DNA REPAIR IN HUMAN XERODERMA PIGMENTOSUM AND CHINESE HAMSTER CELLS

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Aan Ria,
aan onze ouders,
aan Wouter.

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PAPER II B. Zelle and P.H.M. Lohman, Repair of UV-endonuclease-susceptible sites in the 7 complementation groups of xeroderma pigmentosum A through G, Mutation Res., 62 (1979) 363-368.

CHAPTER 1

GENERAL INTRODUCTION

1.1 DNA, DNA damage and DNA repair

Deoxyribonucleic acid (DNA) is the genetic material of pro- and eucaryotic cells. DNA is a copolymer, composed of four different nucleotides, the four letters of the genetic alphabet, each constituted of a base, a sugar and a phosphate group. The four different bases, the pyrimidines thymine and cytosine, and the purines adenine and guanine, form a sequence (the genes) encoding all the proteins that the cell needs in order to maintain itself and to proliferate. In many cases changes in the normal sequence (mutations) can result in the production of a defective gene product which may interfere with the metabolism of the cell and of its daughter cells. Some mutations are incompatible with life and others may be involved in carcinogenesis. Moreover, mutations in germ cells can be transferred to following generations.

A number of agents has been detected that are able to affect the DNA and cause mutations. They include chemicals that can react with DNA, either directly or after modification by the metabolism of the cell or by cells in other tissues of the same organism. Examples of these mutagenic agents are nitrous acid, nitrogen mustard, aflatoxin G1 and benzo(α)pyrene. DNA is also damaged by ionizing radiation, such as X-rays and γ-rays and by some non-ionizing radiations, such as the ultraviolet radiation (UV) present in the spectrum of sunlight.

Not every mutation is caused by environmental factors. The DNA of a living cell can loose bases spontaneously, and also errors can occur when the DNA is replicated before cell division. It is therefore of the utmost importance that cells have the capacity to maintain the integrity of the DNA.

Extensive studies during the last 25 years have revealed the presence of complex mechanisms in the cells that act on damaged DNA and are able to restore the original structure. These processes are the subject of our study.

Insight in the normal course of a metabolic pathway is often obtained by investigating cells in which this pathway is defective. The study of DNA repair processes occuring in mammalian cells is an example of this approach. It may be expected that a defect in the repair of DNA lesions will become apparent by an increased sensitivity of cells to the agent that induces these lesions. In nature, such an agent is ultraviolet radiation. Some individuals in the human population show an abnormal sensitivity to sunlight, which could be the result of defective repair of UV-induced lesions in the DNA of skin cells. In 1968 Cleaver discovered a repair defect in cultured cells derived from patients showing extreme sunlight-sensitivity of

the skin. These patients suffered from the genetic skin disease xeroderma pigmentosum (XP). This discovery provided the basis of our investigations, in which the genetic and biochemical nature of defective DNA repair in XP cells is studied.

1.2 Types of DNA damage

The majority of the DNA lesions that can occur, constitute two groups, viz. DNA strand breaks and base damages (Fig. 1). The type of lesion depends on the chemical and physical properties of the damaging agent. Single-strand DNA breaks occur after exposure to ionizing radiation, e.g. X-rays. When two single-strand breaks are formed opposite each other, or even when they are a few bases apart in the opposite strand, a double-strand break is formed and the DNA looses its continuity. Most single-strand breaks are readily repaired by rejoining, but repair of double-strand breaks is more difficult and these breaks are often lethal to the cell.

A great variety of base damages is known. These damages affect the DNA structure in different ways, depending on the nature of the lesion. Single bases in the DNA can be modified by alkylation, by which an alkyl group is linked to a base. The N-7 position of guanine is the most reactive site for most alkylating agents, but cells can easily overcome this type of lesion during replication (Strauss, 1976). O-6 alkylguanines form a minor alkylation product, but can be more dangerous to the cell as they can be recognized as adenines and cause errors during replication of the DNA (Loveless, 1969). Saturation of the 5,6-double bond of pyrimidine bases also results in single-base damage. Another important group of single-base damages is formed by complete removal of bases, yielding apurinic and apyrimidinic sites. They can be formed as a result of the removal of alkylated bases by N-glycosylases, or even by removal of undamaged bases, by cleavage of the C-N bond that links the base to its deoxyribose.

Damages involving more than one base are also encountered. The most extensively studied DNA lesion, the pyrimidine dimer, is of this type (Fig. 2). This dimer, which is induced by UV radiation, was discovered by Beukers and Berends in 1960 after UV irradiation of a frozen solution of thymine. Soon afterwards it was shown that this photoproduct is also induced in irradiated DNA (Wacker et al., 1961), as the result of covalent binding of two adjacent pyrimidines. Thymine-thymine, cytosine-cytosine and thymine-cytosine dimers can be formed. The wavelength of the radiation determines which of these three types is preferentially induced. At the absorption maximum of DNA (about 260 nm), which is close to the predominant wavelength of the standard laboratory UV source (254 nm), the (di)thymine dimer is the principal lesion. The dimer distorts the DNA structure and presents a block to transcription and replication. Such a block may ultimately result in cell death.

