GENETIC HETEROGENEITY IN THE HUMAN SKIN DISEASE XERODERMA PIGMENTOSUM

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INTRODUCTION TO THE PAPERS

Xeroderma pigmentosum

Xeroderma pigmentosum (XP) is a rare hereditary disease, that has been known as an entity since it was described by Hebra and Kaposi in 1874. The clinical characteristics are an intolerance of the skin and eyes to sunlight. Persistent erythema, abnormal pigmentations with freckling, actinic hyperkeratosis, scarring and premature ageing are found in the skin. Other features of the disease are skin malignancies such as basal and squamous cell carcinomas and melanomas. In addition to malignancies of ectodermal origin, malignancies of mesodermal origin such as sarcomas may also develop. These symptoms may occur even in early childhood.

In 1932 De Sanctis and Cacchione described a syndrome that was characterized by skin abnormalities of ecto- and mesodermal origin, as well as by neurological abnormalities including microcephaly, mental retardation, areflexia, choreoathetosis, cerebellar ataxia and premature closure of cranial sutures, retarded growth and sexual development. This condition was referred to as the De Sanctis-Cacchione (DSC) syndrome and was distinguished from the classical form described by Hebra and Kaposi.

The two forms of XP have never been reported in members of the same family, which might indicate that the two forms of XP are due to genetically distinct defects. Several authors (Hokkanen et al., 1969; Reed et al., 1969) have suggested that the phenotypic differences were the result of the same genetic defect but with variable expressivity; some cases show only the skin changes, while others show mental and somatic symptoms. In a recent report Robbins et al. (1974) pointed out that only very few XP patients have the DSC syndrome although many have one or more of its neurological abnormalities. A strict division of the XP patients into two groups is therefore questionable.

This disease is inherited according to an autosomal recessive pattern. The patients are often offspring of consanguineous marriages. The parents, the presumed heterozygotes, are clinically normal.

DNA repair mechanisms

In 1968 Cleaver discovered that cultivated skin fibroblasts from a classical XP patient and from two related DSC patients had an impaired DNA repair after irradiation with ultraviolet (UV) light (254 nm).

UV light has a potentially deleterious effect on living organisms which is at least for a part of the UV spectrum, due to DNA damage. DNA lesions are induced by that part of the UV spectrum that is absorbed by DNA. This absorption causes several types of photochemical changes in the DNA of which the formation of pyrimidine dimers is generally considered to be the primary lethal event (Setlow and Setlow, 1963).

Three cellular recovery systems have been demonstrated in UV[*] exposed bacteria. These mechanisms are photoreactivation, excision repair and post-replication repair. Photoreactivation can be generated in UV irradiated bacteria by exposure to light in the range of 300 nm-500 nm. This treatment activates a photoreactivating enzyme, which reverses the dimers into monomers. Excision repair, which is often referred to as dark repair because it operates without the need for visible light, is suggested to be a mechanism mediated by at least four enzymatic steps (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964): an incision step which introduces a single-strand break in the DNA strand containing the dimer; an excision step which removes an oligonucleotide containing the dimer and a small number of bases on either side of it; a DNA synthesis step which replaces the deleted DNA, and finally rejoining of the newly synthesized segment with the old strand. The third type of DNA repair, referred to as post-replication repair or recombinational repair, copes with the dimers during DNA replication. If the DNA template contains dimers the daughter strand of DNA produced is of low molecular weight, presumably because it contains gaps opposite each dimer. As incubation proceeds the gaps gradually disappear and DNA of higher molecular weight is formed by a process of recombination between the daughter and parental strands (Rupp et al., 1971).

Since "unscheduled DNA synthesis" (UDS) and "repair replication" were demonstrated in human cells (Rasmussen and Painter, 1964, 1966) it was assumed that an excision repair process existed in human cells. Both phenomena are presumably the result of the same fundamental process: the DNA repair synthesis step of the excision repair mechanism. UDS is the autoradiographic demonstration of uptake of radioactive thymidine (TdR) into DNA by cells which

* In this introduction, UV light means light of 254 nm, unless otherwise indicated, since most experimental work is done with this kind of UV light.

are not in the normal DNA synthesis phase (Djordjevic and Tolmach, 1967). Repair replication shows the non-semiconservative but dispersive pattern of this type of DNA synthesis. In order to demonstrate repair replication, cells which are in S-phase or show the semiconservative DNA synthesis, are labelled with bromodeoxyuridine (BUdR), a heavy analogue of TdR. Subsequently the cells are irradiated with UV light and incubated in the presence of radioactive TdR or BUdR. By centrifugation of the isolated DNA in density gradients a separation of the parental and S-phase synthesized DNA is obtained. Small, newly synthesized pieces are integrated into parental DNA as can be inferred from the gradient profile, showing the presence of radioactive molecules in parental DNA (Pettijohn and Hanawalt, 1964).

A direct proof that dimers are removed to a certain extent (about 50 per cent) from the DNA of human cells was first obtained by a chromatographic procedure which measures the dimer content of DNA (Regan et al., 1968). Recently, more sensitive techniques have been developed to demonstrate dimer excision. An immunological technique using specific antibodies against thymine dimers was devised by Seaman et al. (1972) and Lucas (1972). Another technique uses a dimer-specific endonuclease (isolated from *Micrococcus luteus*) to introduce breaks into UV irradiated DNA *in vitro* . In this way the molecular weight of the DNA, measured in a sucrose gradient, can be related to the number of dimers (Paterson et al., 1973; Wilkins, 1973).

Study of DNA replication during the S-phase of UV irradiated human cells revealed that events, comparable with those described as post-replication repair in bacteria can be observed (Buhl et al. 1972). Gaps in the newly synthesized DNA strand appeared to be filled in by *de novo* synthesis. A recombinational type of process does not seem to be involved in this by-pass mechanism in man.

Demonstration of a photoreactivating enzyme in human cells has been unsuccessful until recently Sutherland (1974) purified a protein from human leukocytes which could monomerize pyrimidine dimers *in vitro* by exposure to visible light. The biological significance of this enzyme in human cells *in vivo* is obscure.

Defective DNA repair in xeroderma pigmentosum

The discovery of impaired DNA repair in XP cells after UV irradiation implicated that XP was a radiation-sensitive mutation in man.

Cleaver (1968, 1970) found that UV exposed XP cells had low or negligible levels of UDS and repair replication which indicated a defect in the

excision repair process. Other investigators confirmed this observation in different XP patients (Bootsma et al., 1970; Kleijer et al., 1973: see appendix, paper I; Robbins et al., 1974). Moreover, no excision of pyrimidine dimers could be shown in these cells (Setlow et al., 1969; Cleaver and Trosko, 1970; Kleijer and Bootsma, 1970; Paterson et al., 1973). If XP cells are exposed to X-rays, normal levels of UDS and repair replication are observed (Cleaver, 1969; Kleijer et al., 1970; Kleijer et al., 1973: see appendix, paper I). X-rays induce single-strand breaks in DNA. The repair of single-strand breaks does not require the initial enzymatic chain scission but involves at least a DNA repair synthesis step and a rejoining step. The latter steps were assumed to be catalyzed by enzymes which also operate during excision repair. From these observations it was inferred that the XP cells were defective in an early enzymatic step, i.e. the incision step of the excision repair process.

The mutation in XP may resemble the mutations which affect the uvrA and uvrB loci in *Escherichia coli* (*E. coli*) . The UV-sensitive uvrA and uvrB mutants are defective in the excision of pyrimidine dimers. Recently Braun and Grossman (1974) purified from wild type *E. coli* cells a protein fraction with endonucleolytic activity specific for UV irradiated DNA and showed that this fraction was absent in uvrA - and uvrB mutants. However, no such UV-specific endonucleolytic enzyme has been isolated from human cells (Bacchetti et al., 1972; Brent, 1972).

Since 1971 evidence has been accumulated that some XP patients do not have a defect in excision repair. Burk et al. (1971) studied a clinically severe classic XP patient whose cells performed normal amounts of UDS after UV irradiation. Later Cleaver (1972) and Kleijer et al. (1973: see appendix, paper I) described similar patients, who had clinically the typical symptoms of classic XP, but whose cells showed apparently normal DNA repair synthesis. Cleaver (1972) called these patients XP variants. Patients, who showed very moderate skin abnormalities resembling those seen in XP, were reported by Jung (1970). Cells from these patients had normal levels of UDS after UV irradiation. In addition Jung inferred from his experiments that the UV treatment had a relatively high inhibitory effect on the progression of these cells through the S-phase. He concluded that this was a new form of molecular defect in XP and called it pigmented xerodermoid. It still has to be elucidated whether the XP variants, the pigmented xerodermoid patients and a patient (XP1RO) described by Bootsma et al. (1970), all having about normal levels of UDS, represent different clinical and genetical entities. Lehmann et al. (1974) have obtained evidence that cells from XP

variants described by Burk et al. (1971) and by Kleijer et al. (1973: see appendix, paper I) have a defect in post-replication repair. Their experiments demonstrated that the conversion of newly synthesized DNA from low molecular weight to high molecular weight was delayed and that this conversion was markedly inhibited by caffeine. These experiments have not as yet been repeated with cells from patients with pigmented xerodermoid and with cells from patient XP1RO described by Bootsma et al. (1970).

Genetic heterogeneity of XP

The laboratory studies discussed so far indicate that the enzymatic defect involved in the variant type of XP is different from that in the other XP patients.

Experimental evidence for genetic heterogeneity within the group of XP patients whose cells are defective in the excision repair process has also accumulated. Different residual levels of DNA repair synthesis have been observed in cells from different patients (Bootsma et al., 1970; Cleaver, 1970; Kleijer et al., 1973: see appendix, paper I; Robbins et al., 1974). The same level of repair synthesis was seen in cells of afflicted members of one family. Thus, the level of DNA repair synthesis may be inherited as a distinct genetic trait. An inverse correlation between the level of DNA repair synthesis and the severity of the clinical symptoms was suggested (Bootsma et al., 1970; Kleijer et al., 1973: see appendix, paper I). However, this correlation was not found in the XP patients studied by Robbins et al. (1974).

Clinical evidence of genetic heterogeneity was found in each of the different phenotypic forms of the disease, DSC and classical XP.

Complementation analysis

The aim of our study, presented in this thesis is to investigate the genetic heterogeneity of XP.

Somatic cell hybridization is a technique which enables us to perform complementation tests comparable with those available for micro-organisms. Complementation takes place if phenotypically normal cells result from fusion of cells with different defects. Intergenic complementation is achieved when mutations in different genes, coding for different polypeptides, are combined in the same cell. Intragenic or interallelic complementation might result from the combination of different mutations affecting the same gene.

Silagi et al. (1969) demonstrated intergenic complementation between

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human heteroploid D98/AH cells which lacked the enzyme hypoxanthine phosphoribosyl transferase (HPRT) and cells from an orotic aciduria patient, which were deficient in orotidine-5'-monophosphate (OMP) decarboxylase and OMP pyrophosphorylase. Intergenic complementation was also observed after fusion of glucose-6-phosphate dehydrogenase (G6PD) deficient human cells with cells from a Lesch-Nyhan patient (HPRT deficient) (Siniscalco et al., 1969). Intragenic complementation was claimed by Nadler et al. (1970), for cells from different galactosemia patients, with galactose-1-phosphate uridyl transferase deficiency. In these complementation experiments mononuclear hybrid cells were produced and isolated by selection procedures. The hybrid character of these selected cells was demonstrated by the presence of marker enzymes and a tetraploid chromosome number.

We used binuclear and multinuclear cells in our experiments looking for complementation between cells originating from different XP patients. Cells of two XP patients, with a reduced activity of DNA repair synthesis were fused. The restoration of the UDS was shown by autoradiography (De Weerd-Kastelein et al., 1972: see appendix, paper II). The hybrid character of these cells could be recognized by choosing cells from XP patients of different sex: the origin of the interphase nuclei in the binuclear cells was identified by sex chromatine fluorescence, using the fluorochrome atebrin (Pearson et al., 1970; Mukherjee et al., 1971).

In addition to the analysis of complementation at the single cell level we developed a biochemical method to study the same phenomenon. The resumption of repair replication following complementation was studied with DNA isolated from fused cell populations. Fusion conditions were such that a high frequency of multinuclear cells and consequently a high frequency of heterokaryons were produced (De Weerd-Kastelein et al., 1973: see appendix, paper IV).

With these experiments we could demonstrate complementation between cells originating from severe classic – and DSC patients. Normal levels of UDS were observed in the nuclei of the hybrid binuclear cells (De Weerd-Kastelein et al., 1972 and 1974 b: see appendix, paper II and III). In the multikaryon analysis the amount of repair replication studied by density gradients could be correlated with the number of nuclei in the same culture showing UDS by autoradiography, indicating complete restoration of repair synthesis following complementation (De Weerd-Kastelein et al., 1973: see appendix, paper IV). Complementation of DNA repair synthesis was also shown in hybrid synkaryotic cells (De Weerd-Kastelein et al., 1974 b: see appendix, paper III).

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Giannelli et al. (1973) demonstrated that cooperation in DNA repair can occur between normal human- and excision repair deficient XP nuclei: in hybrid binuclear cells, formed between normal human - and XP cells, both types of nuclei showed normal levels of DNA repair synthesis. Cooperation in DNA repair was excluded when both cell types were only in the proximity of each other. We carried out similar experiments (De Weerd-Kastelein et al., 1974b: see appendix, paper III) supporting these findings.

A third complementation group was found represented by two related patients with moderately severe symptoms of the disease and whose cells showed intermediate levels of DNA repair synthesis (De Weerd-Kastelein et al., 1974 a: see appendix, paper V). Recently Robbins et al. (1974) performed similar experiments with cells from their XP patients and described four complementation groups. Comparison of these four complementation groups with our three resulted in the identification of five complementation groups in the XP syndrome (see Table I).

TABLE I

Complementation groups in xeroderma pigmentosum

Complementation group (Bethesda nomenclature)	Cell strains from Rotterdam collection	Clinical description in papers from Rotterdam group
A	XP12RO, XP25RO, e.a.	DSC
B	not represented	–
C	XP4RO, XP9RO, XP16RO, e.a.	Classical XP, severe
D	not represented	–
E	XP2RO, XP3RO	Classical XP, light to moderately severe

Each group is characterized by a specific level of DNA repair synthesis (results not presented here).

The existence of five distinct complementation groups in the XP syndrome, among the patients whose cells exhibit defective DNA repair synthesis, indicates intergenic rather than intragenic complementation. The assumed enzymatic defect in these XP cells was a defect in an endonucleolytic enzyme responsible for the incision step of the excision repair process. The affected genes may play a role in the formation of this enzyme, as structural and regulatory genes.

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Another explanation of the complementation observed, could be that the excision repair enzymes act by means of an enzyme complex as was postulated by Kleijer and Bootsma (1971) and by Cleaver et al. (1972). These authors suggested that the different enzymatic steps in the excision repair mechanism are performed in a coordinated way, i.e. in an enzyme complex. This hypothesis implies that mutations affecting one of the enzymatic steps in the excision repair process, prevent the enzyme complex from progression along the DNA strand to the next lesion. This model predicts that all mutations, even those affecting the later steps of the excision repair process will manifest themselves as mutations in an initial step in the excision repair process. Cleaver (1974), using a modified method for velocity sedimentation, obtained different sedimentation profiles with DNA isolated from UV irradiated DSC- and UV irradiated classical XP cells. From these observations he inferred that the DSC cells, in contrast to the classical XP cells, might be able to perform the incision step of the excision repair process but lack a later stage in this repair. The general validity of this observation has not as yet been demonstrated.

Defective DNA repair and cancer

Most of the XP patients studied so far have a defective DNA repair in their cells which is a defect either in the excision repair process or in the post-replication repair process. Therefore it is attractive to postulate a relationship between the outstanding clinical feature of XP, actinic carcinogenesis, and defective DNA repair.

This relationship is supported by the following observations:

At least six different mutations (the five XP complementation groups and some of the XP variants) which all result in defective repair of DNA, coincide with a high risk for actinic carcinogenesis.

Secondly, an inverse correlation has been described between the residual levels of DNA repair in the cells of the patients and the severity of their clinical symptoms (Bootsma et al., 1970; Kleijer et al., 1973: see appendix, paper I). Although it is difficult to quantify the clinical symptoms, the severity is reflected in the age at which the first malignancies develop, the rate of tumor production and the age at death. However, in contrast to the observations on the Dutch XP patients, Robbins and coworkers (1974) did not find this inverse correlation in their comprehensive study of patients from the USA. The discrepancy might be the result of enviromental factors which may influence the phenotypic expression of the XP genotype, e.g. the amount of sun

exposure. A classification of the XP patients according to the severity of the clinical symptoms might therefore reflect differences in the amount of damage administered to the DNA of the cells rather than differences in genotype between the patients.

A third argument is based on the observation that exposure of human cells *in vitro* to various carcinogenic agents generates an excision repair process (Setlow and Regan, 1972; Stich et al., 1973) and probably a mechanism resembling post-replication repair (Buhl and Regan, 1973; Van Den Berg, 1974); studies of DNA repair in bacteria have shown that repair mechanisms are not error-free. Excessive damage to the DNA enhances the chance of mis-repair. Extrapolating to man the high incidence of actinic skin cancer seen in people who have received excessive sun exposure for years (sailor-man's disease) could also be the result of errors made by the DNA repair mechanisms.

