these agents was used in reactions to give quantitative measurements of DNA-repair synthesis mediated by cell-free extracts from human lymphoid cell lines (Hansson and Wood, 1989). Adducts induced by both drugs stimulated repair synthesis in a dose dependent manner (Fig. 8C and D). Measurements by an isopycnic gradient sedimentation method gave an upper limit for the average patch sizes in this in vitro system of around 140 nucleotides. It was estimated that up to 3% of the drug adducts induce the synthesis of a repair patch. The repair synthesis is due to repair of a small fraction of frequent drug adducts, rather than extensive repair of a rare subclass of lesions. Nonspecific DNA synthesis in undamaged plasmids, caused by exonucleolytic degradation and resynthesis, was reduced by repeated purification of intact circular forms. Extracts made from cells representing xeroderma pigmentosum complementation groups A, C, D and G were deficient in repair synthesis in response to the presence of *cis*- or *trans*-diamminedichloroplatinum(II) adducts in DNA.

Repair of site-specifically placed, defined DNA damage

The in vitro system has been extended to study the repair by whole cell extracts of plasmids containing a single adduct at a defined site. Since the lesions occur at a specified site it is possible to characterize a single repair event by examining appropriate restriction fragments. Double-stranded bacteriophage M13 DNA molecules were constructed containing a single specifically placed 2-(acetylamino)fluorene adduct at a specified G residue, or a single 4′-hydroxymethyl-4,5′,8-trimethylpsoralen-thymidine monoadduct. These circular DNA molecules were used as substrates in in vitro assays measuring DNA-repair synthesis by cell extracts from a normal human lymphoid cell line (Hansson et al., 1989). Both types of lesions stimulate DNA-repair synthesis at the site of the adduct. DNA-repair synthesis induced by the 2-(acetylamino)fluorene adduct took place in the damaged strand, and was largely confined to a region within a 31-base-pair restriction fragment around the adduct.

Studies with XP cell extracts

Extracts from XP cell lines of lymphoid origin have generally been found to be deficient in repair synthesis on damaged circular DNA (Wood et al., 1988b). The majority of the studies have been carried out with cell extracts from lymphoid lines representing the most common XP-complementation groups A, C, and D.

Complementation with bacterial proteins. The biochemical defect in most if not all XP-complementation groups appears to lie at the level of DNA incision. Thus, supplementation of XP extracts with enzymes that incise damaged DNA should allow XP extracts to perform DNA-repair replication. This has been found to be true for the pyrimidine dimer–DNA glycosylase/endonuclease from *M. luteus* (Wood et al., 1988b). Furthermore, when UV- or *cis*-DDP damaged plasmid

DNA was pretreated with purified *E. coli* UvrABC proteins, xeroderma pigmentosum cell extracts (complementation groups A, C, D and G) were able to carry out DNA-repair synthesis (Hansson et al., 1990). The ability of *E. coli* UvrABC proteins to complement xeroderma pigmentosum cell extracts indicates that the extracts are deficient in incision, but can carry out later steps of repair. Thus the in vitro system provides results that are in agreement with the incision defect found from studies of xeroderma pigmentosum cells (Hansson et al., 1990). On the other hand, addition of *E. coli* DNA polymerase I or *E. coli* DNA photolyase (in the dark) neither stimulates nor inhibits repair reactions in normal or XP cell extracts.

Complementation by mixing extracts from different XP groups. Mixing of some combinations of crude cell extracts from different repair-defective XP complementation groups can lead to reconstitution of the DNA-repair activity. Presumably, the defective component in one extract is contributed by the other extract, allowing reconstitution of a repair-synthesis reaction. Such biochemical complementation by mixing extracts has been observed when mixing XP-A with XP-C extracts (Wood and Robins, 1989), and XP-D extracts with XP-C or XP-A extracts (D. Coverley, P. Robins and R. Wood, unpublished results). This capacity for in vitro complementation is being used as the basis of an assay for the purification and characterization of protein factors involved in excision repair and defective in XP cells. An example of complementation using a partially fractionated normal cell extract is shown in Fig. 9. Here, whole cell extract from repair-proficient HeLa cells was fractionated by ammonium sulfate precipitation and heparin agarose chromatography. The 0.1 M KCl flow-through fraction and the 1.0 M KCl eluted fraction are each individually incapable of mediating damage-dependent repair synthesis, but repair can be reconstituted by mixing the components (Fig. 9). Furthermore, complementing activity for XP-A extracts is present almost exclusively in the 1.0 M KCl fraction.