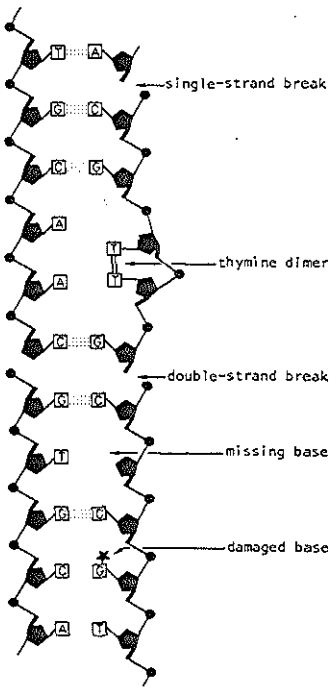

Fig. 1. Schematic representation of a part of a DNA molecule, in which a number of different lesions are shown.

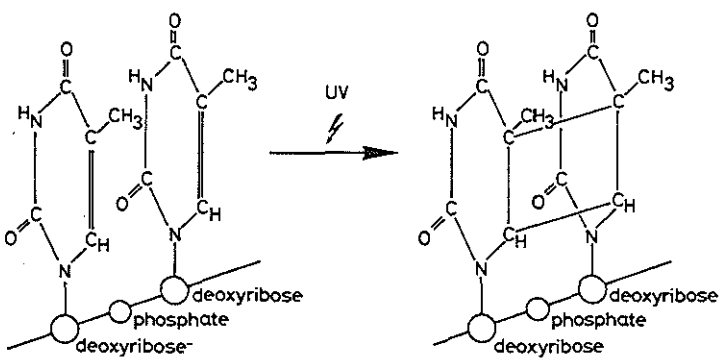

Fig. 2. Dimerization of 2 adjacent pyrimidine molecules in DNA after irradiation with UV. A cis-syn cyclobutane thymine dimer is formed by bonds between their C-5 and C-6 atoms.

A special category of base damages is presented by interstrand cross-links, which are covalent bounds between bases on the two complementing DNA strands (not shown in Fig. 1). They can be produced after reaction of DNA with bifunctional alkylating agents, such as mytomycin C.

1.3 Mechanisms of DNA repair

It has now been well established that cells are able to maintain the integrity of the DNA by their ability to repair many of the lesions described in the previous paragraph. The analysis of repair of UV-induced dimers in the bacterium *Escherichia coli*, has elucidated the enzymatic processes of this repair in great detail. Dimers can be repaired by one of three different repair mechanisms, i.e. photoreactivation, excision repair or postreplication repair.

The most simple way to restore the damaged nucleotides is to repair the damage without cutting the DNA strand. Photoreactivation is such a process. Cells which have been lethally irradiated can be reactivated by exposure to visible light. They possess a photolyase (also called photoreactivating enzyme), which absorbs visible light and uses the energy to monomerize the pyrimidine dimer. The dimer is the only lesion known to be susceptible to the action of a photolyase. A large number of organisms have this enzyme. With regard to mammalian cells only the marsupials (e.g. the rat kangaroo) have been shown to possess a photolyase. It is discutable whether human cells have such an enzyme. From cultured human cells a photoreactivating activity could be isolated (Sutherland, 1974), but this activity is highly dependent on medium conditions of cell culture (Sutherland and Oliver, 1976; Mortelmans et al., 1977). The biological significance of this human photoreactivating activity has not yet been demonstrated.

The second mechanism involved in repair of pyrimidine dimers is excision repair (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). By the use of UV-sensitive mutants the nature of this repair process could be elucidated. It consists of a sequence of steps which are shown in Fig. 3. After recognition of the damage a UV-endonuclease makes a single-strand break adjacent to the dimer, followed by the action of an exonuclease which removes a small piece of the DNA containing the damaged site. The resulting gap is filled by a DNA polymerase. When the gap is completely filled, a ligase forms a phosphodiester bond between the last nucleotide inserted and the unexcised neighbour nucleotide on the same strand. Many other lesions can be excised by a comparable pathway. There is accumulating evidence, however, that different endonucleases may be involved in the recognition and incision step.

The first evidence for the action of an excision repair pathway in mammalian cells was obtained by Rasmussen and Painter (1964). These authors observed incorporation of (^{3}H)thymidine ((^{3}H)TdR) after UV exposure of cells in the G1- or G2-phase of the cell cycle. It has

been shown that this so-called unscheduled DNA synthesis (UDS) represented the filling in of the gaps produced by excision of the UV-induced DNA lesions. The length of these gaps could be estimated and in the case of excision of dimers the size varies between 40 and 100 nucleotides (long-patch repair) (Regan et al., 1971; Regan and Setlow, 1974). Filling of the gaps in the presence of (^{3}H)TdR results in labelling of the DNA, which can be recognized by autoradiography. UDS is a measure for the repair of all lesions subjected to excision repair, and hence is not a lesion-specific instrument for repair analysis. In experiments described in this thesis, we have applied an enzymatic method that measures excision of pyrimidine dimers specifically.