The carcinogenic activity of sunlight must be caused by light of wavelengths greater than 290 nm, since light of UV wavelengths shorter than 290 nm is absorbed by the atmosphere due to the presence of ozone. In our experiments DNA repair was generated by irradiating the cells with UV light of wavelength 254 nm. Evidence is obtained that the deleterious effect of sunlight might be caused, at least partly, by the same DNA lesions as those induced by UV light of 254 nm. Experiments with *E. coli* (Harm, 1969) and with *Saccharomyces cerevisiae* (Resnick, 1970) have demonstrated the role of excision repair in the recovery of these micro-organisms from sunlight-induced killing. The dimer formation (Trosko et al., 1970) and the lethal effect (Harm, 1969) of sunlight is equivalent to the damage induced by a dose rate of 1-2 $erg/mm^2/$ min UV light of 254 nm.

It seems likely that both a defective repair of sunlight-induced DNA lesions and an excess of these lesions in the presence of a normal repair mechanism result in carcinogenesis. The mechanism by which a XP cell transforms into a malignant cell is not known. Whether the etiology of carcinogenesis in XP fits in a viral or mutational theory of carcinogenesis remains to be elucidated.

SUMMARY

Xeroderma pigmentosum (XP) is a rare hereditary disease characterized by a hypersensitivity of the skin to sunlight. Freckles and skin tumors develop on areas of the skin exposed to sunlight. Two clinical forms of the disease have been described: the classical type of XP showing only skin lesions and the De Sanctis-Cacchione (DSC) form of the disease which has neurological complications in addition.

Both forms are inherited according to an autosomal recessive pattern. Heterozygotes are clinically normal.

Cleaver discovered in 1968 that skin fibroblasts from both types of XP patients showed a reduced level of DNA repair synthesis when exposed to ultraviolet (UV) light (254 nm). DNA repair synthesis is one of the enzymatic steps involved in a DNA repair mechanism called excision repair which was first discovered and extensively studied in bacteria. It is a cellular mechanism by which bacteria can recover from otherwise lethal damage such as produced by UV light. UV light absorbed by DNA produces pyrimidine dimers. The excision repair removes the dimers from the DNA by four enzymatic steps: an incision step which interrupts the continuity of the DNA strand, an excision step which removes the DNA damage (pyrimidine dimers), a DNA synthesis step which replaces the removed part of the DNA strand, and a rejoining step which connects the newly synthesized oligonucleotide with the old strand. Evidence has been accumulated that a comparable excision repair mechanism is operative in human cells.

Cleaver's findings were confirmed by several investigators studying different XP patients. Studies of the basic defect in XP at the molecular level showed that XP cells were able to repair single-strand DNA breaks. From this observation it was assumed that the XP cells had a defect in the incision step of the excision repair mechanism. This assumption was supported by the finding that pyrimidine dimers were not removed from the DNA of XP cells.

Studies at the cellular level also indicated genetic heterogeneity in XP since different residual levels of DNA repair synthesis were observed in cells from XP patients of different families. Clinical evidence for genetic heterogeneity is suggested by the two clinical forms of the disease.

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In 1971 Burk et al. first described a XP patient whose cells showed normal excision repair and more such XP patients have been reported. Recently, Lehmann et al. (1974) inferred from their experiments that cells from these XP patients, the variant type of XP, have a defect in post-replication repair. Like excision repair this repair mechanism was first discovered in bacteria. It is a mechanism by which a DNA replicating cell can by-pass DNA lesions such as pyrimidine dimers. The observation that XP variants have a defect in post-replication repair whereas other XP patients have a defect in excision repair also argues for genetic heterogeneity.

The aim of our study was to investigate genetic heterogeneity within the XP syndrome. In more detail, the purpose of our investigations - presented in the appendix - was:

a) to analyse the UV induced excision repair mechanism in cells from healthy persons and different XP patients;

b) to investigate whether the clinical differences between the XP patients as well as the differences in residual levels of DNA repair synthesis are the consequence of different genetic defects; and

c) to study the possibility of cooperation in DNA repair between an excision repair proficient cell and an excision repair deficient cell.

In paper I of the appendix two phenomena of the excision repair mechanism have been studied in normal human cells after UV irradiation: the excision of UV induced pyrimidine dimers and the DNA repair synthesis step. Chromatography was used to demonstrate the excision of pyrimidine dimers from DNA. Two distinct techniques were used to show DNA repair synthesis. One technique is the autoradiographical demonstration of the amount of radioactive thymidine (TdR) incorporated into the DNA of non S-phase cells (unscheduled DNA synthesis or UDS). The other technique to detect DNA repair synthesis is the demonstration of the so-called DNA repair replication by isopycnic centrifugation of DNA in density gradients. The repaired DNA, which has a non-semiconservative but dispersive pattern, is separated from the S-phase or semiconservative synthesized DNA by differences in density, using selective bromodeoxyuridine (BUdR) labelling.

DNA repair synthesis was also studied in UV irradiated XP cells. According to the residual level of DNA repair synthesis in cells from different XP patients, four groups of patients were distinguished:

1. DSC patients with low or undetectable levels of repair synthesis in their cells.

2. Severe classic XP patients showing a low level of repair synthesis in their cells (5-15 per cent of the normal level).

3. Classical XP patients with relatively high residual activities of repair synthesis in their cells (30–50 per cent of the normal level); these activities increase after higher doses of UV light.

4. Classical XP patients who, because of normal levels of repair synthesis in their cells, are referred to as XP variants.

Paper II in the appendix describes hybridization experiments designed to study genetic heterogeneity between DSC - and severe classical XP patients. Cells from two different XP patients were fused and the resulting hybrid binuclear cells were used for the complementation analysis. By choosing XP cells from patients of different sex the sex chromatin diagnosis of the interphase nuclei allowed the recognition of the hybrid cells. DNA repair synthesis was studied with the autoradiographic technique.

These experiments demonstrated that the DSC - and severe classic XP genomes complement each other's defect in excision repair, since normal levels of DNA repair synthesis were observed in both types of nuclei of the hybrid binuclear cells. We concluded that the DSC—and severe classic XP patients represent different mutations.

In paper III the general validity of the complementation studied in paper II, has been tested by extending the number of DSC- and severe classic XP patients in the complementation analysis with binuclear cells. Complementation of DNA repair synthesis was also studied in hybrid synkaryotic cells formed after fusing DSC- with severe classic XP cells. The assay of DNA repair synthesis was performed by autoradiography.

These experiments confirmed our previous finding of complementation between the DSC- and the severe classic XP genome. It was further shown that cell to cell contact between an excision repair proficient (normal) cell and an excision repair deficient (XP) cell did not result in cooperation in DNA repair. When both cell types were combined to form a binuclear cell, a normal level of DNA repair synthesis was observed in both nuclei.

In paper IV we introduced a method to demonstrate complementation with biochemical techniques.

The restoration of the DNA repair synthesis activity, following com-

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plementation, was established with the isopycnic centrifugation of DNA isolated from a fused cell population. For that purpose we used fusion conditions which produced a high frequency of multinuclear cells and consequently a high frequency of heterokaryons. Density profiles of DNA isolated from such a fused cell population showed the resumption of repair replication.

In paper V of the appendix a third complementation group is established, which is represented by two related classical XP patients with moderately severe symptoms and relatively high residual activities of DNA repair synthesis in their cells. The complementation was investigated at the single cell level.

SAMENVATTING

Het syndroom xeroderma pigmentosum (XP), zoals dat oorspronkelijk door Hebra en Kaposi (1874) is beschreven wordt gekenmerkt door een overgevoeligheid van de huid voor zonlicht, uiteindelijk resulterend in huidtumoren. Deze ziekte wordt autosomaal recessief overgeërfd. In 1932 beschreven De Sanctis en Cacchione een erfelijke ziekte, waarbij naast huidafwijkingen, kenmerkend voor het XP syndroom, ook neurologische complicaties optreden.

In 1968 toonde Cleaver aan dat gekweekte huidfibroblasten van mensen lijdend aan het zog. klassieke XP syndroom en fibroblasten van mensen met het De Sanctis-Cacchione (DSC) syndroom na bestraling met ultraviolet (UV) licht (254 nm) een defect DNA herstel vertoonden.

Door onderzoek aan stralingsgevoelige mutanten bij bacteriën is gebleken dat de schadelijke gevolgen van UV licht geheel of gedeeltelijk worden opgeheven door middel van enzymatische processen. De belangrijkste letale schades bleken pyrimidinedimeren in het DNA te zijn. Deze kunnen ontstaan daar waar twee aangrenzende pyrimidinebasen in één DNA streng aanwezig zijn. Het opheffen van de inactiverende werking van UV licht bleek in bacteriën o. a. plaats te kunnen vinden door middel van een zog. excisieherstelmechanisme. Dit proces bestaat uit verschillende enzymatische stappen nl. een incisie stap, waarbij de DNA streng op enkele basen verwijderd van de dimeer in haar continuiteit wordt onderbroken; een excisie stap, waarbij een oligonucleotide van mogelijk enkele honderden basen lang, die de dimeer bevat, uit de DNA streng wordt verwijderd; een herstelsynthese stap, waarbij het verwijderde DNA vervangen wordt door synthese met behulp van de complementaire streng, die als matrijs dient, en tenslotte de zog. "rejoining" stap, waarbij het nieuw gesynthetiseerde oligonucleotide verbonden wordt met de oude DNA streng.

Ook bij hogere organismen zijn aanwijzingen verkregen over het bestaan van DNA herstelprocessen. Men vermoedt dat in menselijke cellen een excisieherstelmechanisme werkzaam is, vergelijkbaar met dat bij bacteriën, daar zowel verwijdering van dimeren als herstelsynthese na bestraling met UV licht aangetoond kan worden. Cellen van de meeste tot nu toe onderzochte XP patienten vertonen echter een verminderde DNA herstelsynthese en een verminderde excisie van dimeren. Deze XP cellen hebben vermoedelijk een defect

in de incisie stap van dit herstelmechanisme. Verder blijkt dat cellen, afkomstig van verschillende patienten, verschillende, maar voor de patient karakteristieke, restactiviteiten in DNA herstelsynthese vertonen. Het niveau van DNA herstelsynthese lijkt genetisch bepaald, daar verschillende XP patienten binnen één familie dezelfde restactiviteit vertonen.

Sinds 1971 zijn XP patienten beschreven, waarbij geen defect in het excisieherstel kon worden aangetoond. Deze patienten worden ook wel aangeduid als XP varianten. Vrij recent hebben Lehmann en medewerkers (1974) aanwijzingen verkregen dat bij deze patienten een ander DNA herstelmechanisme gestoord is.

Het in dit proefschrift beschreven onderzoek heeft tot doel na te gaan of de klinische verschillen tussen de XP patienten als wel de verschillen in DNA herstelsynthese capaciteit tussen de XP cellen, berusten op genetische heterogeniteit. Daartoe werd gebruik gemaakt van de celfusie techniek, ook wel celhybridisatie genoemd. Gekweekte huidfibroblasten afkomstig van steeds twee verschillende XP patienten werden bij elkaar gebracht onder toevoeging van geinactiveerd Sendai virus. Het virus is niet in staat zich in de cellen te vermeerderen, maar kan echter wel celklontering en tenslotte fusie van cellen teweegbrengen. Daardoor ontstaan er meerkernige cellen; sommigen bestaande uit kernen uitsluitend afkomstig van één van de XP patienten, anderen bestaande uit kernen afkomstig van beide XP patienten.

In ons onderzoek werd een methode ontwikkeld waarbij gebruik werd gemaakt van tweekernige cellen. Bij at random fuseren van de twee type cellen worden drie soorten tweekernige cellen verwacht in de verhouding 1 : 2 : 1. Daarbij zullen 50% van de cellen kernen bevatten afkomstig van één van de XP patienten, en de overige 50% zullen kernen van beide XP patienten bevatten. Met het laatste type cellen was het mogelijk een complementatietest uit te voeren, vergelijkbaar met die welke bij micro-organismen worden uitgevoerd: men kan op deze manier toetsen of de verschillende genetische informaties complementair aan elkaar zijn. De hier te toetsen informatie was het vermogen excisieherstel te vertonen. De herstelsynthese stap uit dit mechanisme is op het niveau van de enkele cel aan te tonen. M.b.v. de autoradiografie wordt de incorporatie van radioactief thymidine (TdR) in UV bestraalde G1- en G2-fase cellen zichtbaar ("unscheduled DNA synthesis" of UDS).

Het optreden van complementatie kan ook langs biochemische weg worden onderzocht. De herstelsynthese kan in een dichtheidsgradient aangetoond worden door gebruik te maken van het dispersief, niet semiconservatief karakter

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van deze synthese. Door de S-fase cellen, welke een semiconservatieve DNA synthese vertonen, te labellen met bromodesoxyuridine(BUdR) en vervolgens na bestraling met UV licht de cellen te incuberen met radioactief BUdR of TdR, kan na isolatie van DNA en centrifugering in een dichtheidsgradiënt een goede scheiding van de twee soorten synthesen worden verkregen. De op deze manier zichtbaar gemaakte DNA herstelsynthese wordt ook wel herstelreplicatie genoemd. In dit proefschrift wordt een methode beschreven om fusies uit te voeren welke veel meerkernige cellen opleveren, zulks met het oogmerk een zo groot mogelijk aantal kernen aan de eventuele complementatie te laten deelnemen. Cultures resulterend uit dergelijke fusies werden op hun herstelreplicatiecapaciteit getoetst.

Het eerste artikel van de appendix bevat een inventarisatie van XP cellijnen – afkomstig van verschillende patiënten – onderzocht op celkinetische aspecten van het excisieherstelproces. Naar restactiviteiten in UDS en herstelreplicatie konden we vier groepen XP patiënten onderscheiden:
1. DSC patiënten met geen meetbare restactiviteit.
2. Ernstige klassieke XP patiënten met geringe restactiviteit.
3. Klassieke XP patiënten met duidelijke restactiviteit, welke bij toenemende UV dosis toeneemt.
4. Klassieke XP patiënten met normale niveaus van DNA herstelsynthese en als XP varianten aangeduid.

In het tweede artikel van de appendix wordt het onderzoek beschreven naar de genetische heterogeniteit tussen DSC- en ernstige klassieke XP patiënten. De complementatieanalyse werd in de enkele cel uitgevoerd. Door bij de fusie gebruik te maken van cellen afkomstig van XP patiënten van verschillend geslacht, werd het mogelijk de interfase kernen in de tweekernige cel te identificeren met behulp van hun sexchromatine.
De beide chromatines vertonen een karakteristieke fluorescentie met het fluorochroom atebrine. De herstelsynthese werd onderzocht met behulp van de autoradiografie. Het complementatie effect in tweekernige cellen, gevormd door fusie van cellen afkomstig van ernstige klassieke XP patiënten met cellen afkomstig van DSC patiënten, toonde voor het eerst aan, dat binnen het syndroom XP verschillende mutaties een rol kunnen spelen.

Het derde artikel van de appendix geeft een overzicht van alle fusies welke tussen cellen afkomstig van DSC- en ernstige klassieke XP patiënten werden uitgevoerd, waarmee de algemene geldigheid van het complementatie

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effect werd aangetoond. In alle fusiecombinaties, waarbij beide type cellen met elkaar werden gefuseerd, werd complementatie gevonden. Complementatie kon ook worden aangetoond in synkaryotische cellen, ontstaan na fusie van een DSC- met een ernstige klassieke XP cel. In dit artikel worden ook experimenten be- schreven, welke aantonen dat bij cellulair contact tussen cellen afkomstig van een ernstige klassieke XP patient en cellen van een gezond individu geen coöperatie in DNA herstel mogelijk is. Daarentegen was wel coöperatie in DNA herstelsynthese waar te menen, wanneer beide type cellen gefuseerd werden tot een tweekernige cel.

In het vierde artikel van de appendix wordt het complementatie effect, ontstaan na fusie van cellen afkomstig van een ernstige klassieke XP patient met cellen van een DSC patient met behulp van biochemische technieken aange- toond. De fusieprocedure hier toegepast stelde ons in staat een biochemische analyse uit te voeren op de cultuur resulterend uit de fusie, waarin niet voor de hybride cellen was geselecteerd. Het weer terugkeren van het vermogen DNA herstelsynthese uit te voeren, als gevolg van complementatie, werd met behulp van het aantonen van herstelreplicatie in de gefuseerde cultuur gedemon- streerd.

In het vijfde artikel van de appendix wordt, met behulp van analyse op het niveau van de enkele cel, aangetoond dat tenminste twee klassieke XP patienten een derde complementatiegroep vormen. Beide patienten, die fami- lie van elkaar zijn, vertonen minder ernstige klinische verschijnselen. De cellen van deze patienten hebben een duidelijke restactiviteit in DNA herstelsynthese.

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NAWOORD

Na het behalen van het einddiploma h.b.s. B in 1961 aan de Gemeentelijke h.b.s. voor meisjes te Arnhem begon ik in hetzelfde jaar met de studie in de biologie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen (k) legde ik in 1965 af; het doctoraalexamen met hoofdvak genetica, nevenrichting microbiologie, en bijvakken endocrinologie en plantenfysiologie in 1968. Van oktober 1964 tot oktober 1966 was ik als student-assistent verbonden aan het Zoölogisch Laboratorium te Utrecht.