It is important to verify the genetic specificity of the in vitro complementation assay. XP-A activity providing complementation in the in vitro assay has also been detected in calf-thymus tissue,

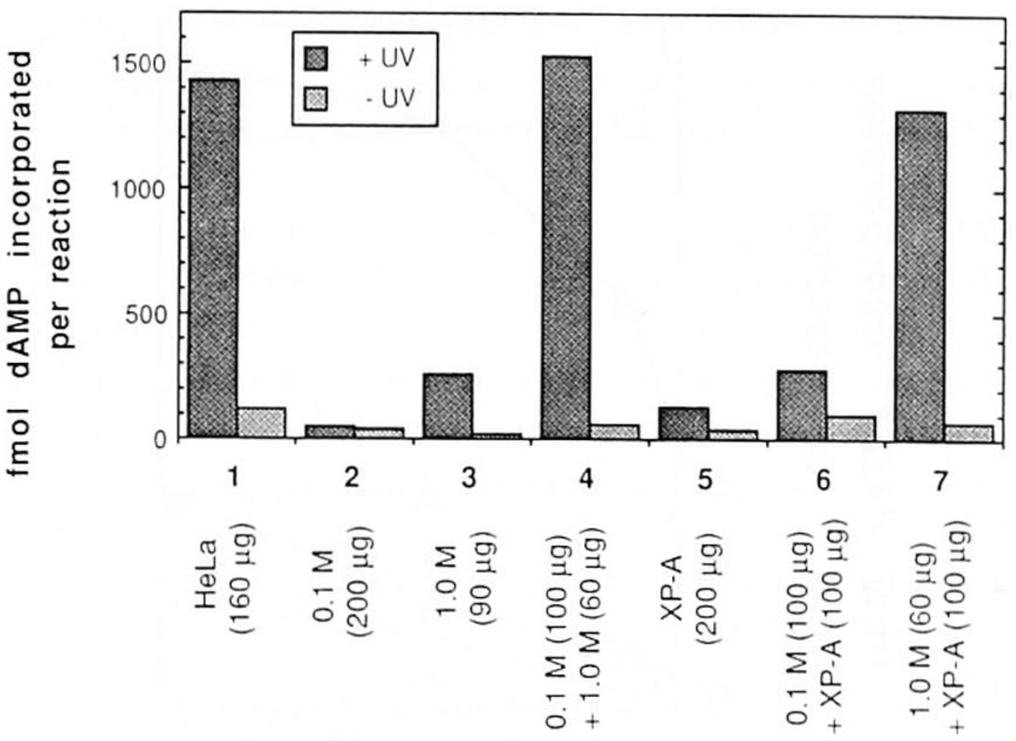

Fig. 9. Complementation of XP-A cell extract for repair synthesis, using partially fractionated HeLa cell extract. The high-speed supernatant of a HeLa extract, prepared by the method of Manley et al. (1980) was dialyzed to 0.1 M KCl and applied to a heparin–agarose column which was washed sequentially with buffer containing 0.1 M KCl and buffer containing 1.0 M KCl. Fractions containing the bulk of the protein were concentrated by ammonium sulfate precipitation. Repair reactions were carried out with a mixture of UV-irradiated pAT153 (+UV) and unirradiated pBR322 (−UV), using: Column 1, HeLa whole cell extract; Column 2, 0.1 M heparin agarose fraction; Column 3, 1.0 M heparin agarose fraction; Column 4, a mixture of the two fractions; Column 5, XP-A extract; Column 6, XP-A extract supplemented with 0.1 M heparin agarose fraction; Column 7, XP-A extract supplemented with 1.0 M heparin agarose fraction.

as well as in HeLa cells or lymphoid cell lines. However, XP-A factor is not present in extracts from XP-A cells, while the XP-C and XP-D factors are both present (unpublished data).

Comparison of the in vivo and in vitro systems

It is apparent from the foregoing that the cell-free repair assay is particularly useful for the characterization of those excision-repair steps that proceed in this system. It can be manipulated easily. Defined DNA substrates containing specific lesions can be utilized which permit detailed analysis of repair events. Repair proteins of other organisms can be introduced, as well as antibodies against specific components. This system is not expected to reflect all aspects and complexities of the in vivo process. DNA is introduced into the reactions in the form of 'naked' DNA rather than chromatin, and active transcription does not normally take place in the extracts. Moreover, in the cell, components of the repair apparatus can be continually synthesized or modified, whereas in