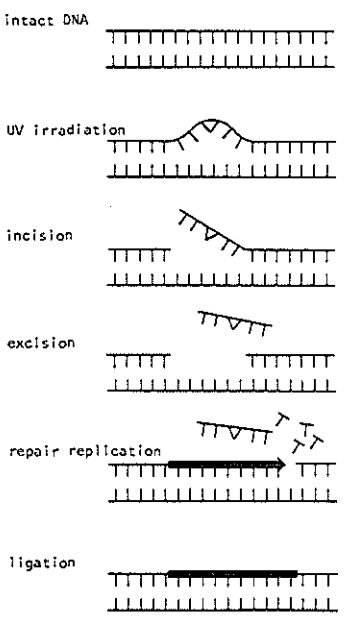

Fig. 3. Schematic representation of the steps involved in excision repair of damaged DNA.

In addition to long-patch repair the excision of some DNA lesions results in much smaller repair patches (only a few nucleotides). This is the case after X-irradiation, when damaged bases are excised (short-patch repair) (Regan and Setlow, 1974). Whether excision repair results in short or long repair patches depends on the nature

of the lesion, thus lesions can be classified based on this criterium (as UV- or X-ray-like).

For some base damages an alternative pathway has been proposed (Lindahl et al., 1974; Duncan et al., 1978). In that model the first step involves removal of the damaged base by cleaving its C-N bond with its deoxyribose by an N-glycosylase, which results in an apurinic or apyrimidinic site (AP site). This AP site is recognized and incision is made by an apurinic-endonuclease, after which excision takes place as described above.

Although DNA damage that is not removed by photoreactivation or by excision repair may interfere with DNA replication, it does not provide an absolute block. The processes facilitating the formation of intact daughter strands on a template that contains lesions are known as postreplication repair (PRR) processes and were first detected in *E. coli* (Rupp and Howard-Flanders, 1968). Gaps are left in the daughter strand opposite the damaged region. By single-strand recombination a piece of DNA from the parental strand of the sister-duplex is inserted and the remaining discontinuity is filled in by normal repair replication (Rupp et al., 1971). In about half the recombinational exchanges of UV-irradiated DNA the strand containing the dimer is exchanged, so that the dimers become equally distributed among parental and progeny strands (Ganesan, 1974). Mammalian cells are also able to deal with damaged DNA during replication, but recombinational events could not be detected. Also in mammalian DNA gaps are left opposite the damaged region with the same length of about 1000 nucleotides as in bacterial DNA, but they appeared to be filled in by *de novo* synthesis (for review, see: Lehmann, 1976).

The application of bacterial DNA repair models in the description of DNA repair in mammalian cells is complicated by the difference in structure of the chromosomes of pro- and eucaryotic cells. In mammalian chromosomes, DNA is associated with histones in a complex structure, the chromatin, whereas bacterial chromosomes contain DNA in a more or less native configuration. The chromatin can be considered as a alternate sequence of core- and linker-DNA (for review, see: Felsenfeld, 1978). Each piece of core-DNA (140 base-pairs) is wrapped around a globular histone complex and this forms a nucleosome. The nucleosomes are joined together by pieces of linker-DNA (about 50 base pairs). There is evidence that the rate of repair of DNA lesions depends on their position in this chromatin structure (Bodell, 1977; Cleaver, 1977). Moreover, it can be envisaged that the accessibility of the DNA for repair enzymes requires "factors" which do not exist in procaryotic cells (Mortelmans et al., 1976).

1.4 Xeroderma pigmentosum, a genetic DNA repair deficiency in man

The elucidation of repair pathways in mammalian cells has been facilitated by the isolation of mutants being defective in DNA repair. These mutants can be obtained in different ways. One approach is to

isolate mutants from wild type cells, after treatment with mutagenic agents, and to select them on the basis of increased sensitivity to DNA damaging agents (Klimek et al., 1977; Sato and Hieda, 1979). The diploid character of mammalian cells complicates a successful isolation of mutants from wild type cells. Most mutations will not be expressed, as the cell has still a wild type allele on the homologous chromosome. Expression of a mutation is possible when the cell is hemizygous for that specific gene, or when both genes on the two homologous chromosomes have been mutated, resulting in homozygosity of the mutation. As hemizygosity will be a rare event and mutation in the two alleles of a gene is rather infrequent, the isolation of repair mutants from established cell lines will be difficult to achieve. It has not been shown that the sensitive cell lines which so far have been isolated, have a defect in the repair of DNA damages. A more successful approach has been the isolation of cell strains from patients being extremely sensitive to DNA damaging agents in the environment. The sensitivity may be the result of defective DNA repair. The criteria for the selection of these patients are the genetic nature of the disease and the abnormal sensitivity to environmental mutagens and carcinogens, as judged from chromosomal instability or predisposition to cancer. Such diseases are xeroderma pigmentosum, ataxia telangiectasia, Fanconi's anaemia, Bloom's syndrome and Cockayne's syndrome (for review, see: Setlow, 1978).