In februari 1969 trad ik als wetenschappelijk medewerker in dienst bij de Medische Faculteit te Rotterdam; eerst aan de afdeling Celbiologie II en vanaf oktober 1972 bij de afdeling Genetica. Het in dit proefschrift beschreven onderzoek werd sinds 1969 op de hiervoor genoemde afdelingen verricht.

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APPENDIX

PAPER I

UV-INDUCED DNA REPAIR SYNTHESIS IN CELLS OF PATIENTS WITH DIFFERENT FORMS OF XERODERMA PIGMENTOSUM AND OF HETEROZYGOTES

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SUMMARY

UV-induced DNA repair synthesis, as measured by autoradiography as well as by isopycnic centrifugation methods, was studied in a large number of cell strains from patients with the classic form of xeroderma pigmentosum (XP) or the De Sanctis-Cacchione syndrome (DSC) and several of their heterozygous parents. On the basis of the kinetics of repair synthesis in the cultured skin fibroblasts we have recognized four distinct groups of XP patients: (*1*) classic XP patients with low residual repair capacities, (*2*) classic XP patients with intermediate, but dose-dependent, levels of repair synthesis relative to the normal level, (*3*) patients, diagnosed as having classic XP, with a normal or only slightly reduced repair capacity and (*4*) DSC patients with a complete deficiency of repair synthesis. Complementation studies reported elsewhere have shown that different mutations are responsible for the defect in at least three of these groups. Cell strains of each of the four XP types were able to rejoin single-strand DNA breaks induced by X-rays. Most of the cell strains derived from heterozygotes showed normal repair activities. However, in the parents some of the of DSC-patients a significant reduction of the level of repair synthesis was found.

INTRODUCTION

The biological significance of an excision repair process in mammalian cells has been demonstrated in studies of DNA repair in cells from patients having the autosomal recessive disease XP. The UV-sensitive cells[7,12], originating from XP patients have a reduced capacity to perform DNA repair synthesis after UV irradiation[5,1,4] and are unable to excise pyrimidine dimers from their DNA[25,10,15].

Several observations have indicated that the genetic basis of XP is not similar

Abbreviations: BUdR, 5-bromodeoxyuridine; DSC, De Sanctis-Cacchione; FUdR, 5-fluorodeoxyuridine; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum.

in all patients. Two clinically distinct forms of this disease are known: the classic type of XP and the DSC syndrome[23]. Both forms are characterized by hypersensitivity of the skin of the patients to UV-irradiation, leading to severe skin lesions in sun-exposed regions of the body; in addition, DSC-patients suffer from neurological complications. In previous autoradiographic studies, BOOTSMA *et al.*[1] found various levels of UDS in a number of classic XP strains, but similar levels in genetically related patients. Genetic evidence for the involvement of different mutations was obtained from complementation studies by DE WEERD-KASTELEIN *et al.*[27,28,18]. Complementation, leading to the restoration of the capacity to perform DNA repair synthesis, was demonstrated first in heterokaryons obtained after fusion of classic XP and DSC cells. Similarly, we have recently found evidence for the presence of two complementation groups in classic XP (DE WEERD-KASTELEIN, unpublished results).

From biochemical data it has been suggested that neither classic XP nor DSC cells perform an initial step of the repair process[6,26,17]. A further biochemical characterization of XP cell strains is required to reveal the defective enzymatic function in each of the distinct XP forms. For that purpose we have compared the kinetics of UDS (by autoradiography) and repair replication (by density-gradient analysis) in normal human cells and in cell strains from a large number of XP patients and several of their heterozygous parents. The group of patients includes cases of classic XP with clinical symptoms varying from mild to severe and cases of the DSC syndrome.

MATERIALS AND METHODS

(a) Cell strains and culture techniques

Fibroblast cultures were started from skin biopsies in Carrel flasks using F12 medium supplemented with 20% foetal calf serum. Before the fibroblasts were subcultured in tissue culture bottles, the serum concentration was changed to 15% foetal calf serum. HeLa cells and T cells (established human kidney cell line) used in the excision experiments were grown in F12 medium with 10% newborn calf serum. For UDS experiments, cells were seeded in small petri dishes (Falcon, diameter 3.5 cm) containing a coverslip, whereas for repair replication and excision experiments large petri dishes (Greiner, diameter 9 cm) were used.

Details on the XP cell strains are presented in Tables I, II and III. The designations used in this paper for XP-cell strains anticipate a proposal by CLEAVER AND BOOTSMA for the standardization of the nomenclature for XP cell strains. All strains from individuals clinically diagnosed as XP patients are called XP (including classic XP, DSC and variant patients with a normal repair ability). The cell strains from heterozygotes are called XPH. The strains are further characterized by a serial number or by two letters (*e.g.* the initials of the patient) given in the institute where the cells had been brought into culture and by two letters that denote the city where this institute is situated (*e.g.* RO for Rotterdam, SF for San Francisco, *etc.*).

(b) Irradiation

UV irradiation (254 nm) of the washed and drained cultures was performed with a Philips TUV lamp (15 W) at either 7.5 or 9 erg/mm²/sec. X-irradiation conditions were as described previously[17].

(c) Unscheduled DNA synthesis

The cultures were labelled with [³H]thymidine (10 μCi/ml; 2.0 Ci/mmole for 1 h before UV irradiation to label cells in S-phase and for 2 h after irradiation. Fixation of the coverslip cultures, autoradiography, staining and the analysis of the autoradiograms were performed as described previously[1].

(d) Repair replication

The cells were incubated in medium containing BUdR (1.5 μg/ml) and FUdR ($10^{-6}M$) for 2 h before UV irradiation. After irradiation, incubation was continued in medium containing [³H]thymidine (10 μCi/ml; 20 Ci/mmole), BUdR (1.5 μg/ml), FUdR ($10^{-6}M$) and hydroxyurea ($10^{-3}M$) for 2 h (or other periods indicated under RESULTS). After the cells were harvested ($1-2 \cdot 10^6$ for each experimental point), the DNA was isolated and analysed by sodium iodide density gradient centrifugation as previously described[20].

(e) Excision of pyrimidine dimers

Cells, grown in petri dishes, were labelled for 20 h with [³H]thymidine (2.5 μCi/ml; 26 Ci/mmole). After post-UV-incubation periods of 0, 8 and 24 h the cells were collected and washed. DNA preparations, free of protein and containing relatively low amounts of RNA, were obtained by the phenol extraction procedure described by KIRBY AND COOK[14]. The DNA was hydrolysed in formic acid. The radioactive products, thymine and thymine-containing pyrimidine dimers, were separated by two-dimensional paper chromatography as described by SETLOW AND CARRIER[25]. The pyrimidine dimer content of the DNA was determined by counting the radioactivity in the thymine and the dimer regions of the chromatogram, and is expressed as the percentage radioactivity in dimers of the total radioactivity ($\widehat{XT}/T\%$). The amount of radioactivity recovered from the dimer regions of each chromatogram was 400–1200 cpm.

(f) Rejoining of X-ray-induced single-strand breaks

The numbers of single-strand breaks in the DNA of X-irradiated cells were calculated from the molecular weights of the DNA as determined by sedimentation in alkaline sucrose gradients. The conditions used for cell lysis and centrifugation of the DNA were as described earlier[17].

RESULTS

(a) The time course of repair replication in UV-irradiated normal human cells

Fig. 1 shows an example of the separation in NaI-density gradients of repaired DNA with a normal density (Fig. 1b; fractions 10–15) from semi-conservatively synthesized DNA with a higher density (fractions 1–5). The peak representing repaired DNA was found after UV irradiation and labelling of normal cells as described in MATERIALS AND METHODS, but was absent if the cells used are not irradiated (Fig. 1a). A quantitative measure for the amount of repair replication is given by the specific radioactivity of repaired DNA (cpm/μg DNA). This value was calculated from the radioactivity and the amount of DNA (as measured by fluorescence of the ethidium-bromide–DNA complex[20]) in the three top fractions of the peak containing the re-
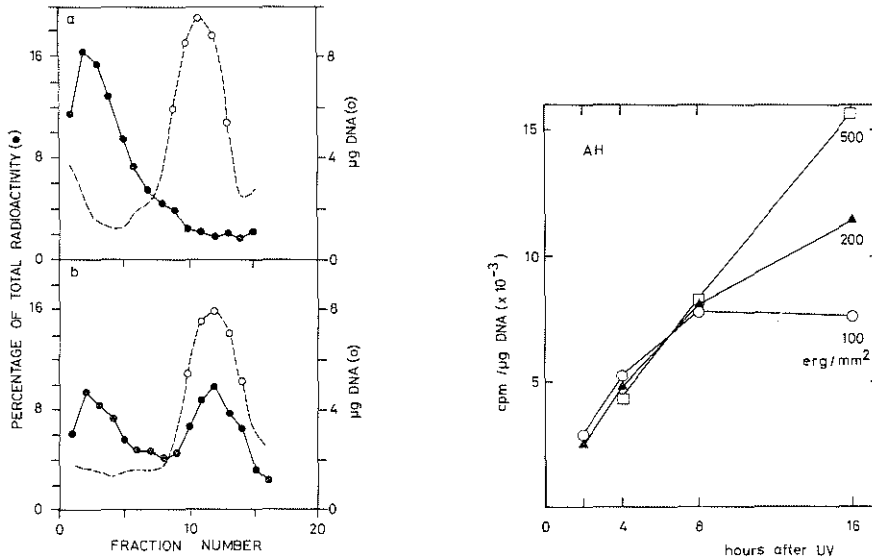
Fig. 1. Repair replication in human cells demonstrated with the NaI isopycnic centrifugation technique. The cells were grown for 2 h in medium containing BUdR and FUdR before UV irradiation. After irradiation, incubation was continued for 2 h in medium containing [³H]-thymidine, BUdR, FUdR and hydroxyurea. Distributions in NaI isopycnic gradients of the total amount of DNA (○) and of tritium-labelled DNA (●) from normal human cells (AH). Total amount of radioactivity per gradient: $2–3 \cdot 10^4$ cpm, (a) 0 erg/mm²; (b) 100 erg/mm².

Fig. 2. The amount of repair replication as a function of time after UV irradiation in normal human cells (AH) determined by NaI isopycnic gradient analysis.

paired DNA. The standard deviation of specific radioactivity measurements was 10% in control experiments in which different cell samples were irradiated, labelled and analysed simultaneously under identical conditions.

The amounts of repair replication occurring in normal cells following UV doses of 100, 200 and 500 erg/mm² are given in Fig. 2. The repair process seemed to be completed at about 8 h after a dose of 100 erg/mm², whereas after 200 erg/mm² a considerable amount of repair took place between 8 and 16 h. After 500 erg/mm² the amount of repair replication increased almost linearly with time, which suggests that the repair replication rate remains constant for at least 16 h.

(b) Excision of pyrimidine dimers

The excision of pyrimidine dimers from the DNA was studied by the determination of the radioactivity in dimers ($\widehat{TT}$ and $\widehat{CT}$) as a percentage of the total radioactivity in the DNA of cells at various time intervals after UV irradiation (200 erg/mm²). A decrease in the dimer content ($\widehat{XT}$/T%) of DNA of about 25% after 8 h and 50% after 24 h post-irradiation incubation was found in experiments with T cells, HeLa cells and normal human skin fibroblasts (Fig. 3).

(c) Unscheduled DNA synthesis and repair replication in XP cells

The amounts of UDS and repair replication performed during the 2 h following various UV doses were measured in a series of classic XP and DSC cell strains. In
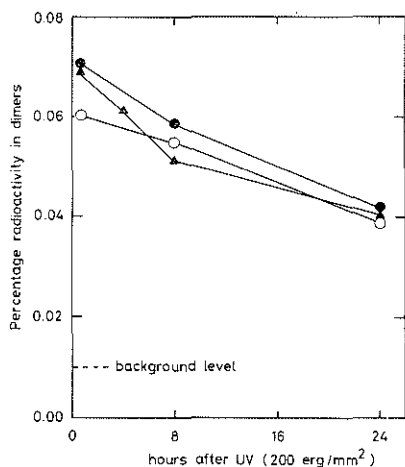
Fig. 3. Excision of pyrimidine dimers in normal human primary fibroblasts (AH) (○), T cells (●) and HeLa cells (▲) during incubation after UV irradiation (200 erg/mm²). The pyrimidine dimer content ($\widehat{XT}/T\%$) of the DNA was determined by measuring the radioactivity in the dimers ($\widehat{TT} + \widehat{CT}$) and thymine (T) after chromatography.

TABLE I

DNA REPAIR CAPACITIES OF CELLS FROM CLASSIC XERODERMA PIGMENTOSUM PATIENTS

Cell strain[a]	Case			Unscheduled DNA synthesis[b]	Repair replica- tion[b]	Rejoining of DNA breaks[c]
	Sex	Age	Clin. symp. (Severity)			
XP4RO[d]	Female	16	Severe	10–15	5–10	n
XP9RO	Male	9	Severe	10–15		n
XP16RO	Male	9	Severe	5–10	5–10	
XP19HO[e]	Female	14	Severe	10–15		
XP20RO	Male	3	Severe	10–15		
XP21RO[d]	Male	15	Severe	10–15		
XP12SF[f]	Female	12	Severe	10–15		
XP2RO[g]	Female	34	Moderate	40	35	n
XP3RO[g]	Female	29	Light/mod.	45		
XP5RO[h]	Female	25	Light/mod.	25	22	n
XP6RO[h]	Female	18	Light	31		
XP7RO[h]	Female	18	Light	28		
XP1RO	Male	48	Very light	75	70–100	n
XP11RO	Female	45	Very light	100		
XP7TA[i]	Female	20	Severe	99		
XP30RO[j]	Male	30	Light/mod.	88	110	n

[a] A new nomenclature for XP cell strains is used. See under MATERIALS AND METHODS.
[b] Expressed as a percentage of the levels found in normal cells after a UV dose of 100 erg/mm².
[c] n (normal), means that more than 70% of the breaks were rejoined within 20 min after X-irradiation (20 krad), which was also found in normal cells.
[d] XP4RO and XP21RO are siblings.
[e] XP19HO provided *via* Dr. R. M. HUMPHREY, Houston.
[f] XP12SF, provided by Dr. J. E. CLEAVER, San Francisco.
[g] XP2RO and XP3RO are second cousins.
[h] XP5RO, XP6RO and XP7RO are siblings.
[i] XP7TA, provided by Dr. H. SLOR, Tel Aviv.
[j] XP30RO, skin biopsy provided by Dr. V. DER KALOUSTIAN, Beirut.
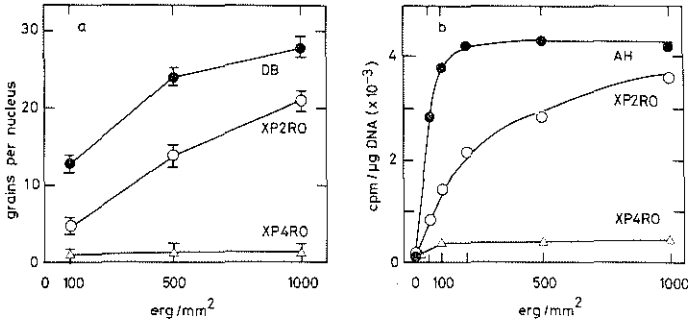
Fig. 4. DNA repair synthesis as a function of UV dose in normal human fibroblasts (DB and AH) cells from a moderately severe classic XP patient (XP2RO) and cells from a severe classic XP patient (XP4RO). (a) Unscheduled DNA synthesis; [³H]thymidine labelling for 3 h after UV. Autoradiographic exposure time: 3 days. (b) Repair replication; [³H]thymidine labelling (in the presence of BUdR, FUdR and hydroxyurea for 2 h after UV; NaI isopycnic gradient analysis.

Table I, UDS and repair replication (at 100 erg/mm²) in the classic XP strains are given as a percentage of the levels in normal human cells, which were measured simultaneously as controls in each experiment. In a group of 7 cell strains, all originating from severe cases of classic XP (the first group in Table I), low residual repair activities were observed. Cell strains from patients in 2 unrelated families (the second group in Table I) showed intermediate repair activities. In 4 cases the levels of DNA repair synthesis were normal or only slightly reduced (the third group in Table I). Data on UDS obtained for the strains XP (1–7)RO and XP9RO have been described earlier[1].

Autoradiography and density gradient data obtained with the strains XP2RO and XP4RO are shown in more detail in Fig. 4. The amount of repair replication observed in XP2RO cells was strongly reduced at low doses of UV, but increased with the dose up to at least 1000 erg/mm² and approached the level in normal cells, which reached a maximum at 100–200 erg/mm² (Fig. 4b). A corresponding increase in the relative repair level was apparent from the dose–response curves for UDS in XP2RO (Fig. 4a) and XP3RO cells (see ref. 1) and possibly also for XP5RO cells. In other strains (*e.g.* XP4RO, Fig. 4) the level of repair synthesis relative to that in normal cells was not dose-dependent.
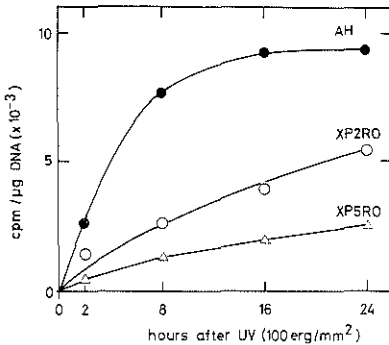

Fig. 5. The amount of repair replication as a function of time after UV irradiation (100 erg/mm²) in normal cells (AH) and two cell strains from moderately severe classic XP patients (XP2RO and XP5RO), determined by NaI isopycnic gradient analysis.