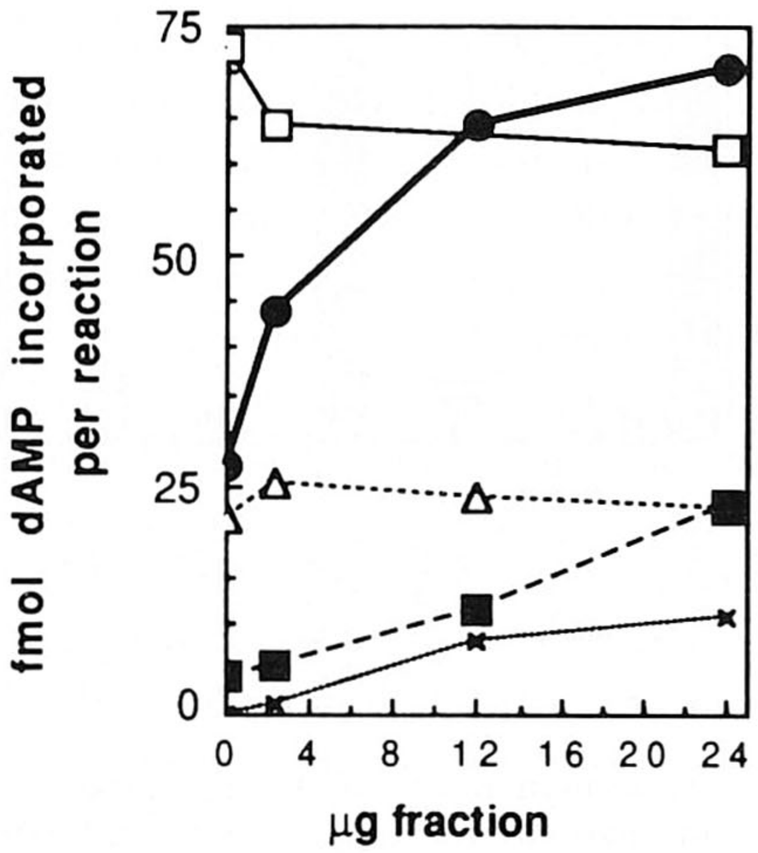

Fig. 10. Enhancement of DNA-repair synthesis in UV-damaged plasmid DNA by addition of protein fraction from calf thymus. Partially purified XPAC protein from calf thymus obtained as described in the text was added in the amounts indicated to 100 μg extract from GM1953 normal cells (□), 100 μg extract from GM2345 XP-A cells (●), 100 μg extract from GM2249 XP-C cells (■), 100 μg extract from XP3BR SV clone 15 XP-G cells (△), or calf thymus fraction only (×). Repair synthesis in nondamaged plasmid DNA did not increase upon addition of partially purified XPAC protein, and amounted to less than 10 fmoles dAMP incorporated per reaction.

soluble cell extracts constituents of the repair apparatus may be rate-limiting or even absent, so that only a part of the total repair process is observable. However, it should be possible to study repair of damaged chromatin or actively transcribing DNA by modifying the cell-free system appropriately. In contrast to the cell-free repair assay, the in vivo microinjection system is much less manipulatable and only a few (single cell) repair endpoints can be measured; but it entails all intricacies of the cellular repair machinery.

An initial experiment has been performed to test the biochemical specificity of the in vitro repair synthesis assay, using XP-A factor partially purified using the in vivo microinjection assay. In agreement with the in vivo results, an XP-A correcting preparation from calf thymus was found to significantly enhance repair synthesis in an XP-A cell extract, much less in XP-C, and not in an XP-G cell extract (Fig. 10). The XP-A factor does not appear to be present in rate-limiting amounts in a normal cell extract, since addition of the XP-A complementing fraction did not enhance repair synthesis in the normal cell extract (Fig. 10). 1–2 μg of the calf-thymus fraction was necessary to visualize complementation of the XP-A extract by the repair-synthesis assay, whereas the same fraction could be diluted more than 100-fold and would still show complementation in the microinjection assay.

These and other data demonstrate that both assays specifically score for the correcting factors of the XP complementation groups tested. However, there are complementation group-dependent differences. For example, rapid and substantial correction can be achieved both in vivo and in vitro for XP-A, whereas with XP-C a significantly smaller degree of complementation is observed in the microinjection assay as compared to cell-free extracts. This may in part be due to the very slow rate of complementation of the XP-C defect found in cell and cytoplast fusion experiments. The rate limiting step of complementation present in the living cell may not be operative in vitro.

In both systems, the effect of adding exogenous repair proteins has been examined. Comparable results were obtained with the cyclobutane pyrimidine dimer-specific DNA glycosylase/endonuclease of *M. luteus*. This enzyme efficiently bypasses the incision defect in XP cells and extracts. In contrast, the stimulation of repair synthesis by UvrABC proteins in the cell-free extracts, but not in the microinjection experiments may be due to differences in stability of the proteins (as discussed above) as well as the absence of intracellular compartmentalization and the lack of extensive higher-order chromatin structures in the soluble extracts. Studies to photoreactivate cyclobutane pyrimidine dimers also yielded quantitatively different results. A more pronounced reduction of repair synthesis was found in vivo than in vitro. The underlying reason for this difference may reside in the structural and enzymatic distinctions in complexity between cells and cell-free extracts, as noted above. Thus the challenge for the future of in vitro systems will be to more closely approach the complexity of the cell, while at the same time dissecting each individual step in the repair process.

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