Cleaver (1968) was the first to discover a repair defect after UV-irradiation in cultured cells of xeroderma pigmentosum (XP) patients. XP is a rare, autosomal recessive heriditary, human disease first described by Hebra and Kaposi in 1874. The clinical symptoms are hypersensitivity to sunlight, expressed in a dry atrophic appearance of the skin, abnormal pigmentation and malignancies in the sun-exposed areas of the skin. De Sanctis and Cacchione described in 1932 a syndrome which, in addition to the skin lesions, also was associated with microencephaly, retarded growth, abnormal sexual development and abnormalities of the nervous system, such as hearing loss, reduction or absence of reflexes and mental retardation. Among XP patients only a few show the complete set of symptoms of this De Sanctis-Cacchione (DSC) syndrome, others have only some of the neurological abnormalities associated with DSC, whilst a number of patients is free of neurological complications (Robbins et al., 1974).

Cleaver (1968) reported that skin fibroblasts obtained from XP patients were defective in the repair of UV-induced DNA damage. These cell strains showed decreased levels of repair DNA synthesis as measured by the uptake of (^{3}H)TdR in parental DNA by isopycnic centrifugation analysis of isolated DNA and by autoradiography of UV-irradiated cells (UDS). Evidence for a defect in the excision of pyrimidine dimers was obtained by chromatographic analysis of the dimer content of the DNA of XP cells as a function of time after UV exposure (Setlow et al., 1969). A sensitive test system (UV-endo assay) was devised by Paterson et al. (1973) and is described in more detail in chapter 3.2 and paper II of the appendix (Zelle and Lohman,

1979).

The biological consequences of deficient repair have also been demonstrated in *in vitro* studies. After a certain dose of UV less XP cells survived than normal cells (Cleaver, 1970; Goldstein, 1971). XP cells were less able to reactivate UV-irradiated SV40, herpes simplex and vaccinia virus (host-cell reactivation) (Aaronson and Lytle, 1970; Lytle et al., 1972; Abrahams and Van der Eb, 1976). Maher and coworkers (1976) showed that after a certain dose of UV more XP cells than normal human human cells were mutated.

A difficulty in understanding the etiology of the syndrome has been the detection of XP patients with normal levels of repair (Burk et al., 1971; Kleijer et al., 1973). Cells of these so-called XP variants were found to be slightly more sensitive than normal human cells to UV, as observed in UV-survival experiments (Cleaver, 1972; Lehmann et al., 1977). Evidence for a repair defect in these cells was obtained by Lehmann et al. (1975). These authors found a defect in postreplication repair (PRR). In normal human cells, semi-conservative replication of DNA yields relative short pieces of DNA shortly after irradiation, which are subsequently linked together to attain higher molecular weights. In the XP variant cells the DNA synthesized shortly after irradiation did not attain the same high molecular weight as in normal cells, during a post-irradiation incubation period of a few hours. It is postulated that lesions present in the parental DNA delay the chain elongation of the daughter strands in these cells to a greater extent than in normal cells. The clinical symptoms of these XP patients indicate the biological significance of the processes operating in normal cells to facilitate the replication of DNA on damaged templates.

The detection of the XP variants provided the first indication of biochemical heterogeneity within xeroderma pigmentosum. Heterogeneity was also observed in UDS studies of different excision repair-deficient XP patients (Bootsma et al., 1970). At the clinical level heterogeneity is clearly indicated as described earlier in this paragraph. The question whether biochemical and clinical heterogeneity were based on genetic differences was studied by De Weerd-Kastelein et al. (1972). They fused cells from different XP patients and determined the repair capacity of hybrid binuclear cells (heterokaryons). In several combinations of XP strains these authors found restoration of DNA repair in the heterokaryons, most probably as a result of complementation between the repair defects of the XP cells involved in the fusion. As a result of these studies (De Weerd-Kastelein et al., 1972, 1974; Kraemer et al., 1975; Arase et al., 1979; Keijzer et al., 1979) at the present time 7 different complementation groups could be identified in XP. The last one (the G group) was recently detected in our laboratory and is described in chapter 2.1 and paper I of the appendix (Keijzer et al., 1979). The genetic basis of complementation in XP is still obscure. The 7 groups may represent mutations in 7 different genes involved in excision repair (intergenic complementation). However, the possibility of

complementation between different mutations in the same gene (intragenic complementation) cannot be ruled out. We have approached this problem indirectly by studying complementation in the case of repair of lesions produced by the carcinogen 4-nitroquinoline-1-oxide (4NQO), for which XP cells are also hypersensitive (chapter 2.2). Furthermore, we investigated the kinetics of complementation, which may throw light on the biochemical characteristics of the gene product(s) involved in complementation (chapter 2.3).

All 7 complementation groups have decreased levels of UV-induced UDS. In order to elucidate the biochemical nature of the defects in these 7 groups, we have studied the excision of pyrimidine dimers by using the UV-endo assay devised by Paterson et al. (1973). This analysis is described in chapter 3.2 and paper II of the appendix (Zelle and Lohman, 1979). The results of our experiments indicate that in all 7 complementation groups the defect is located in the incision step of the excision repair pathway.