TABLE II

DNA REPAIR CAPACITIES OF CELLS FROM DE SANCTIS-CACCHIONE PATIENTS

| Cell strain[a] | Case | | | Unscheduled DNA synthesis[b] | Repair replication[b] | Rejoining of DNA breaks[c] |
	Sex	Age	Clin. symp.			
XP12RO[d]	Male	13	Severe	<5	<5	n
XP4LO[e]	Male	6	skin lesions	<5		
XP17SF[f]	Female	9	and	<5		
XP25RO[d]	Male	1	neurological	<5	<5	n
XP26RO[d]	Female	7	complications	<5	<5	
XPPKSF[f]	Female	9		<5		

[a,b,c] See footnotes a, b, c to Table I.
[d] XP12RO is genetically related to the siblings XP25RO and XP26RO. Skin biopsies provided by Dr. V. DER KALOUSTIAN, Beirut.
[e] XP4LO, provided by Dr. J. M. PARRINGTON, London.
[f] XP17SF and XPPKSF, provided by Dr. J. E. CLEAVER, San Francisco.

To investigate whether XP cells with appreciable residual repair capacities can ultimately perform as much repair as normal cells, the repair process was followed for extended periods in XP2RO and XP5RO cells. Fig. 5 shows that the repair process in these cells after a dose of 100 erg/mm² continued for at least 24 h, whereas in normal cells the repair was almost completed after 8 h.

Table II summarizes the results of experiments on UDS in 6 DSC strains and repair replication in 2 of these strains. In no strain did the amount of repair synthesis at various UV doses up to 1000 erg/mm² exceed significantly the background level observed in unirradiated cells.

(d) DNA repair synthesis in cells from XP heterozygotes

Repair synthesis was studied in 7 cell strains derived from the parents of classic

TABLE III

DNA REPAIR CAPACITIES OF CELLS FROM HETEROZYGOTE PARENTS OF CLASSIC XERODERMA PIGMENTOSUM AND DE SANCTIS-CACCHIONE PATIENTS

| Cell strain[a] | Case | Unscheduled DNA synthesis[b] | | | Repair replication[b] | | |
| | | 100 | 500 | 1000 | 100 | 500 | 1000 |
		(erg/mm² UV)			(erg/mm² UV)		
XPH8RO	Mother of XP5RO XP6RO, XP7RO (classic XP)	100	100	100			
XPH10RO	Mother of XP9RO (classic XP)	100	100	100			
XPH14RO[c]	Mother of XP12RO (DSC)	81	71	83	51	60	56
XPH15RO[c]	Father of XP12RO (DSC)	74	67	72	76	73	61
XPH17RO	Mother of XP16RO (classic XP)	100	100	100	127	98	78
XPH27RO[c]	Mother of XP25RO XP26RO (DSC)	86	83	81			
XPH28RO	Father of XP25RO XP26RO (DSC)	95	88	94	128	88	97

[a,b] See footnotes a, b to Table I.
[c] Skin biopsies were provided by Dr. V. DER KALOUSTIAN, Beirut.

XP and DSC patients. In 2 heterozygous strains (XPH14RO and XPH15RO) from both parents of a DSC patient (XP12RO) a significant reduction of the repair synthesis level was found with the autoradiographic technique (Table III and ref. 2) as well as with the density-gradient technique (Table III). The same mutation is probably present in the heterozygotes XPH27RO and XPH28RO because of family relationships. However, the cells of these individuals did not show a consistent decrease of the repair level, although a slight decrease is indicated in the UDS data. The cell strains derived from parents of classic XP patients performed normal amounts of repair after doses up to 1000 erg/mm² (Table III).

(e) Rejoining of X-ray-induced single-strand DNA breaks

After X-irradiation (20 krad) normal and XP cells were incubated for 0, 10, 20, 40 and 60 min. The molecular weight of the DNA at the various post-irradiation periods was determined by sedimentation in alkaline sucrose gradients as described previously[17], and the number of breaks relative to DNA from unirradiated cells was calculated. The time courses of rejoining for all cell strains studied (normal and XP, Tables I and II) were similar to those published earlier for XP4RO and a normal strain[17].

DISCUSSION

Repair replication (Fig. 2) and excision of pyrimidine dimers (Fig. 3 and refs. 22, 26) in UV-irradiated human cells followed time courses consistent with the hypothesis that the two phenomena represent steps in the same repair process. A considerable fraction of the dimers is still found in the DNA at a time when the cell ceases repair replication. Similar observations of PATERSON *et al.*[21], who used UV endonuclease purified from *Micrococcus luteus* to detect dimer sites, show that the remaining dimers are situated in the DNA indeed and not in large excised oligonucleotides, as might be supposed in the chromatographic method that we used. Moreover, the incompleteness of dimer excision can not be attributed to an early inactivation of the repair system, or even of the whole cell, by the UV dose used (200 erg/mm²), because at higher doses (*e.g.* 500 erg/mm²) the repair process appeared to continue for longer times. Therefore, it is suggested that part of the dimer sites is less accessible to the excision–repair system; the cell might cope with these dimers by a post-replication repair mechanism (refs. 19, 3).

The amount of repair replication, performed in normal cells during incubation for 2 h as a function of the UV dose, reached a maximum between 100 and 200 erg/mm² (Fig. 4b). This can be ascribed to saturation of the repair system in this dose range, rather than to completion of repair at the lower doses, because the rate of repair replication after 100 erg/mm² remained constant for a longer time than the 2 h labelling period (Fig. 2). For the same reason the amount of repair synthesis measured in UV-irradiated normal and XP cells after labelling periods of 2 h was proportional to the initial rates of repair synthesis.

The XP patients in this study belonged to two main groups according to their clinical symptoms: classic XP and the DSC syndrome. Among the classic XP patients there were differences in the severity of the clinical symptoms. As described by BOOTSMA *et al.*[1] and confirmed by the present extended investigations, the severity of

the clinical symptoms of the patients and the levels of repair synthesis in the cells seemed to be inversely correlated. The various patients can be divided into four groups according to the different kinetics of the UV-induced DNA repair synthesis in their cells, as follows.

(1) Cases of classic XP with low, but detectable, residual repair capacities (Table I, first group; 5–15% of the control).

(2) Cases of classic XP with intermediate levels of repair synthesis (Table I, the second cousins XP2RO and XP3RO, 35–50%; the siblings XP5RO, XP6RO and XP7RO, 20–30%). However, in XP2RO cells we observed a marked increase with dose in the relative repair replication rate from 29% of the control rate at 100 erg/mm^2 to 85% at 1000 erg/mm^2 (Fig. 4b). Like the XP lymphocytes investigated by BURK *et al.*[4] and ROBBINS AND KRAEMER[24], XP2RO and XP5RO cells continued the repair process for a longer time than did normal cells (Fig. 5); so eventually these XP cells may perform as much repair as normal cells.

(3) Cases of classic XP with normal or only slightly reduced repair capacities (Table I, third group). Patients having symptoms comparable to those of XP but with normal DNA repair synthesis have also been reported by JUNG[13], BURK *et al.*[4] and CLEAVER[8]. The defect involved in these patients is probably not associated with any DNA repair system, including post-replication repair[3], as was suggested by CLEAVER[8] because of his finding that cells of such patients have a normal UV sensitivity.

(4) DSC patients with a complete deficiency of the repair capacity (Table II, 6 cases from 4 unrelated families). This complete deficiency is probably not characteristic for the DSC syndrome since DSC patients with residual repair capacities have been reported[7,12,24].

In the study of cell strains from the heterozygous parents of XP patients a reduced repair activity is indicated in the parents of the DSC patients in our series. In contrast, the only two cases of heterozygotes having a reduced repair level reported by CLEAVER[8] were the parents of a patient with the classic form of XP. In most cases so far investigated, normal repair levels have been observed, which is consistent with the recessive nature of the disease. Apparently the enzyme that is defective in the XP-cells is present in excessive rather than in rate-limiting amounts in normal cells. These results indicate that the detection of heterozygotes by means of the present techniques is not reliable.

We have previously demonstrated that classic XP and DSC cells can complement each other[27,28,18]. Recently we have found complementation between the XP2 RO cells (group 2) and either DSC cells or classic XP cells of group 1 (unpublished results). These observations indicate that different mutations are involved in the three forms of the disease. As discussed above, three of the four groups of XP patients were defective in excision repair. These three groups (1, 2 and 4), distinguished by their distinct kinetics of repair synthesis, coincided with the three complementation groups. The results do not indicate whether the mutations are located within one gene or in different genes coding for polypeptides of either the same enzyme or different enzymes.

Thus far four arguments have been presented for the hypothesis that classic XP as well as DSC cells are defective in an initial step of excision repair, probably incision.

(1) The normal repair replication[6,17] and rejoining of single-strand breaks[17] (Tables I and II) after X-irradiation in XP cells.

(2) The inability of XP cells to excise pyrimidine dimers[26,10,15].

(3) The demonstration that dimer sites in the DNA, which are susceptible to attack by UV-endonuclease from *Micrococcus luteus*, remain in XP cells, whereas they disappear from normal cells[21].

(4) The absence of accumulated breaks, which might be expected in the DNA of UV-irradiated XP cells as a result of incision if one of the steps following incision were defective[16].

However, none of these results is conclusive for the hypothesis of a defective incision function in XP cells. The first argument only holds if the same enzymes are involved in the repair replication and rejoining steps in the repair of UV and X-ray damage. The other three arguments are no longer valid if the repair enzymes operate in a coordinated way[15,9,16] (*e.g.* by a systematic scanning of the DNA by an enzyme complex), since a defect in any step would then block the whole repair process. If the coordinated repair hypothesis is correct it cannot be excluded that steps in excision repair other than the incision step are defective in XP. Moreover, in a complex model one can also envisage a mutation that affects the rate at which the complex advances along the DNA strands from one lesion to the next; such a defect might cause a deviating dose–response curve for repair replication similar to that found for the XP2RO strain (Fig. 4b).

As described earlier, normal levels of repair synthesis per nucleus were found in most of the heterozygous (XP) strains and also in the heterokaryons obtained by fusion of normal cells with XP cells[11] or by fusion of different types of XP strain[27,28]. These observations can be explained in the complex model if we assume that repair complexes can start the scanning and repair process only at a limited number of sites on the DNA. One single complex could then operate effectively in a DNA region belonging to one "initiation site for repair"; subsequent complexes entering the same region would be redundant because the DNA has already been repaired. A reduction of the normal concentration of repair complexes would not affect the repair capacity as long as the concentration remains high enough to bind one complex to each initiation site. A coordinated repair mechanism, as proposed here, seems to permit an efficient repair of the DNA in the complicated chromosomal structure and would avoid the presence of single-strand DNA regions or breaks for relatively long periods during the repair process.

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PAPER II

Genetic Heterogeneity of Xeroderma Pigmentosum demonstrated by Somatic Cell Hybridization

XERODERMA pigmentosum is an autosomal recessive disease characterized by hypersensitivity of the skin to ultraviolet radiation resulting in severe skin lesions. DNA repair replication after ultraviolet irradiation is absent or markedly reduced in cultivated fibroblasts from patients with xeroderma pigmentosum (XP cells) compared with normal cells[1,2]. Using the dark repair mechanism in microorganisms as a model, evidence has been presented that XP cells are defective in the incision step of DNA repair[3-5].

There are some indications of a genetic heterogeneity in the basic defect of XP. Different clinical symptoms have been described. In the classic form of XP, the patients show skin lesions only, whereas in the De Sanctis-Cacchione syndrome, a rare variant of XP, there are also severe neurological abnormalities and mental deficiency. At the molecular level, heterogeneity is suggested by the finding of different rates of DNA repair replication in fibroblast cultures from patients with XP of different families[2,6-8].

In our study, the existence of a genetic heterogeneity in the basic defect of XP is shown by detection of complementation after application of cell fusion techniques.

Table 1 Origin of Cell Strains and Repair DNA Synthesis following Exposure to 100 erg/mm² Ultraviolet Light

Strain	Origin	Repair DNA synthesis Grain count *	% Labelled nuclei †
C2	Control, male	18.0 ± 0.5	96
C3	Control, female	17.2 ± 0.5	94
XP4	Severe case of XP, female	3.6 ± 0.2	3
XP9	Severe case of XP, male	5.3 ± 0.4	15
XP12	De Sanctis-Cacchione, male	2.3 ± 0.3	3
XP16	Severe case of XP, male	3.4 ± 0.5	4

* The average number of grains per nucleus and the standard error of the mean. Only nuclei with 50 or less grains were included.

† Only nuclei with 0–50 grains were counted; nuclei with 8–50 grains were considered as labelled.

The origin of the cell strains is given in Table 1. Fibroblast cultures were grown in thymidine-free F12 medium supplemented with 15% foetal calf serum. To study unscheduled

DNA synthesis the cells were seeded in small Petri dishes containing a sterile coverslip. 2- to 4-day-old cultures were labelled for 1 h with 10 μCi ^{3}H-thymidine (^{3}H-TdR, specific activity 2.0 Ci/mmol) per ml. medium, washed with a balanced salt solution, drained and exposed to 100 erg/mm^2 of ultraviolet light (a 15 W 'Philips' TuV low pressure mercury tube, dose rate 9 erg/mm^2/s, predominantly 254 nm). Subsequently the cultures were incubated again in ^{3}H-TdR containing medium (10 μCi/ml.) for 2 h, rinsed in saline and fixed with Bouin's fixative. Autoradiographic preparations were made using Kodak AR10 stripping film and an exposure time of one week. Cells in S-phase were heavily labelled; cells in the process of repair DNA synthesis after ultraviolet exposure, but not in S-phase, could easily be distinguished as lightly labelled cells[3].

Repair DNA synthesis is very slow or even absent in the four XP cell strains both in terms of grain counts and of percentages of weakly labelled cells (Table 1). The frequency distribution of grain counts in one normal (C2) and two XP strains (XP4 and XP12) is presented in Fig. 1. On the basis of the distribution in normal cells (Fig. 1a and 1b) we adopted the grain numbers of 8 and 50 as the limits of unscheduled DNA synthesis (Table 1).

The occurrence of complementation was investigated in binuclear cells to avoid the rather complicated isolation of a pure mononuclear hybrid cell line. In the fusion experiments, cells of two different XP strains were trypsinized, mixed in a ratio of 1 : 1 and incubated in the presence of ultraviolet-inactivated Sendai virus according to the method of Harris et al.[9]. After fusion, the cell suspension was seeded in Petri dishes on coverslips. After 1, 2 or 3 days' growth, the cultures were exposed to 100 erg/mm^2 ultraviolet light to study repair DNA synthesis as described for the parental cell strains. In Table 2 the results are presented of an experiment in which XP4 cells were fused with XP12 cells. Fusions with only one cell type (either XP4 or XP12) served as controls.

The fused and irradiated populations of XP4/XP12 resulted in preparations in which 25–35% of the binuclear cells were weakly labelled. These labelled binuclear cells are only found in the hybrid fusion and are absent in the ultraviolet irradiated fusions of XP4/XP4 and XP12/XP12 as well as in the unirradiated populations.

Using four xeroderma cell strains (XP4, XP9, XP12 and XP16), six different fusions were performed to study the specifity of the occurrence of unscheduled DNA synthesis in binuclear cells (Table 3). In those combinations involving XP12 cells (De Sanctis–Cacchione), 30–40% of the binuclear cells were found to be lightly labelled over each of both nuclei,

2

whereas binuclear cells obtained after fusion of cells from different patients, each with the classic form of xeroderma pigmentosum, did not show any increase in labelling on
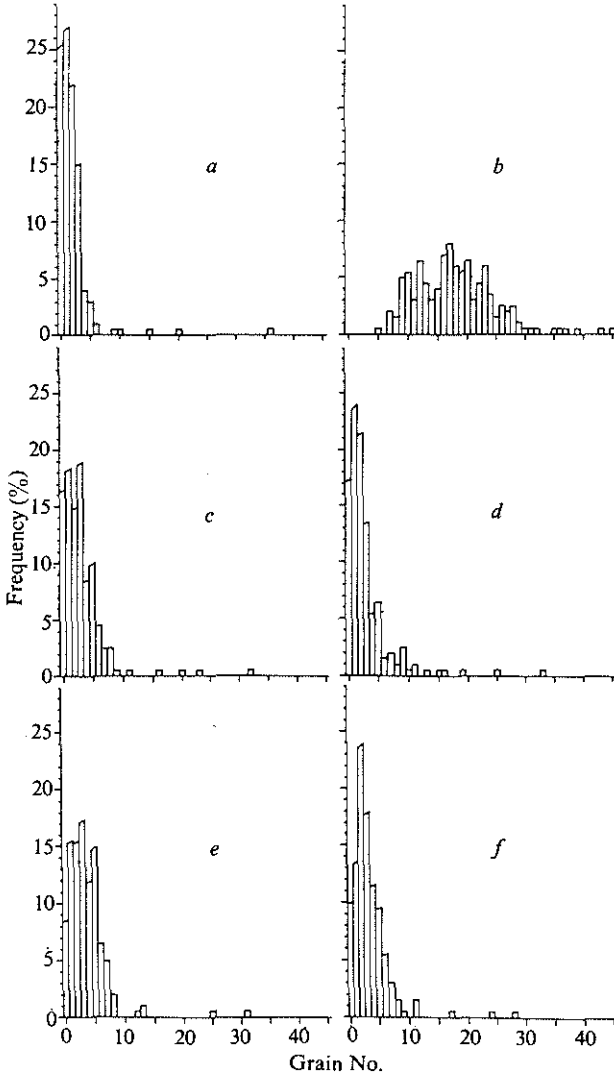

Fig. 1 Repair DNA synthesis in fibroblast cultures of normal individuals and patients with xeroderma pigmentosum. The frequency distributions of grain numbers over nuclei in auto-radiographic preparations after ultraviolet exposure and ^{3}H-TdR labelling are presented. Only cells with 50 or less grains per nucleus were counted. *a, c* and *e,* Unirradiated cells of the control (C2), the XP4 and the XP12 cell strain respectively. *b, d* and *f,* C2, XP4 and XP12 cells after exposure to 100 erg/mm^2 of ultraviolet irradiation.