In most studies of the genetic defect(s) in XP, the investigators use a laboratory UV source which predominantly emits UV with a wavelength of 254 nm. UV emitted by the sun and reaching the surface of the earth does not contain radiation with wavelengths below 290 nm, as this is absorbed by the ozon layer in the atmosphere. The clinical features of XP cannot be caused by this part of the UV spectrum (far-UV) and have to be attributed to longer wavelengths (near-UV). To investigate the effects of the UV region of sunlight we performed a study of the biochemical and biological consequences of exposition to near-UV radiation by using cultured chinese hamster cells (chapter 4).

1.5 Other genetic diseases associated with DNA repair deficiencies

Much less is known about the nature of the deficiencies in other genetic disorders in which a defect in DNA repair is indicated. The X-ray treatment of cancers in some cases of ataxia telangiectasia (AT) revealed hypersensitivity of normal tissues to ionizing radiation. This sensitivity was confirmed in *in vitro* survival experiments, in which AT cells were exposed to ionizing radiation (Taylor et al., 1975). AT cells are also characterized by high levels of spontaneous and X-ray-induced chromosomal aberrations. DNA repair studies performed by Paterson et al. (1976) have shown that some cultured AT cell strains have a reduced capacity to remove ionizing radiation-induced DNA lesions. Although the precise nature of these lesions has not been elucidated, they act as substrate for incision by a bacterial γ-endonuclease. Paterson et al. (1976) also found reduced levels of repair DNA synthesis after γ-irradiation in some AT strains, although other strains showed a normal response.

The clinical symptoms of Fanconi's anaemia (FA) are haematological abnormalities, which are ultimately lethal as a result of haemorrhage or haematological failure (Fanconi, 1967). Lymphocyte and fibroblast cultures of affected individuals show high levels of

chromosome aberrations (German, 1972). Treatment of FA cells with bifunctional alkylating agents results in abnormal high levels of chromosome aberrations (Latt et al., 1975). A defect in the repair of interstrand DNA cross-links is indicated by the studies of Fujiwara and Tatsumi (1975).

Bloom's syndrome (BS) is a rare condition showing pre- and postnatal growth retardation, hypersensitivity to sunlight and predisposition to cancer (Bloom, 1954). This genetic disease is associated with an abnormal high number of spontaneous chromosome aberrations (German et al., 1965) and sister-chromatid exchanges (Chaganti et al., 1974). It has been reported that cultured BS cells are hypersensitive to UV and that abnormalities exist in the semi-conservative replication of DNA (Giannelli et al., 1977). The UV sensitivity could not, however, be confirmed in other studies (Arlett and Harcourt, 1978).

Cultured cells from patients suffering from Cockayne's syndrome (CS) (Cockayne, 1936) are also hypersensitive to UV on the basis of *in vitro* UV survival experiments (Wade and Chu, 1979). The molecular background of this sensitivity is still obscure.

So far, in none of these syndromes the primary genetic defect has been identified. And although the involvement of DNA repair in XP is well documented, it cannot be ruled out that even in this disease the impairment of repair is not the primary but a secondary consequence of a defect in another, still unknown cellular function.

CHAPTER 2

GENETIC ANALYSIS OF DEFECTIVE DNA REPAIR IN XERODERMA PIGMENTOSUM

2.1 Complementation analysis following UV-exposure

A joint survey among European and North-American XP strains revealed in 1975 the existence of 5 different complementation groups, designated A-E (Kraemer et al., 1975). Recently in Japan a patient, XP230S, was found whose cells showed complementation with each of the 5 existing complementation groups. This proved the existence of a 6th complementation group (Arase et al., 1979). This Japanese patient, a 45 year old woman, was diagnosed to have XP, based on freckles in sun-exposed areas of the skin and on her history of skin lesions since the age of six. Clinically this patient is of interest because of the absence of skin tumours. She had no neurological abnormalities.

Subsequently a 7th group, G, was detected, represented by XP2BI (Keijzer et al., 1979). The details of this study are presented in paper I of the appendix. This strain was derived from a 17 years old girl, with marked skin symptoms, but also without skin tumours. She showed progressive mental retardation starting at the age of 11. The repair studies performed revealed an almost complete absence of DNA repair after 254 nm UV. At 5 $J.m^{-2}$ no removal of dimers, assayed by the UV-endonuclease test system, was observed. Recently also another strain, XP3BR, was assigned to group G (A.R. Lehmann and C.F. Arlett, unpublished). This strain too has almost no repair activity, as judged from its level of UDS. The patient shows skin lesions without tumours, but in contrast to XP2BI, no neurological complications, which may be related to the age of the patient (4 years).

The complementation analysis has not resolved the clinical heterogeneity observed in XP. Although most of the XP patients with severe neurological abnormalities were assigned to the groups A, D and G, heterogeneity is still apparent within each of these groups. Most patients classified into the A group have severe neurological complications, whereas a few patients, classified into the same group, did not show neurological symptoms.

2.2 Complementation analysis following exposure to 4-nitroquinoline-1-oxide[a]

2.2.1 Introduction

XP cells are not only impaired in the repair of UV-induced lesions, but also in the removal of damage induced by a variety of other agents. On the other hand, also DNA damages can be induced which are repaired at a normal rate. Compounds that induce DNA damage that is not repaired as efficient as in normal cells can be recognized by the reduced level of UDS after treatment in comparison with normal cells. Examples of these compounds are: 4-nitroquinoline-1-oxide (4NQO), 4-hydroxylaminoquinoline-1-oxide (4HAQO) (Stich and San, 1971), N-acetoxy-2-acetylaminofluorene (NAAF) (Stich et al., 1972), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Cleaver, 1973), 2-methyl-4-nitroquinoline-1-oxide, 3-methyl-4-nitropyridine-1-oxide (Stich et al., 1973), Benz(α)antracene 5,6-epoxide (Stich and San, 1973), aflatoxin B1, aflatoxin G1, aflatoxicol and sterigmatocystin (the last 4 compounds after metabolic activation with a microsomal liver preparation) (Stich and Laishes, 1975). Examples of compounds that induce a normal repair in XP cells are: ionizing radiation such as X-rays (Cleaver, 1969), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methanesulphonate (MMS) (Cleaver, 1971), and N-methyl-N-nitrosourea (MNU) (Cleaver, 1973).

The classification of agents on the criterium of repair induction in XP cells is complicated by the fact that most agents, if not all, produce each a number of different DNA lesions. The repair of a fraction of these lesions can be impaired in XP cells, while the remainder is normal. For example, alkylating agents produce N7-alkylguanine and O6-alkylguanine residues. The first type is removed efficiently in XP cells, but the repair of the latter type is deficient (Goth-Goldstein, 1977).

The DNA damaging agent 4-nitroquinoline-1-oxide (4NQO) is a potent carcinogen (Nakahara et al., 1957). The enhanced sensitivity to 4NQO of XP cells in comparison with normal cells was demonstrated by Takebe et al. (1972) who observed an increased cytotoxicity of this compound in XP cells, while Stich and San (1971) and Cleaver (1973) established that 4NQO-treated XP cells show a relatively low level of repair DNA synthesis.

The various processes resulting in the formation of DNA adducts by 4NQO have been characterized to some extent (Nagao and Sugimura, 1976). Via an enzymatic reduction the nitro-group is first converted into the hydroxylamino substituent, which is then aminoacylated by aminoacyl-tRNA synthetase. The final product reacts with guanine and

a) This paper by B. Zelle and D. Bootsma is submitted for publication in Mutation Research.

adenine bases in the DNA under the formation of 4NQO-purine adducts. Several products are formed which differ in chemical stability. Three major stable purine adducts were identified; these lesions were found to be removed from the DNA in normal human cells. No removal of these lesions was detected, however, with the SV40-transformed XP strain from complementation group A (Ikenaga et al., 1977). A less stable guanine adduct did disappear from the XP DNA, but the rate of removal was lower than in normal cells (Ikenaga et al., 1977).

The analogy between the effects of 4NQO and UV on XP cells suggests that the same repair process, which in these cells is (partially) blocked, is involved in the removal of the lesions induced by the two agents. The existence of several complementation groups of XP strains with regard to the repair of UV-damage is usually taken to indicate that the products of a number of genes are necessary for an efficient removal of the damage. If 4NQO-lesions are (predominantly) removed via the same pathway as the pyrimidine dimers, in dependance of the same gene products, it is to be expected that XP strains will classify in the same complementation groups that were found with UV, when the repair of 4NQO damage is studied in various combinations of fused XP cells.

In this study we analyzed whether the genetic heterogeneity of XP with respect to the repair of UV-induced DNA lesions is indeed the same as to the repair of 4NQO-induced lesions. Repair was studied by determination of the extent of unscheduled DNA synthesis in the exposed cells, by means of autoradiography.

2.2.2 Materials and methods

Cell strains and tissue culture

The primary human fibroblast strain used were AH and HAN, derived from healthy individuals; the primary XP fibroblast strains were: from complementation group A: XP1BI, XP8LO and XP25RO; from complementation group B: XP11BE; from complementation group C: XP1TE, XP4RO and XP20RO; from complementation group D: XP3NE; and from complementation group E: XP2RO.

Monolayer cultures were routinely grown in 75 cm^2 plastic tissue culture flasks (Costar, USA) in Ham's F10 medium, supplemented with 15% (v/v) foetal calf serum, 30 µM hypoxanthine, 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. When cells became confluent they were subcultured at a ratio of 1:2. The absence of mycoplasma (PPLO) infections was checked by the method of Peden (1975). Cells to be used for experiments were seeded in tissue culture dishes and incubated at 37°C in a humidified 5% CO_2-atmosphere.