Table 2 Tritiated Thymidine Labelling following Ultraviolet Exposure of Fused Cell Populations

Fusion	Time after fusion (days)	% Labelled binuclear cells * after	
		0 erg/mm^2	100 erg/mm^2
XP4/XP4	1	0	3
	2	3	4
	3	0	5
XP12/XP12	1	0	0
	2	< 2	< 2
	3	< 2	0
XP4/XP12	1	1	25
	2	1	36
	3	0	31

* Only cells with 0–50 grains over each of both nuclei were counted; cells with 8–50 grains over each of both nuclei were counted as labelled. Each value is based on counting of 100–300 binuclear cells.

irradiation. The 8% labelled binuclear cells observed after fusion of XP4 with XP9 cells might be the result of a relatively high residual activity of the repair process in XP9 cells (Table 1).

The population of XP4/XP12 cells should contain three types of binuclear cells XP4/XP4, XP12/XP12 and XP4/XP12. To determine which class of binuclear cells shows the labelling pattern indicative for repair replication, the technique of identification of sex chromosomes in interphase nuclei was used. Fusion was performed between XP4 and XP12 cells, while XP4/XP4 and XP12/XP12 fusions served as controls. Two days after fusion, ultraviolet exposure and ^{3}H-TdR labelling were carried out as described earlier. The cells were fixed in ethanol–ether (1 : 1) for 2.5 h, air dried and stained for 5 min in 0.5% aqueous atebrin (G. T. Gurr). After rinsing and mounting in Na-K phosphate buffer (pH 5.5) the preparations were examined by fluorescence microscopy. After the atebrin staining the Y chromosome in male interphase nuclei[10] and the Barr body in female nuclei[11] are visible as fluorescent spots. Four classes of binuclear cells in the female XP4/male XP12 fusion were observed, characterized by two nuclei each with a Barr body (Fig. 2a), two nuclei each with Y chromatin (Fig. 2b), one nucleus with a Barr body and one with Y chromatin (Fig. 2c), and one or two unidentifiable nuclei. The positions of about 100 binuclear cells of each of the first three classes were recorded. After autoradiography the labelling pattern of the same cells was studied (Fig. 2).

In the ultraviolet irradiated cultures 87% of the binuclear cells having one nucleus with Y chromatin and the other nucleus with a Barr body (XX/XY) showed 8–50 grains over each nucleus (Table 4). Weak labelling was found in only
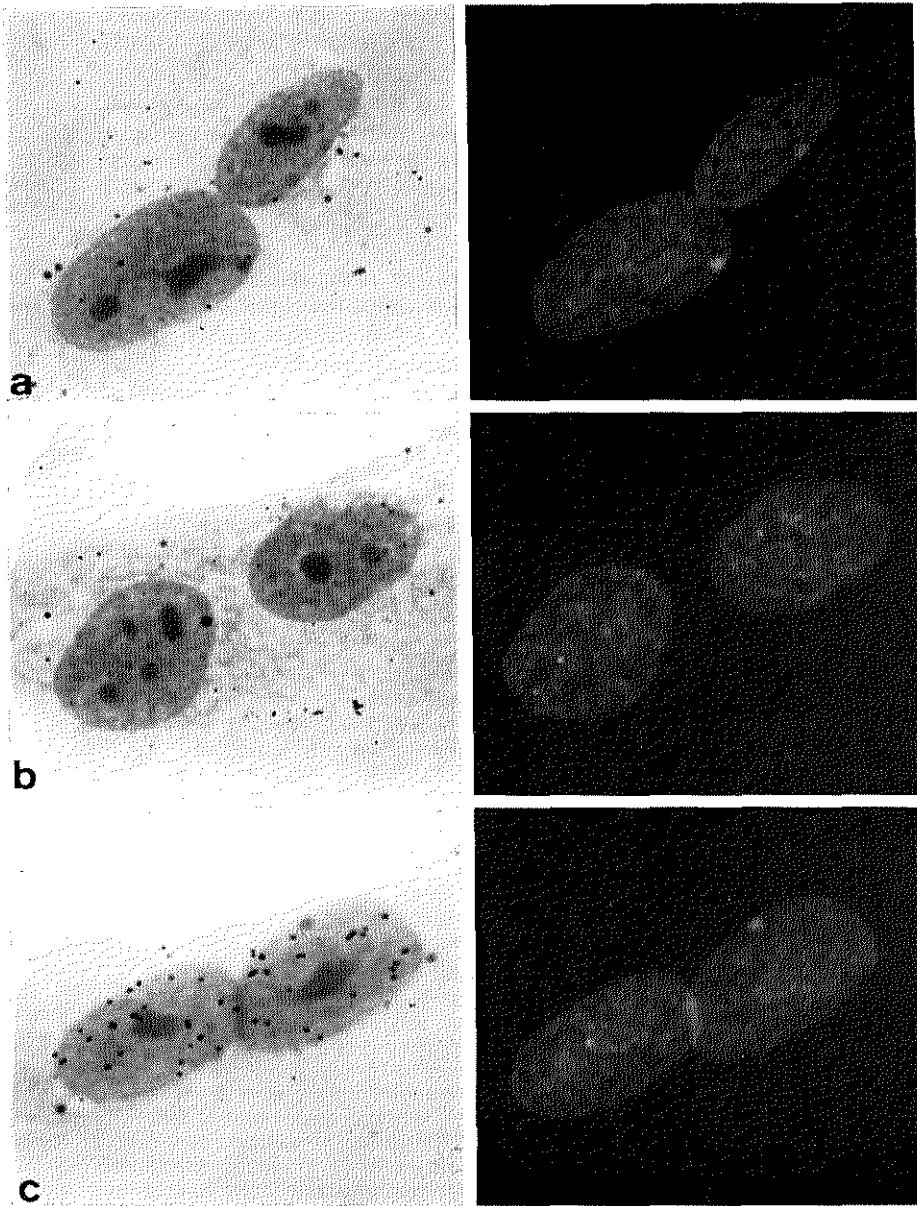
Fig. 2 Repair DNA synthesis in binuclear cells after fusion of XP4 (classic form of XP) and XP12 (De Sanctis-Cacchione) cells. Autoradiograms (on the left) and fluorescence after atebrin staining (on the right) are shown. *a*, Binuclear cell having two nuclei with a Barr body (XP4/XP4); *b*, binuclear cell showing Y chromatin in both nuclei (XP12/XP12); *c*, binuclear cell having one nucleus with a Barr body and another nucleus with Y chromatin (XP4/XP12). Only the XP4/XP12 binuclear cell shows repair replication. Magnification is 1100x.

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8 % of the XX/XX and 2 % of the XY/XY binuclear cells. The frequency distributions of grain counts (Fig. 3) clearly indicate that, based on atebrin characterization of the binuclear cells, two different classes can be distinguished: first, XX/XX (Fig. 3a) and XY/XY (Fig. 3b) binuclear cells showing a grain count distribution comparable with that in the parental cell strains (Fig. 1d and 1f); second, XX/XY binuclear cells (Fig. 3c) showing the same labelling pattern as normal cells after ultraviolet exposure (Fig. 1b).

The average number of grains per nucleus found over the XX/XY binuclear cells (Table 4) is about the same as observed for control cells showing repair DNA synthesis after ultraviolet exposure (Table 1, cell strains C2 and C3).
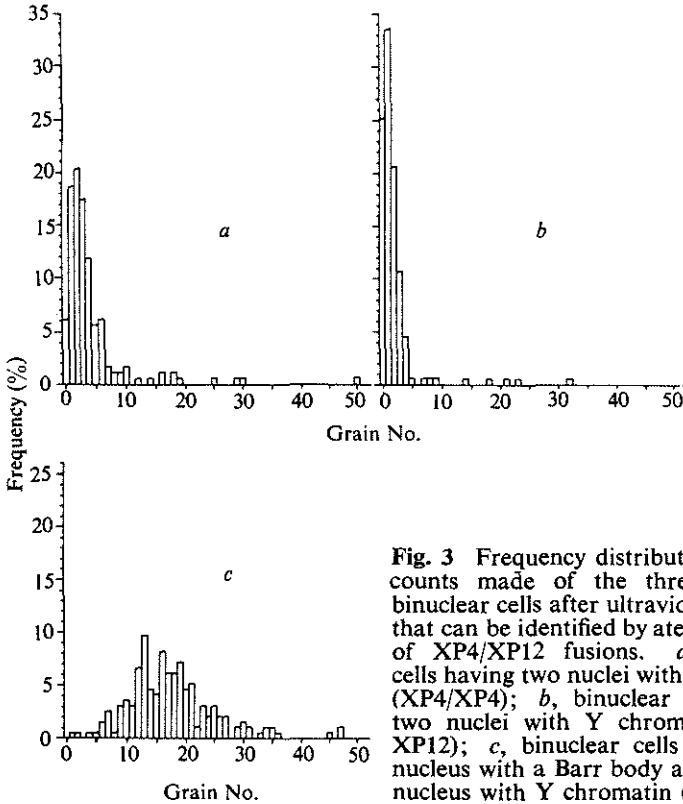

Fig. 3 Frequency distribution of grain counts made of the three types of binuclear cells after ultraviolet exposure that can be identified by atebrin staining of XP4/XP12 fusions. a, Binuclear cells having two nuclei with a Barr body (XP4/XP4); b, binuclear cells having two nuclei with Y chromatin (XP12/XP12); c, binuclear cells having one nucleus with a Barr body and the other nucleus with Y chromatin (XP4/XP12).

Table 3 Tritiated Thymidine Labelling following Ultraviolet Exposure of Fused Cell Populations using Different Cell Strains of Xeroderma Pigmentosum

Fusion	% Labelled binuclear cells *	
	0 erg/mm^2	100 erg/mm^2
XP4/XP9	<1	8
XP4/XP12	1	36
XP4/XP16	<1	2
XP9/XP12	<1	30
XP9/XP16	<1	4
XP12/XP16	<1	36

* See footnote to Table 2. The number of binuclear cells counted varied from 50–200.

The present results strongly suggest that the ^{3}H-TdR labelling in the binuclear hybrid cells after ultraviolet irradiation represents repair DNA synthesis as a result of complementation. This assumption is supported by the fact that the labelling in binuclear cells is found only after ultraviolet exposure; labelling is absent in fusions between cells belonging to the same XP cell strain; only those binuclear cells are labelled which have the hybrid combination of nuclei (classic form of XP and De Sanctis-Cacchione syndrome); in these binuclear cells the labelling pattern is identical to that of ultraviolet irradiated control cells.

Table 4 Tritiated Thymidine Labelling of Binuclear Cells identified by Atebrin Staining following Exposure to 100 erg/mm^2 Ultraviolet Light

Fusion	Atebrin diagnosis of sex chromosomes in binuclear cells	Grain count *	% Labelled binuclear cells †
XP4/XP4	XX/XX	4.4 ± 0.2	0
XP12/XP12	XY/XY	1.8 ± 0.2	0
XP4/XP12	XX/XX	4.2 ± 0.4	8
	XY/XY	2.0 ± 0.3	2
	XX/XY	17.5 ± 0.5	87

* The average number of grains per nucleus in binuclear cells and the standard error of the mean. 100 binuclear cells were counted in each group. Only nuclei with 50 grains or less were included.

† See footnote to Table 2.

The simplest interpretation of these data is that two different genes are involved in the basic defect of the De Sanctis-Cacchione and the classic xeroderma pigmentosum syndromes

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(intergenic complementation). As a consequence normal gene product will be present in the binuclear hybrids. Other cases of intergenic complementation have been described by Siniscalco et al.[12] and Silagi et al.[13] in mononuclear human hybrid cells. Our results show that this phenomenon can be studied also in binuclear hybrid cells.

As has been found in fungi heterokaryons[14], however, as well as in mononuclear human hybrid cells[15], interallelic complementation might have occurred. Therefore, further studies are required on the properties of the enzyme involved in this repair process in the binuclear hybrids as compared with normal cells.

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PAPER III

.

INTERACTION BETWEEN NUCLEI IN HYBRID BINUCLEAR CELLS RELATED
TO DNA REPAIR MECHANISMS

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SUMMARY

By extending the number of different severe classic xeroderma pig-
mentosum (XP)- and De Sanctis-Cacchione (DSC) strains in our complementa-
tion test, we have confirmed our previous finding that different mutations are
involved in the two forms of XP. Complementation is demonstrated in binuclear
and hybrid synkaryotic cells. A XP nucleus situated with a control nucleus in
one cell resumes unscheduled DNA synthesis (UDS) undistinguisable from a
control nucleus, demonstrating that the DNA repair enzymes can migrate into
the XP nucleus. Transfer of DNA repair enzymes, presumably affected in the
XP cells, is not possible via the cytoplasmic membrane, when a XP cell is in
cellular contact with a control cell.

INTRODUCTION

The human hereditary disease XP is characterized by a hypersensi-
tivity of the skin for UV light. Two clinical forms in the XP syndrome have
been distinguished: the classic form, in which skin lesions are seen in the sun
exposed regions of the body and the DSC syndrome in which neurological com-
plications occur in addition to the skin lesions[1]. Both forms are inherited as
autosomal recessive diseases.

In 1968 Cleaver has demonstrated that cultivated skin fibroblasts from
XP patients were unable to perform DNA repair synthesis after irradiation
with UV light[2]. In previous reports we have shown that the capacity to perform
DNA repair synthesis after UV irradiation was restored in multikaryons con-

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taining nuclei of both clinical forms of XP[3,4]. These first observations demonstrated that the repair deficiency in each of the two distinct forms of the disease is caused by different mutations. We have now extended our studies to a larger number of classic XP and DSC cell strains to examine the general validity of our previous findings. Complementation is studied in binuclear cells and synkaryotic cells obtained after fusion of classic XP cells with DSC cells.

Our experiments and those of Darżynkiewicz on DNA repair synthesis in chicken erythrocyte nuclei after fusion with HeLa cells or rat fibroblasts[5], indicate that cooperation in DNA repair can occur between genetically distinct nuclei. A direct proof of this cooperation was reported by Giannelli et al. in his study of binuclear cells produced by fusing normal human cells with XP cells[6]. Cooperation in DNA repair via cytoplasmic contact was excluded in experiments of Giannelli et al.[6].

Our present experiments do support Giannelli's findings on the occurence of cooperation between the nuclei in one cytoplasm and the absence of cooperation when the cells are in cytoplasmic contact.

MATERIALS AND METHODS

Cell strains and culture conditions

The different fibroblast cell strains used are summarized in Table I. The XP strains are described in more detail elsewhere[7]. The fibroblast strain C4 originating from a healthy woman, served as a control strain. The cells were grown in thymidine-free F12 medium supplemented with 15 per cent foetal calf serum and incubated at 37°C. Cells were seeded in small Petri dishes provided with a sterile coverslip and incubated in air containing 5 per cent CO_2.

Cell fusion procedure

Virus mediated fusion was performed according to the method of Harris et al.[8]. Cells of two parental cell strains were trypsinized and mixed in a 1:1 ratio. 1×10^6 Cells of this mixed suspension were incubated with 125 HAU β-propiolactone-inactivated[9] Sendai virus in a final volume of 1 ml. After 4 to 6 min at 4°C and 20 min at 37°C the cells were seeded in the Petri dishes.

Determination of DNA repair synthesis

The DNA repair synthesis known as UDS was measured by autoradio-

graphy. Two-days-old coverslip cultures were incubated for 1 h in medium containing ^{3}H-methyl thymidine (^{3}H-methyl TdR, 10 μ Ci/ml, spec. act. 2.0 Ci/mmole), washed with a balanced salt solution, drained and exposed to 100 erg/mm^2 UV light (predominantly 254 nm) using a 15 W Philips TUV low pressure mercury tube (dose rate: 9 erg/mm^2/sec). Subsequently the cultures were incubated for 2 h in medium with ^{3}H-methyl TdR (10 μ Ci/ml), rinsed in saline and fixed in Bouins fixative or ethanol-ether ($1:1$ ^{V}/v). Labelling prior to irradiation was carried out to ensure that cells in S-phase could unambiguously be identified as heavily labelled cells.

Autoradiographs were prepared with Kodak AR 10 stripping film. After exposure for 1 week autoradiographs were developed and stained with hematoxylin and eosin. For each estimation the average number of grains per nucleus was established by counting the grains over 50-100 nuclei in G1- or G2- phase of the cell cycle. Frequency analysis of these grain countings were made using a Digital- 8/1 computer. Based on the distribution of grains over control cells under these labelling conditions (presented in a previous paper[3]) we adopted the grain numbers 8 and 50 as the limits of UDS: Cells having between 8 and 50 grains over their nuclei were referred to as labelled. Binuclear cells were considered to be labelled when both nuclei had between 8 and 50 grains. Only nuclei with 0-50 grains were counted. In the experiments concerning the tetraploid mononuclear cells, nuclei with 100 or less grains were included in the countings.

Atebrin staining and fluorescence microscopy

The coverslip cultures fixed in ethanol-ether were used for atebrin staining. After fixation for 2.5 h the preparations were air dried and incubated for 5 min in 0.5 per cent aqueous atebrin (G.T. Gurr) rinsed in running tap water and destilled water and mounted in Na-K phosphate buffer (pH 5.5). The slides were examined with a Leitz orthoplan microscope with vertical illumination. A xenon light source XBO 150 W, a BG12 excitation filter and a KP 500 interference filter provided the excitation light. Selection of the emission light was performed with a interference dividing plate λ H 495, a barrier filter K 495 and an additional K 530 barrier filter. Observations were made using an oil immersion objective 54, 0.95 N.A.

After examination of the atebrin stained preparations and recording the positions of cells of interest, the slides were rinsed in destilled water and processed through the autoradiographic procedure.

RESULTS

DNA repair synthesis in control- and XP strains after UV exposure

In all the strains of the severe classic XP patients a very low residual
UDS activity was found. Under the conditions used an average number of grains
between 3 and 6 per nucleus was observed, whereas a control cell demonstrated
18 to 23 grains per nucleus. The frequency analysis of the grain countings of
the severe classic XP strains revealed a small percentage of nuclei having
between 8 and 50 grains per nucleus (2 to 14 per cent, Table I), which we refer
to as labelled cells.

TABLE I

Origin of cell strains and Unscheduled DNA Synthesis following exposure to
100 erg/mm^2 ultraviolet light.

Strain	Origin	Unscheduled DNA Synthesis[c]	
		Mean Grain counts per nucleus	% labelled nuclei
C4	control	18–23	96–99
XP4RO[a]			
XP9RO			
XP16RO	severe cases of	3–6	2–14
XP20RO	classic XP		
XP21RO[a]			
XP12SF			
XP12RO[b]			
XP4LO			
XP17SF			
XP25RO[b]	DSC cases of XP	1–3	0–5
XP26RO[b]			
XPPKSF			

[a,b] genetically related

[c] determined from 1 to 8 independent experiments and expressing the lowest
and highest value found for the different types of cell strains. Only nuclei
with 0–50 grains were counted; nuclei having between 8 and 50 grains were
considered as labelled. Autoradiographic exposure time: 1 week.

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Under the same conditions the DSC cell strains had a mean grain number of 1 to 3 grains per nucleus, which is not different from unirradiated nuclei. Frequency analysis of the grain countings in the irradiated DSC cell strains showed that 0 to 5 per cent of the cells were labelled (Table I).

DNA repair synthesis in binuclear cells formed after fusion of different XP strains

8 Different classic XP/classic XP fusions and 9 different DSC/DSC fusions were performed, combining in each fusion different XP strains. In the same way 8 different classic XP/DSC fusions were carried out. Two days after fusion the cultures were UV irradiated and labelled. The fixed preparations were analysed after autoradiography.

In the cell populations obtained by fusion of either different classic XP-, or different DSC strains, the percentages of labelled binuclear cells after UV exposure (Table II) did not exceed the percentages in the corresponding parental XP strains shown in Table I. In all fusions combining a classic XP strain with a DSC strain 30-44 per cent of the binuclear cells were labelled after irradiation, approximating the expected percentage of 50 per cent hybrid binuclear cells (Table II).

DNA repair synthesis demonstrated in hybrid synkaryotic cells.

In order to examine the possibility of complementation in hybrid synkaryotic cells the female/male fusion XP4RO/XP25RO was performed. Two days after fusion the culture was irradiated and labelled.

After fixation three types of mononuclear cells were localized with the aid of atebrin staining: mononuclear cells showing 2 Barr bodies, mononuclear cells showing 2 Y chromatin spots and mononuclear cells showing a Barr body and an Y chromatin spot. The first two categories of mononuclear cells are assumed to be XP4RO and XP25RO cells respectively, having at least a tetraploid chromosome set. In the last category of mononuclear cells (Fig. 1a) the genetic constitutions of XP4RO and XP25RO nuclei are supposed to be combined, resulting in a hybrid synkaryotic cell having presumably a tetraploid set of chromosomes. From each class 30-40 cells were recorded. After autoradiography grain countings were performed for the same cells. Fig. 2 shows the frequency distribution of grain numbers found over the three types of irradiated mononuclear cells.

The nuclei with 2 Barr bodies demonstrated a grain number between 3 and 27 with a mean of 9 grains per nucleus (Fig. 2a), which is about 2 times

TABLE II

Unscheduled DNA Synthesis following ultraviolet exposure of fused cell populations using different xeroderma pigmentosum cell strains

Fused populations		% labelled binuclear cells[a]	
		0 erg/mm^2	100 erg/mm^2
Classic XP/Classic XP:	XP4RO/XP9RO	0	8
	XP4RO/XP16RO	0	2
	XP4RO/XP20RO	0	4
	XP4RO/XP21RO	0	4
	XP4RO/XP12SF	0	0
	XP9RO/XP16RO	0	4
	XP9RO/XP20RO	0	0
	XP16RO/XP20RO	2	0
DSC/DSC	XP12RO/XP4LO	0	0
	XP12RO/XP25RO	0	0
	XP12RO/XPPKSF	0	0
	XP4LO/XP17SF	0	0
	XP4LO/XP25RO	0	0
	XP4LO/XPPKSF	0	0
	XP17SF/XP25RO	0	0
	XP25RO/XP26RO	0	0
	XP26RO/XPPKSF	0	0
Classic XP/DSC	XP4RO/XP12RO	0	36
	XP4RO/XP4LO	0	38
	XP4RO/XP25RO	0	36
	XP9RO/XP12RO	0	30
	XP16RO/XP12RO	0	36
	XP16RO/XP4LO	0	36
	XP20RO/XP12RO	0	44
	XP12SF/XP25RO	0	38

[a] Only cells having between 8–50 grains over each of both nuclei were counted as labelled. Each value is based on countings of 50 binuclear cells. Autoradiographic exposure time: 1 week.

as high as found over mononuclear cells in the parental XP4RO cell strain (Table I). This finding is in agreement with the assumed tetraploidy of the nuclei. The grain numbers observed over nuclei with 2 Y chromatin spots varied between 0 and 68 per nucleus (Fig. 2b). The frequency distribution of grain counts indicates that 4 cells had exceptional high grain numbers over their nuclei, possibly representing mismatched cells. The presence of these cells contributes to a higher mean grain number per nucleus (9 grains per nucleus) than was expected from tetraploid XP25RO nuclei (Table I).
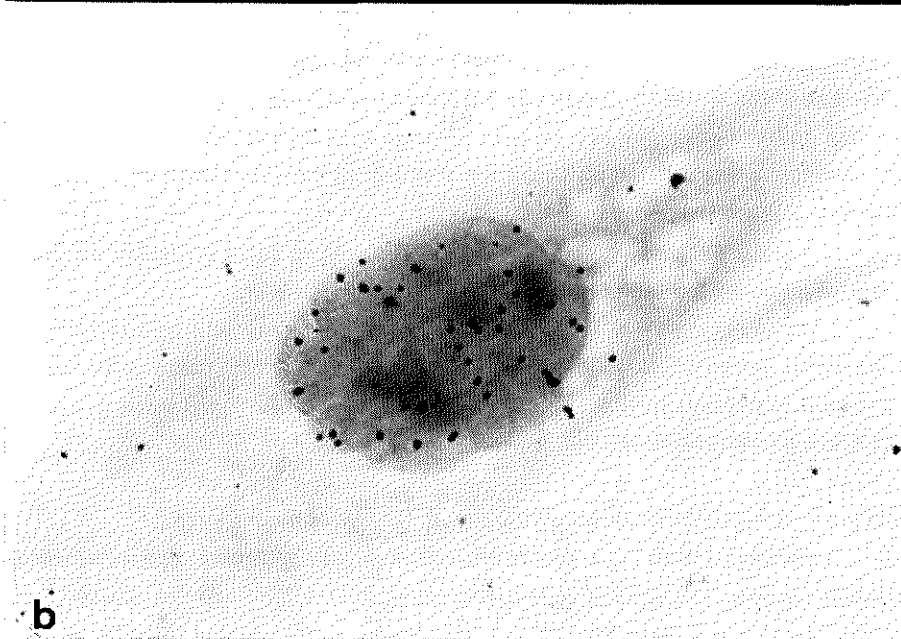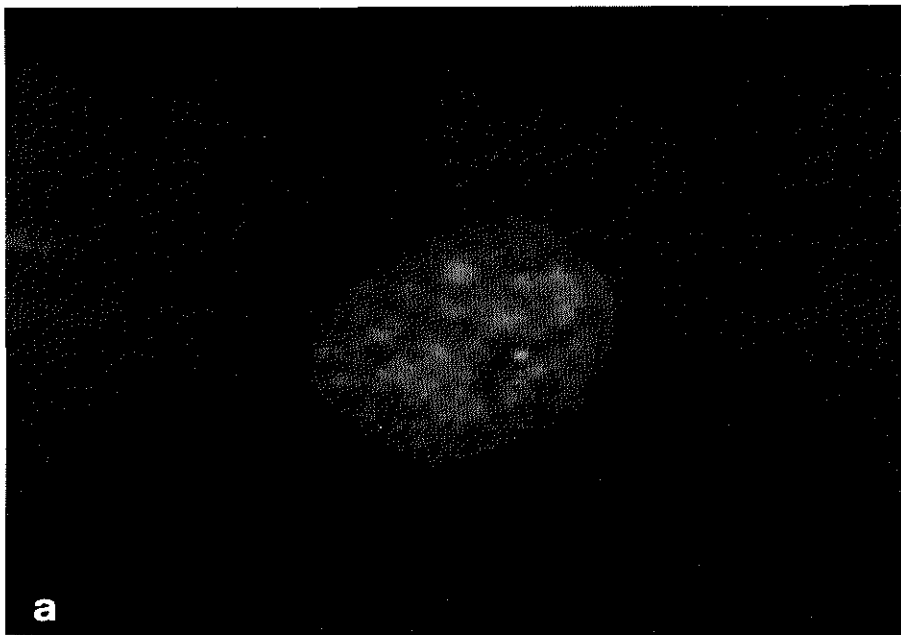
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Fig. 1. Atebrin staining (a) and autoradiography (b) of a hybrid synkaryotic cell formed by nuclear fusion of a female classic XP nucleus and a male DSC nucleus. Irradiated with 100 erg/mm^2 UV light. Autoradiographic exposure time: 1 week. Magnification is 1800 x.
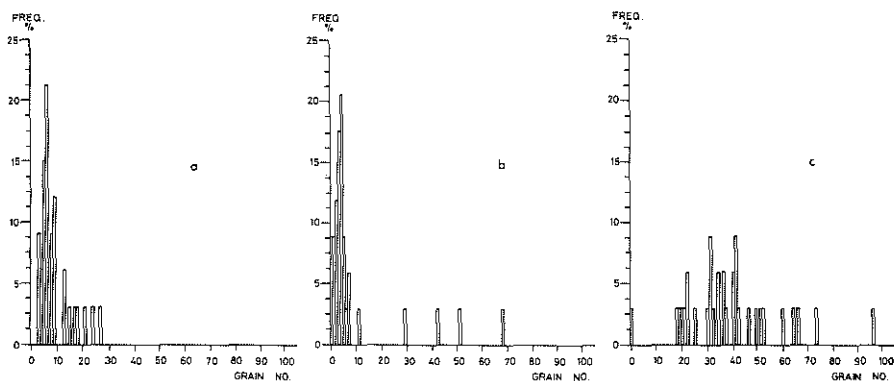
Fig. 2. Frequency distributions of grain numbers found over three types of mononuclear cells showing two sex chromosomes in their interphase nuclei
a) 33 nuclei with 2 Barr bodies
   (Mean $\pm$ SEM = 9 $\pm$ 1)
b) 34 nuclei with 2 Y chromatin spots
   (Mean $\pm$ SEM = 9 $\pm$ 3)
c) 34 nuclei with 1 Barr body and 1 Y chromatin spot
   (Mean $\pm$ SEM = 39 $\pm$ 3)
Cells were irradiated with 100 erg/mm^2 UV light. Autoradiographic exposure time: 1 week.

Mononuclear cells with one Barr body and one Y chromatin spot showed between 0 and 96 grains per nucleus (Fig. 1b and Fig. 2c). The average number of grains per nucleus in these synkaryotic cells was 39 grains per nucleus, which is about 2 times the mean grain number found over normal cells under similar conditions (Table I).

Among the three diagnosed types of tetraploid cells irregular nuclear forms were observed indicating fusion of interphase nuclei.

An attempt was made to estimate the frequency of this type of hybrid synkaryotic cells (XX, XY) in the population. The number of these cells was expressed as percentage of all the nuclei in the culture and found to be 0.3 per cent.after scoring about 20.000 nuclei.

DNA repair synthesis in XP nuclei after fusion of XP cells with normal cells

Cooperation in DNA repair between a repair proficient and a repair deficient nucleus was studied in heterokaryons formed by fusion of control female C4 cells and male XP16RO cells. Two days after fusion the culture was irradiated and labelled. In the fixed culture three types of binuclear cells – C4/C4, XP16RO/XP16RO and C4/XP16RO – were identified by means of atebrin staining as binuclear cells having two female nuclei (XX/XX), two male nuclei

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TABLE III

Unscheduled DNA Synthesis in parental, fused and mixed cell populations, obtained from female control- and male XP cells, following a dose of 100 erg/mm^2 ultraviolet light.

(a) *Unscheduled DNA Synthesis in parental cells*

Cell strain	Grain counts per nucleus (mean ± SEM[d])	Number of nuclei counted
C4(XX)	22,6 ± 0,8	100
XP16RO(XY)	5,4 ± 0,3	100

(b) *Unscheduled DNA Synthesis following cell fusion*

Fusion	Type of nucleus in diagnosed binuclear cells	Grain counts per nucleus (mean ± SEM[d])	Number of nuclei counted
C4/XP16RO	XX in XX/XX	23,1 ± 0,9	88
	XY in XY/XY	8,1 ± 0,9	82
	XX in XX/XY	23,1 ± 0,8	90
	XY in XX/XY	21,6 ± 1,0	90

(c) *Unscheduled DNA Synthesis in mixed cultures*

Mixed population	Type of nucleus in diagnosed cell to cell contact	Grain counts per nucleus (mean ± SEM[d])	Number of cells counted
C4 + XP16	XX in XX + XX	23,6 ± 1,1	52
	XY in XY + XY	3,5 ± 0,4	46
	XX in XX + XY	21,6 ± 1,1	46
	XY in XX + XY	4,2 ± 0,4	46

[d] SEM stands for standard error of the mean.
For each estimation one slide was used: the slide to slide variation was usually small as was evaluated from other experiments in which each estimation was based on 2 or more slides; the slide to slide variation found contributed less than 5 per cent in the eventual SEM, whereas when atebrin staining was used for these estimations a slide to slide variation was found contributing less than 2 per cent in the SEM.
The background level of UDS in this experiment, measured as the mean number of grains over unirradiated nuclei, was 2.3 ± 0.2 to 2.9 ± 0.2 (100 nuclei counted in each strain). Autoradiographic exposure time: 1 week.

(XY/XY) and one female and one male nucleus (XX/XY) respectively. The position of about 50 XX/XX, 50 XY/XY and 100 XX/XY cells were recorded. After autoradiography the labelling patterns of the same cells were studied.

The UDS level in the nuclei of the XX/XX cells was comparable with the level seen in control cells (23 grains per nucleus, Table III a and b). The

mean grain number per nucleus in the XY/XY cells was at the same level as in the parental XP16RO cells (5 – 8 grains, Table III a and b). In the XX/XY binuclear cells both the female control and the male XP nuclei were labelled to the level of the control C4 cells in the parental culture.

DNA repair synthesis in XP cells which are in cytoplasmic contact with control cells

In the same experiment as described above female C4- and male XP16RO cells were mixed in a ratio 1:1 to investigate whether metabolic cooperation through cytoplasmic contact could occur between control and XP cells. Two days after mixing the cells, the culture was irradiated and labelled. Table III c summarizes the results of grain countings in the autoradiographs of the irradiated control – and XP cells in the three possible contact combinations (C4 with C4, XP16RO with XP16RO and C4 with XP16RO) which were identified with the aid of atebrin staining (Fig. 3a). The level of repair synthesis in XP cells was not increased by direct cell to cell contact with control cells (Fig. 3b and Table IIIc).

DISCUSSION

Our previous observations of complementation in DNA repair[3,4] between severe classic and DSC cells were extended by using 6 severe classic XP cell strains and 6 DSC cell strains from different origin. The present results support our earlier finding that these two classes of patients represent two different complementation groups. The data on UV stimulated ^{3}H-methyl TdR incorporation in hybrid synkaryotic cells, obtained by fusion of the two types of XP, are indicative for the same phenomenon.