Cell fusion

Trypsinized cells of 2 different strains were mixed in a 1:1 ratio and approximately $3x10^5$ cells were seeded in a tissue culture dish (diameter: 3.5 cm, Greiner), containing a sterile coverslip (18x18 mm). After 1 day of incubation in 1 ml of F10 medium the populations were treated with polyethylene glycol (PEG) 1000 to induce cell fusion, as described by Davidson and Gerald (1976) and with modifications according to Davidson et al. (1976) and Norwood et al. (1976). Cells were washed with serum-free F10 medium and 1 ml of PEG 1000 solution (see below) was added to a carefully drained plate. After 60 s the PEG solution was sucked off and the plates were washed three times with serum-free medium, after which the cells received fresh medium with serum and were incubated again at 37^0C. The PEG was sterilized by autoclaving 50 g of solid PEG 1000 at 120^0C for 15 min. After several minutes of cooling of the melt, until the first PEG crystals appeared, 68.5 ml serum-free F10 medium containing 15% (v/v) dimethylsulfoxide (DMSO) and 1.5 ml of a 70 mM $NaHCO_3$ solution were added and a homogeneous solution was obtained by shaking. This solution was used at a temperature of about 30-35^{0}C.

UV-irradiation

The UV source was a single 15 W low-pressure mercury, germicidal lamp (Philips TUV lamp) with a dose rate at the exposition area of 0.39 $W.m^{-2}$ of 254 nm radiation as determined by chemical actinometry using the procedure of Parker and Hatchard (Calvert and Pitts, 1966) and corrected for the contribution of radiations with longer wavelengths, the intensities of which were given by the manufacturer. Cells to be irradiated were thoroughly washed free of medium with phosphate-buffered saline (PBS), consisting of 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.7 mM KCl. Irradiation was performed at room temperature through a thin layer of PBS (1 ml/plate).

4-Nitroquinoline-1-oxide

4-Nitroquinoline-1-oxide was dissolved at 0.1 M in DMSO and diluted in serum-containing medium to a final concentration of 10 µM. Only freshly prepared solutions were used.

Unscheduled DNA synthesis

One or two days after fusion the cells (unfused populations to be used for control experiments were inoculated simultaneously with the populations meant to be fused) were incubated for 1 h in fresh medium containing (^{3}H)thymidine ((^{3}H)TdR; 10 µCi/ml, 19 Ci/mmol; The Radiochemical Centre, U.K.) to label cells in S-phase. The cells were then either exposed to 5 $J.m^{-2}$ of UV radiation or incubated for 1 h with 10 µM of 4NQO at 37^0C, or they served as controls and received no

damaging treatment. Subsequently, the cells were again incubated in medium with (^{3}H)TdR (10 µCi/ml) for 2 h. Finally they were washed twice with PBS, fixed with Bouins fixative (aqueous solution saturated with picric acid, 35% formaldehyde solution and glacial acetic acid at a volume ratio of 15:5:1), washed with water and dried.

Autoradiography

The coverslips containing the cells were mounted onto microscope slides and autoradiograms were prepared by dipping the slides in Ilford K2 nuclear research emulsion (diluted with distilled water at a 1:1 (v/v) ratio) and exposure for 4 days at 4^{0}C. After development and fixation the preparations were stained with May-Grünwalds modified solution and with Giemsa's solution. Grains over 50 nuclei of non-S-phase cells were counted; cells having more than 120 grains over their nuclei were considered to be S-phase cells.

2.2.3 Results

The UV-dose and the conditions of exposure to 4NQO were chosen so that the two treatments resulted in levels of UDS in normal control cells which were almost equal and high enough to give distinct differences in grain counts between control and XP cells. After a dose of 5 J.m^{-2} of 254 nm UV radiation the average number of grains per nucleus, corrected for the background, was 48 and after the exposition to 4NQO (10 µM in serum-containing medium at 37^{0}C for 60 min) the average was 53. The background level, as counted over the nuclei of untreated cells, ranged among the experiments between 0.7 and 4.5.

The relative UDS levels of the XP strains studied are shown in Table I. The results indicate that all XP strains used are considerably reduced in their ability to excise UV damage, and in all but one strain also after treatment with 4NQO the level of UDS is substantially depressed. With the exception of strain XP8LO, all strains show a higher relative level of UDS after 4NQO than after UV. In XP2RO cells the UDS level after treatment with 4NQO was so high that under the conditions of exposure chosen for these experiments, complementation studies with this strain could not be carried out.

In each fusion the grain numbers over both nuclei of 50 binuclear cells were counted. The results of these grain counts, without correction for a background UDS level, were plotted in scatter diagrams as described before (Kraemer et al., 1975). Fig. 1 shows 4 of the resulting diagrams. The solid lines in each graph represent the 95% probability limits as calculated from the UDS data of the unfused parental and control cell strains, assuming a normal distribution. The abscissa being the grain number over one nucleus and the ordinate that over the other. If a considerable fraction of the points is located beyond the 95% probability limits of the 2 parental XP

strains, complementation is indicated.