Recently we have found a third complementation group[12] represented by two XP patients having intermediate levels of DNA repair in their cells and relatively mild symptoms of the classic form of the disease. Robbins et al.[13] obtained evidence for four complementation groups in the XP syndrome. Comparison of the different groups are in progress.

At present it is uncertain whether the complementation described in this paper is a result of intragenic or intergenic complementation i.e. whether the different mutations in both forms of XP affect the same or different genes. One example of interallelic complementation observed by somatic cell hybridization has been claimed by Nadler et al.[14] for cells originating from different patients with galactosemia. At present the possibility cannot be excluded that
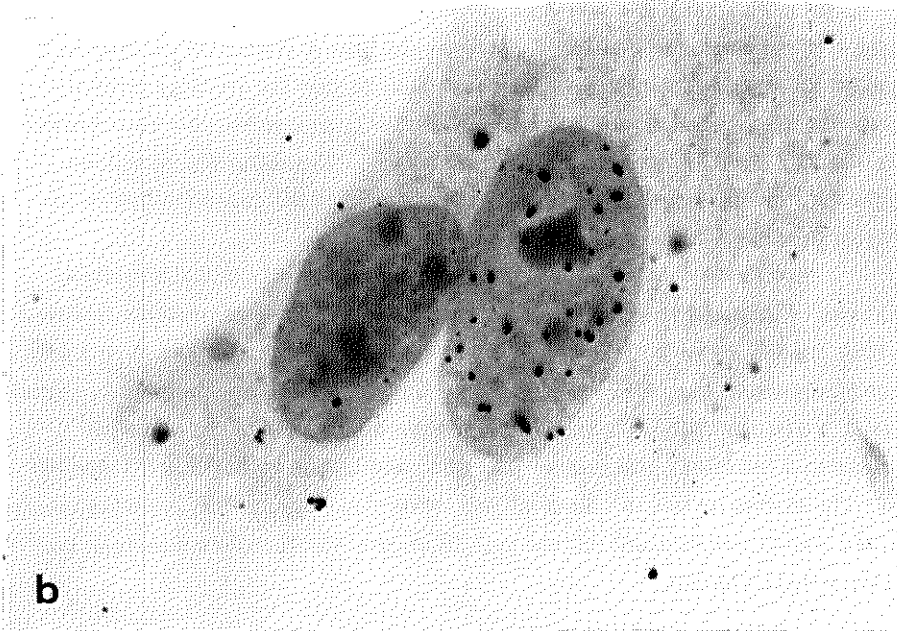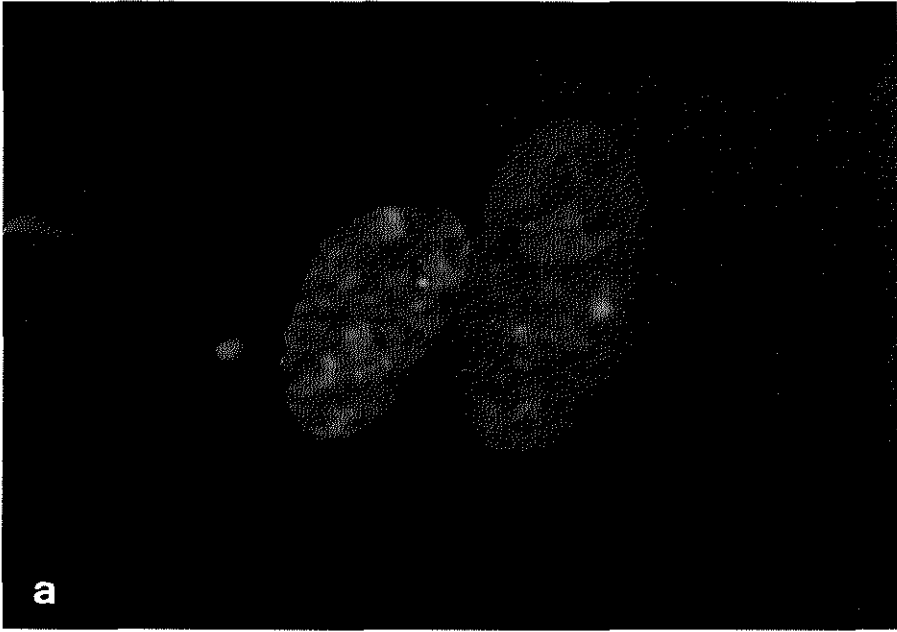
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Fig. 3. Atebrin staining (a) and autoradiography (b) of two cells, which are in cytoplasmic contact with each other: a female control cell and a male classic XP cell. Irradiated with 100 erg/mm^2 UV light. Autoradiographic exposure time: 1 week. Magnification is 1800 x.

the galactose-1-phosphate uridyl transferase enzyme is determined by more than one locus, in which case intergenic rather than interallelic complementation might have occured. Recently complementation is reported in maple syrup urine desease after fusing cells of different patients[15]. The defective enzyme in this disease, BCKA decarboxylase, might well be a complex heteropolymeric protein suggesting that the restored enzyme activity found after cell fusion might also be the result of intergenic complementation.

Little is known about the enzymes involved in the repair of UV damage in mammalian cells. The excision repair model postulated for microorganisms[16] has been extrapolated to mammalian systems in order to explain phenomena as UDS, repair replication and excision of pyrimidine dimers after UV light. The UDS deficient XP cells do not remove the pyrimidine dimers from their DNA[17,18,19]. Evidence has been presented that the XP cells are presumably affected in an endonuclease responsible for the incision in the DNA molecule adjacent to the dimer[20,21]. A dimer specific endonuclease has not yet been isolated from human cells.

If in both complementation groups the same enzymatic step is affected one might assume that the mutations are present in two different genes involved in the UV endonuclease synthesis. They might have a structural as well as a regulatory function. Another explanation could be that the repair enzymes, involved in the excision of UV lesions in the DNA, are acting in a coordinated way e.g. by means of a repair enzyme complex[22]. The different mutations might then inhibit the action of different enzymes, in all cases leading to inactivation of the total enzyme complex.

The basic condition for a complementation test is that the protein products of mutual genetic information can mix. Transfer of proteins involved in DNA repair seems not to occur via the cytoplasmic membranes during cell to cell contact of control – with XP cells. On the other hand the data of Giannelli et al.[6] and our data on cooperation between a control nucleus and a XP nucleus in a binuclear cell, prove that this condition is fulfilled in a binuclear cell. These data as well as those of Darżynkiewicz[23] imply that proteins involved in DNA repair can migrate via the cytoplasm into the xeroderma nucleus and perform their function.

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PAPER IV

REPAIR REPLICATION IN HETEROKARYONS DERIVED FROM DIFFERENT REPAIR-DEFICIENT XERODERMA PIGMENTOSUM STRAINS

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SUMMARY

Repair replication was studied in UV-irradiated cell populations obtained after fusion of cell strains originating from different xeroderma pigmentosum (XP) patients. The capacity to perform repair replication appeared to be restored completely in multinucleate heterokaryons resulting from fusion between a classic XP-strain and a De Sanctis-Cacchione (DSC) strain. In cell populations obtained by fusion of either two different classic XP strains or two different DSC strains no repair replication was observed.

These results, obtained with the technique of density labelling and isopycnic centrifugation of DNA, confirm our previously reported results of autoradiographic studies of unscheduled DNA synthesis. The occurrence of complementation between a classic XP strain and a DSC strain indicates that the defect in the two forms of the disease is caused by different mutations.

INTRODUCTION

Two clinically different forms of the hereditary disease XP have been recognized: the classic form of XP and the DSC syndrome[9]. Both forms of XP are characterized by hypersensitivity of the skin to sunlight, leading to the development of severe skin lesions. DSC patients are distinguished from patients with the classic form of XP by the involvement of neurological complications.

Studies on skin fibroblasts[1,2] and lymphocytes[2] from patients of each XP type have indicated an impaired ability of these cells to perform repair DNA synthesis after UV irradiation, probably owing to a defective initial step in excision repair[4,7,10]. In cell strains from different patients various levels of repair DNA synthesis were found ranging from 0–100% of that in normal human cells[1]. Cell strains from genetically related patients performed similar amounts of repair DNA synthesis[1]. These

Abbreviations: BUdR, 5-bromodeoxyuridine; DSC, De Sanctis-Cacchione; FUdR, 5-fluorodeoxyuridine; HAU, hemagglutinating units; TCA, trichloroacetic acid; XP, xeroderma pigmentosum.

findings demonstrated that the degree of reduction of the repair activity is genetically determined.

The different characteristic levels of repair activity in the XP strains and the occurrence of two clinically distinct forms of XP suggest a genetic heterogeneity in this disease. This hypothesis was supported by autoradiographic studies of repair DNA synthesis in heterokaryons obtained by fusion of different XP strains, described in a previous paper[11]. In binucleate heterokaryons derived from two repair-deficient XP strains, one of the classic type and the other of the DSC type, the capacity to perform unscheduled DNA synthesis appeared to be restored. In this report the complementation between a classic XP and a DSC strain is confirmed by the demonstration of repair replication in the fused cells, using the technique of BUdR density labelling and isopycnic centrifugation of DNA[8].

MATERIALS AND METHODS

Cell strains and culture techniques

The cell strains used were the following primary fibroblast strains: AH, originating from a normal individual; XP4 (ref. 1) and XP16 (ref. 11), from two severe classic XP patients; and two strains from DSC patients, XP24 (provided by Dr. Cleaver and designated by him XP17) and XP25 (the biopsy was provided by Dr. Der Kaloustian). Cultures were grown in F12 medium supplemented with penicillin (100 I.U./ml), streptomycin (0.1 mg/ml) and 15% foetal calf serum.

Fusion procedure

The fusions were performed according to the procedure of HARRIS *et al*.[5]. Cells of two different XP strains were mixed in a ratio of 1:1 at a final concentration of $2 \cdot 10^6$ cells per ml. β-Propiolactone-inactivated Sendai virus[6] was added (final titre 500 HAU per ml). This mixture was kept during 4 min at 4° and after shaking at room temperature incubated for 20 min at 37°. After fusion the cells were diluted and plated in plastic petri dishes (Greiner, diameter 9 or 14 cm for density gradient experiments, or Falcon, diameter 3.5 cm, containing a coverslip for autoradiography).

Irradiation and labelling of the DNA

UV irradiation (254 nm) of washed and drained cultures was performed with a Philips TUV lamp (15 W) at 7.5 erg/mm²/sec, two days after fusion.

The cultures to be analysed in density gradients were incubated in medium containing BUdR (1.5 μg/ml) and FUdR (10^{-6} *M*) for 2 h before irradiation, and [³H]BUdR (10 μCi/ml; spec.act. 15.3 Ci/mmole), FUdR (10^{-6} *M*) and hydroxyurea (10^{-3} *M*) for 2 h after irradiation. The cultures to be processed for autoradiography were incubated during 1 h before and 2 h after irradiation in medium containing [³H]thymidine (10 μCi/ml; spec.act. 2 Ci/mmole).

Extraction and isopycnic centrifugation of DNA

Approx. $2 \cdot 10^6$ control cells and cells of the fused XP populations (a number equivalent to $2 \cdot 10^6$ cells in the mononucleate situation before fusion) were collected by scraping them from the dishes, washed twice in cold saline, centrifuged and stored as cell pellets at —70°. The frozen cells were resuspended in 1 ml buffer (0.15 *M*

NaCl, 0.01 M EDTA, 0.01 M Tris, pH 9.5, 0.1% sarkosyl (Geigy)) and incubated for 30 min at 60°. The DNA was extracted by shaking with an equal volume of chloroform–isoamyl alcohol (24:1).

5 ml of a solution containing the DNA extract, 0.01 M sodium bisulphite and ethidium bromide at 20 μg/ml was added to 4.650 g NaI (final density 1.5300 g/cm³) in tubes which were then filled to the top with mineral oil. The tubes were centrifuged in a Spinco fixed-angle rotor (40) for 60 h at 33000 rev./min and 20°.

The technique of isopycnic centrifugation of DNA in NaI gradients has been described and discussed in more detail in a separate paper[8].

Fractionation, DNA concentration and radioactivity assays

The gradients were sampled from the bottom in 15–17 fractions of 0.3 ml. Fluorescence at 590 nm (excitation wavelength, 520 nm) was measured in each fraction using a Baird Atomic spectrofluorometer. The amount of DNA was determined by comparison with a calibration curve made with a solution containing NaI, sodium bisulphite and ethidium bromide in the same concentrations as in the gradients and with various known concentrations of DNA[8]. The DNA in each fraction was precipitated with 10% TCA–0.01 M sodium pyrophosphate (0°), collected on Whatman GF/C glass-fibre paper and washed with 5% TCA–0.01 M sodium pyrophosphate and 96% ethanol respectively. The radioactivity in the dried filters was counted in toluene–PPO (6 g/l)–POPOP (0.1 g/l) in a liquid scintillation counter (Nuclear Chicago, mark 2).

Autoradiography

Autoradiographs of the cultures grown on coverslips were made as described earlier[1]. For the determination of the percentage of weakly labelled nuclei (*i.e.* 8–50 grains per nucleus), 2000 nuclei were counted.

RESULTS AND DISCUSSION

Repair replication was studied in cell populations resulting from fusions between a classic XP strain and a DSC strain (XP4/XP25), between two genetically unrelated classic XP strains (XP4/XP16) and between two unrelated DSC strains (XP24/XP25).

In the population of cells obtained after fusion of two different strains three main classes of cells may be expected, namely: unfused parental cells, fused cells containing nuclei of only one of the parental strains (homokaryons) and fused cells with nuclei of both parental strains (heterokaryons). Complementation and therefore also repair replication may be expected only in heterokaryons. To promote a maximal amount of repair replication, fusion conditions were applied that gave a high percentage of multinucleate cells and consequently also a high percentage of heterokaryons.

Fig. 1 shows a detail of an autoradiographic preparation of an XP4/XP25 population after UV irradiation and labelling with [³H]thymidine. Part of the binucleate cells and most of the multinucleate cells were weakly labelled, whereas a very small number of grains was found over all mononucleate cells (except for heavily labelled S-phase cells). In three experiments 50–70% of all nuclei were weakly labelled.

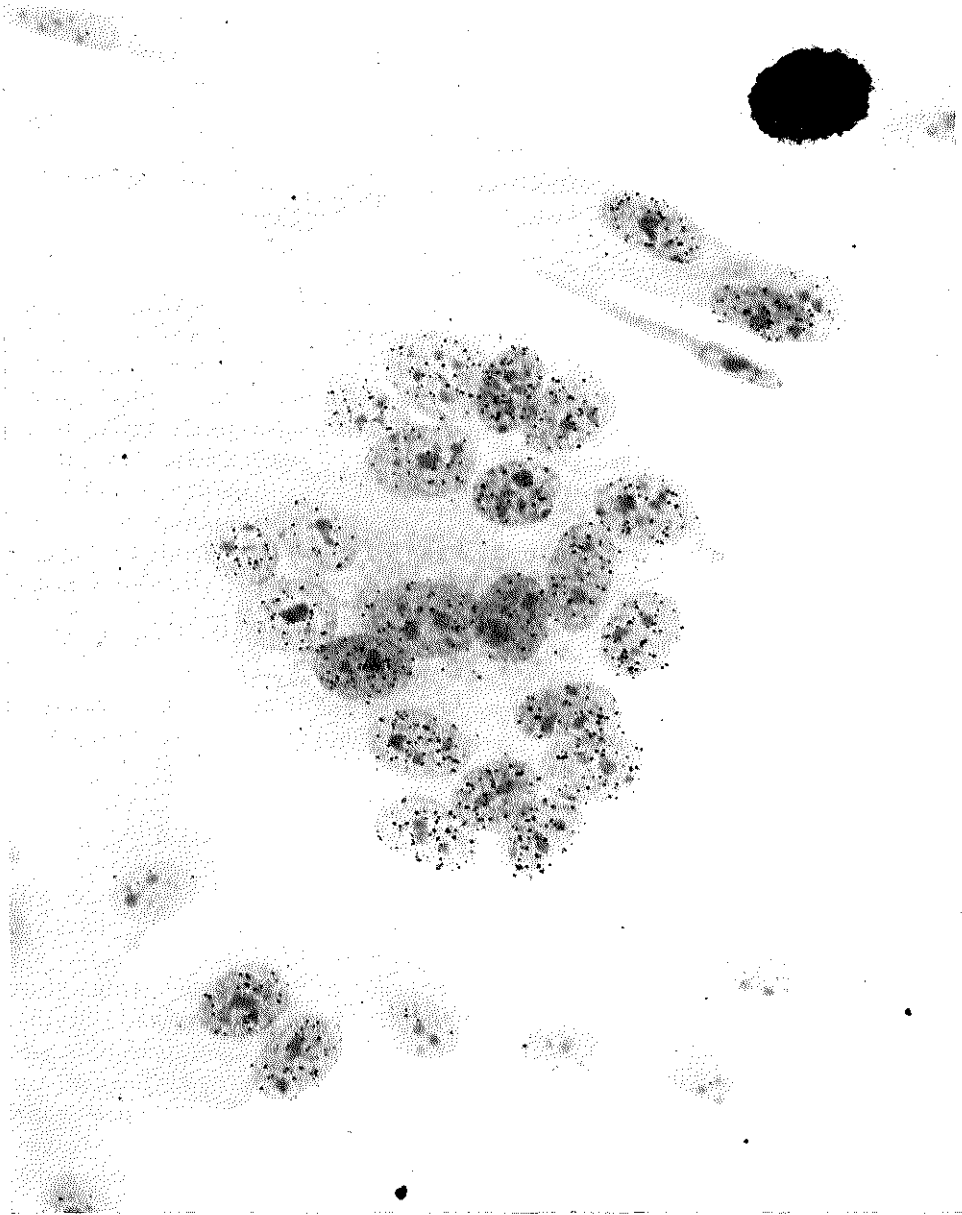Cell populations from the same fusions as used for autoradiography were

Fig. 1. Autoradiograph of a cell population after fusion (XP4/XP25). Two days after fusion the cells were irradiated with 100 erg/mm² UV-light. [³H]thymidine labelling was performed 1 h before and 2 h after UV irradiation. Autoradiographic exposure time: 1 week. The large multinucleate cell, the trinucleate and the binucleate cell shown are labelled. The unfused XP cells are not labelled except one heavily labelled S-phase cell.
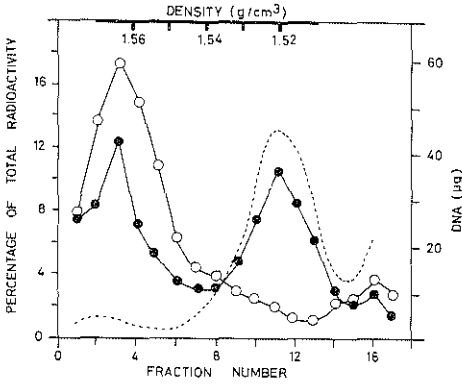
Fig. 2. NaI isopycnic gradients of DNA from cells of an XP4/XP25 population. Two days after fusion the cells were grown for 2 h in BUdR, irradiated with 100 erg/mm² UV-light (●), or not irradiated (○), and labelled with [³H]BUdR. The DNA-distribution as measured by fluorescence (– – –) is shown only for irradiated cells because the profile for unirradiated cells was similar. The total amount of radioactivity in the gradient for irradiated cells was 44 000 cpm.

analysed for repair replication. The profiles in Fig. 2 show the distribution in the NaI gradient of DNA from unirradiated and irradiated cell populations after fusion (XP4/XP25). The distribution of DNA, as measured by the fluorescence of the ethidium bromide–DNA complex shows one peak at the normal density for DNA (approx. 1.522 g/cm³ in NaI). The radioactivity profiles obtained for both unirradiated and irradiated cells show a peak at a higher density (1.565 g/cm³) representing semiconservatively replicated DNA that had acquired a hybrid density by BUdR substitution in one of the strands. For irradiated cells there is an additional peak coinciding with the peak in the distribution of the total amount of DNA. The radioactivity in this peak at the normal density of DNA demonstrates that [³H]BUdR has been incorporated in the DNA dispersively and indicates repair replication. A quantitative measure for repair replication is given by the specific radioactivity (cpm/µg DNA) of the DNA of normal density. The average specific radioactivity was calculated from the three top fractions of the peak of repaired DNA. The standard deviation of the specific radioactivity was 10% in control experiments in which separate normal human cell cultures were irradiated (100 erg/mm²), labelled and analysed simultaneously and under identical conditions.

Fig. 3a presents the results of an experiment in which repair replication in the XP4/XP25 population was compared with that in a normal human strain (AH) and two populations of fused parental cells (XP4/XP4 and XP25/XP25) which served as controls. The level of repair replication in the XP4/XP25 population appeared to be 61% of the level in normal cells, whereas in the XP4/XP4 and XP25/XP25 populations only very low levels were found. In the populations obtained after fusion of either two classic XP strains (XP4/XP16) or two DSC strains (XP24/XP25) no repair activity was detected (Fig. 3b).

It is evident from these results that the repair capacity has been restored by complementation in the XP4/XP25 population. Since only a fraction of all nuclei, namely those in heterokaryons XP4/XP25, will have contributed to the measured amount of repair replication, the repair activity in these nuclei relative to normal (AH) nuclei will be higher than the 61% found for the whole population. The fraction
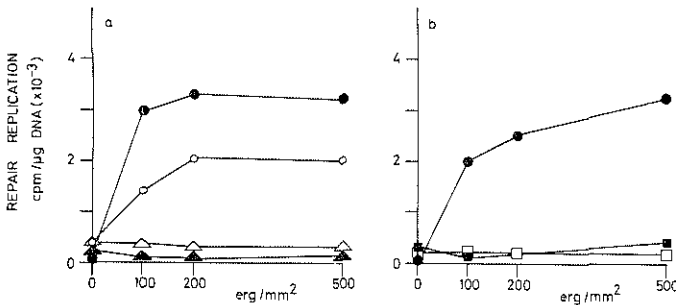
Fig. 3. Repair replication as a function of UV-dose, expressed as specific radioactivity (cpm/μg DNA) of a normal human cell strain (AH) (●), and cell populations obtained after fusion; (a) XP4/XP25 (○), XP4/XP4 (△) and XP25/XP25 (▲); (b) XP4/XP16 (□) and XP24/XP25 (▨).

of repair-positive nuclei in the present experiment was obtained by the addition of two distinct fractions of nuclei, counted in the autoradiographic preparation: (*a*) a large fraction (61 ± 2%) consisting of the repair-positive nuclei in G_1 or G_2 phase, recognized as weakly labelled nuclei, and (*b*) a small fraction (2%) consisting of the probably repair-positive S-phase nuclei, situated in multinucleate cells. (12% of all nuclei were in S phase; 18% of these heavily labelled nuclei were found in multi-nucleate cells.) The level of repair replication in repair-positive nuclei relative to that in normal cells (AH) is obtained by dividing the level found for the whole population (61%) by the fraction of repair-positive nuclei (a+b), *i.e.* 61/63 × 100 = 97%. It is suggested therefore that in those cells where complementation occurs, the repair capacity is restored to the normal human cell level.

The restoration of the repair-replication capacity after fusion of a classic XP and a DSC cell strain indicates that, in the two forms of XP, different mutations are responsible for the defect. These results are in agreement with our earlier findings on UV-induced [³H]thymidine incorporation occurring in classic XP/DSC hetero-karyons[11]. The dispersive character of the UV-induced DNA synthesis in these hetero-karyons, shown in the present report, confirms that the incorporation of [³H]thymidine represents repair DNA synthesis.

ACKNOWLEDGEMENTS

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PAPER V

A third complementation group in xeroderma pigmentosum

As previously described complementation is observed in binuclear cells originating from fusions between cells of two types of XP, the classic and the DSC form of the disease[8]. In both types of XP severe skin lesions are produced following exposure to sunlight. DSC patients have severe neurological abnormalities in addition to the skin lesions. Both forms of the disease are inherited in an autosomal recessive way. In 1968 CLEAVER demonstrated that cultivated skin fibroblasts of these patients were not able to perform UDS after UV light exposure[2]. A DNA repair mechanism operating in human cells after UV-irradiation, which can be demonstrated by UDS, is impaired in cells of XP patients. The ability to perform repair was restored after fusion of cells of classic XP patients with cells of DSC patients, indicating that different mutations are involved in these two forms of the XP syndrome[8,9].

In the present paper we report a third complementation group represented by two patients (XP2RO and XP3RO) with moderately severe and relatively mild symptoms of the classic form of the disease[1,5]. In both cases the first skin lesions were observed relatively late in childhood: Patient XP2RO was 14 years old when the first skin tumors developed.

In contrast to the cells of patients belonging to the earlier reported complementation groups (*e.g.* Fig. 1; XP4RO) having low or negligible levels of UDS after UV light exposure, cultivated cells of these patients have a 50% UDS activity in response to 100 erg/mm^2 UV light and slightly more after higher doses when compared with normal human fibroblasts[1,5] (Fig. 1). To study complementation, fusions were performed between XP2RO cells and cells of a classic XP patient (XP16RO) and between XP2RO cells and cells of a DSC patient (XP25RO).

Hybridizations were performed as previously described[8]. Recognition of the hybrid binuclear cell was carried out by means of atebrin staining of the sex chromosomes in the interphase nuclei in a female/male fusion[8]. The labelling procedure, UV
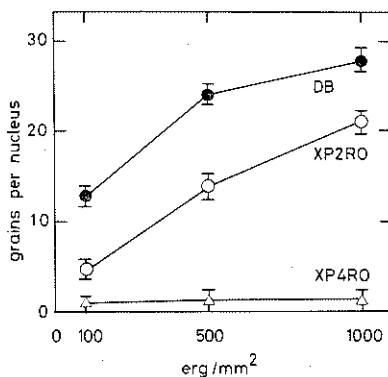

Fig. 1. Repair DNA synthesis after UV exposure of control human fibroblasts (DB), cells from patient XP2RO (moderately severe case of XP) and patient XP4RO (classic form of XP). Exposure time: 3 days.

Abbreviations: DSC, De Sanctis-Cacchione; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum.

exposure conditions and preparation of the autoradiographs were the same as described earlier[8].

Table I presents the results of the fusion between the female XP2RO cells and the male XP16RO cells of the classic XP type.

Unfused XP2RO cells exhibited 50% of the UDS of normal human fibroblasts, while XP16RO cells had much lower UDS activity after a dose of 100 erg/mm^2 UV light. In the fused cell population XP2RO/XP16RO, binuclear cells having two female (XX/XX) or two male (XY/XY) nuclei were labelled at a level as seen in the parental XP2RO and XP16RO cells, respectively. In the hybrid binuclear cells with a female and a male nucleus (XX/XY), the mean grain number per nucleus (26 and 28 grains over female and male nuclei respectively) was comparable with the level of UDS seen in control cells (26 grains per nucleus).

The same result was obtained after fusion of XP2RO cells with male XP25RO DSC cells (Table II). In this experiment the parental XP2RO cells have about 65% UDS compared with control human fibroblasts after a dose of 100 erg/mm^2 UV light. UDS in the DSC cells did not exceed the background level. In the fused cell population

TABLE I

UNSCHEDULED DNA SYNTHESIS FOLLOWING UV LIGHT EXPOSURE OF PARENTAL AND FUSED CELL POPULATIONS, USING TWO DIFFERENT TYPES OF CLASSIC XERODERMA PIGMENTOSUM

The cells were exposed to 100 erg/mm^2 UV light. The exposure time of the autoradiographs was 1 week. C6 is a control human fibroblast strain, whereas XP2RO cells originate from a moderately severe classic case of XP and XP16RO cells are from a severe classic case of XP. The designations used for the XP cell strains follow a proposal by CLEAVER AND BOOTSMA for the standardization of the nomenclature for XP cell strains. The strains are characterized by a serial number or by two letters (*e.g.* the initials of the patient) given in the institute where the cells have been brought into culture. The last two letters denote the city where this institute is situated (*e.g.* RO for Rotterdam). The average number of grains per nucleus and the standard error of the mean (SEM) in parental mononuclear cells was estimated by including only cells with 50 or less grains over their nuclei. In the fused binuclear cells this estimation was performed by counting only cells with 0–50 grains over each of both nuclei. For each estimation one slide was used. However, for those estimations concerning one type of nucleus in a binuclear cell, diagnosed by means of atebrin staining, 2 to 4 slides of the same culture were used. The slide to slide variation as evaluated from these estimations was very small, contributing less than 2% in the SEM as quoted here. The background level of UDS in this experiment, measured as the mean number of grains over unirradiated nuclei, was 2.0 ± 0.2 to 2.2 ± 0.3 (50 nuclei counted in each strain).

(a) Unscheduled DNA synthesis in parental cells

Cell strain	Grain counts per nucleus (mean ± SEM)	Number of nuclei counted
C6	26.1 ± 0.7	100
XP2RO (XX)	13.7 ± 0.6	100
XP16RO (XY)	3.9 ± 0.2	100

(b) Unscheduled DNA synthesis following cell fusion

Fusion	Type of nucleus in diagnosed binuclear cells	Grain counts per nucleus (mean ± SEM)	Number of nuclei counted
XP2RO/XP16RO	XX in XX/XX	15.0 ± 0.7	96
	XY in XY/XY	4.2 ± 0.3	88
	XX in XX/XY	26.4 ± 0.8	94
	XY in XX/XY	28.0 ± 0.8	94

TABLE II

UNSCHEDULED DNA SYNTHESIS FOLLOWING UV LIGHT EXPOSURE OF PARENTAL AND FUSED CELL POPULATIONS, USING A MODERATELY SEVERE CLASSIC AND A DE SANCTIS-CACCHIONE FORM OF XERODERMA PIGMENTOSUM

XP25RO cells are from a DSC patient. The background level of UDS in this experiment, measured as the mean number of grains over unirradiated nuclei, was 2.3 ± 0.3 to 2.7 ± 0.2 (50 nuclei counted in each strain). For other details see legend to Table I.

(a) Unscheduled DNA synthesis in parental cells

Cell strain	Grain counts per nucleus (mean ± SEM)	Number of nuclei counted
C6	21.9 ± 1.1	50
XP2RO (XX)	15.4 ± 0.7	50
XP25RO (XY)	2.4 ± 0.3	50

(b) Unscheduled DNA synthesis following cell fusion

Fusion	Type of nucleus in diagnosed binuclear cells	Grain counts per nucleus (mean ± SEM)	Number of nuclei counted
XP2RO/XP25RO	XX in XX/XX	13.5 ± 0.5	96
	XY in XY/XY	5.3 ± 0.9	86
	XX in XX/XY	20.8 ± 0.8	95
	XY in XX/XY	22.2 ± 0.7	95

XP2RO/XP25RO, binuclear cells, in which both nuclei were either of the female (XX/XX) or the male (XY/XY) parental origin, exhibited UDS comparable with the parental cells (14 and 5 grains per nucleus, respectively). UDS found in hybrid binuclear cells, with a female and a male nucleus (XX/XY) was at the level found in control human fibroblasts (about 21 grains per nucleus).

A second cousin of patient XP2RO also suffered from the disease, showing relatively mild symptoms and intermediate levels of UDS (patient XP3 in ref. 1; XP3RO in Table III).

Cells of this patient were hybridized with XP2RO cells and the control fusion XP2RO/XP2RO as well as XP3RO/XP3RO were performed. Identification by atebrin staining of the three types of binuclear cells in the fused cell population XP2RO/XP3RO was not possible because both parental strains were of female origin. In the three fused populations 100 binuclear cells were randomly selected and the number of grains per nucleus following a UV light dose of 100 erg/mm^2 was counted (Table III).

The number of grains counted above the nuclei of the parental cells XP2RO and XP3RO were not significantly different. The grain numbers found over the nuclei of binuclear cells in the fused populations XP2RO/XP2RO and XP3RO/XP3RO were comparable with the numbers found over the nuclei of their respective parental cells. Finally, no difference was observed between the number of grains counted above the nuclei of binuclear cells in the fused population XP2RO/XP3RO and the number found over both parental cells. These data suggest that the same mutation is present in these two XP patients, which is expected because of the family relationship. However, the recognition of small changes in the number of grains over the hybrid binuclear cells, might have been hampered by the presence of binuclear cells

TABLE III

UNSCHEDULED DNA SYNTHESIS FOLLOWING UV LIGHT EXPOSURE OF PARENTAL AND FUSED CELL POPULATIONS, USING CELLS OF TWO RELATED PATIENTS, BOTH CLASSIC MODERATELY SEVERE CASES OF XERODERMA PIGMENTOSUM

C_3 is a control human fibroblast strain, whereas XP3RO cells originate from a second cousin of XP2RO patient, having relatively mild symptoms of the classic form of the disease. The background level of UDS in this experiment, measured as the mean number of grains over unirradiated nuclei, was 3.2 ± 0.3 to 3.5 ± 0.3 (50 nuclei counted in each strain). For other details see legend to Table I.

(a) Unscheduled DNA synthesis in parental cells

Cell strain	Grain counts per nucleus (mean ± SEM)	Number of nuclei counted
C_3	19.0 ± 0.5	100
XP2RO (XX)	13.5 ± 0.5	100
XP3RO (XX)	14.7 ± 0.6	100

(b) Unscheduled DNA synthesis following cell fusion

Fusion	Grain counts per nucleus in binuclear cells (mean ± SEM)	Number of nuclei counted
XP2RO/XP2RO	12.7 ± 0.4	200
XP3RO/XP3RO	15.2 ± 0.5	200
XP2RO/XP3RO	14.8 ± 0.5	200

originating from one parent, which have been included in our calculations. Since the standard error of the mean of the grain number per nucleus above binuclear cells is similar in all three fused populations, and the frequency distribution of these grain countings in the fused population XP2RO/XP3RO showed one peak (not presented here) there is no evidence of a complementation effect.

From these experiments we conclude that a third complementation group in XP is represented by the XP2RO and XP3RO cell strains, which show intermediate levels of UDS. Biochemical analyses of these cell strains sofar[5] have indicated that an early step of DNA repair is defective in each of the three complementation groups. These data are in agreement with prior work on the classical XP[7] and the DSC syndrome[3,4].

Recently KRAEMER et al.[6] presented evidence for at least three different complementation groups in XP. Experiments are in progress to compare the three groups presented in this paper and those reported by KRAEMER et al.

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