TABLE I
UNSCHEDULED DNA SYNTHESIS OF A VARIETY OF XP CELL STRAINS AFTER UV-IRRADIATION OR 4NQO-TREATMENT

Normal human fibroblasts (controls) and cells taken from various XP strains were either UV-irradiated (5 $J.m^{-2}$) or exposed to 4NQO (10 μM, 60 min, 37^0C) and then UDS was determined. The results of 50 non-S-phase cells were averaged and related to the number of grains counted over the nuclei of control cells after background correction. The average (± S.E.M.) of the number of grains over the nuclei of control cells in all experiments was 48.2 ± 4.6 after UV-irradiation and 53.0 ± 4.1 after 4NQO-exposure.

Complementation group	Strain	UDS (% of normal)	
		UV-induced	4NQO-induced
-	controls	100	100
A	XP1BI	6	15
	XP8LO	34	26
	XP25RO	1	11
B	XP11BE	1	9
C	XP1TE	20	26
	XP4RO	14	16
	XP20RO	10	20
D	XP3NE	11	22
E	XP2RO	52	95

Fig. 1A and 1B give the results of a fusion using 2 XP strains of the C group; the lack of complementation, both after UV-irradiation and after 4NQO treatment, is obvious. A fusion of 2 strains from different complementation groups, A and D, resulted in the diagrams presented in Fig. 1C and 1D. In both cases a substantial number of points is present outside the XP-95% probability areas. So the 2 strains complement each other, not only for the repair of UV-lesions, but also for the removal of 4NQO-induced DNA damage. The results of all fusion experiments performed are compiled in Table II. These are presented in the form of a complementation-index. Without complementation an index in the order of 5% is expected. Values considerably exceeding this percentage indicate that complementation

occurs. It is evident that XP strains that are classified into the same complementation group on the basis of their repair of UV-damage do not complement each other after 4NQO treatment either, whereas strains that belong to different complementation groups because they do complement each other for the excision of UV-lesions, also show complementation for the repair of 4NQO-induced damage.

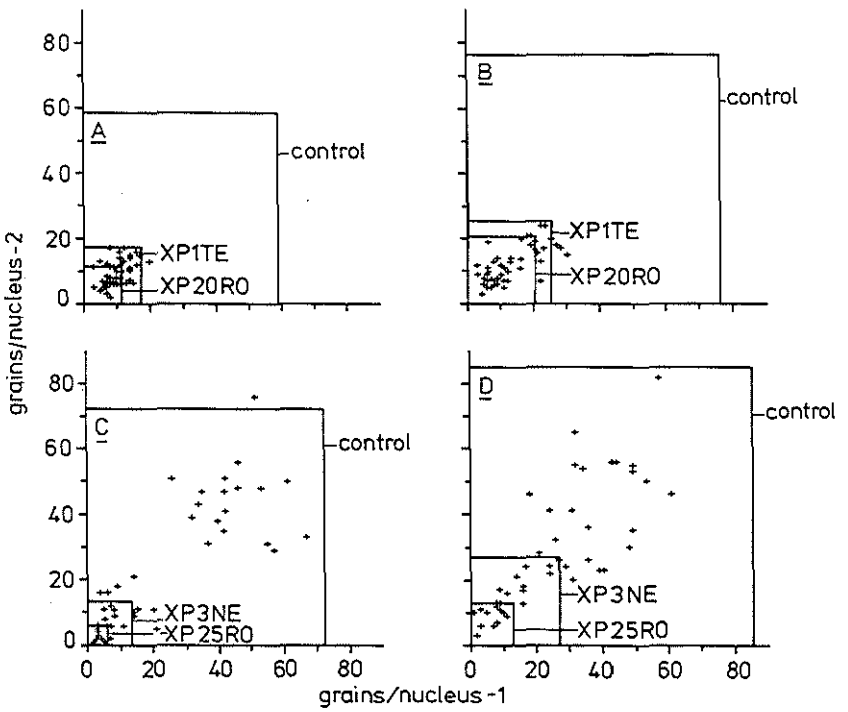

Fig. 1. Complementation analysis of XP strains, after UV-irradiation (A and C; 5 $J.m^{-2}$) or after 4NQO-exposure (B and D; 10 µM, 60 min, 37.0°C). Each point represents the unscheduled DNA synthesis of one binuclear cell; its position is determined by the numbers of grains over the two nuclei, plotted on abscissa and ordinate, respectively. Each square indicates the area that is statistically expected to comprise 95% of the grain numbers found over nuclei of unfused cells of the strain indicated.
A and B: fusion XP1TE/XP20RO (both C group).
C and D: fusion XP25RO/XP3NE (A and D group, respectively).

TABLE II
RESULTS OF COMPLEMENTATION ANALYSIS WITH VARIOUS XP STRAINS AFTER UV-IRRADIATION OR 4NQO-TREATMENT

Fusion	Complementation groups	Complementation-index[a] after	
		UV	4NQO
XP1BI/XP8LO	A/A	3	1
XP1BI/XP25RO	A/A	10	3
XP8LO/XP25RO	A/A	8	1
XP1TE/XP4RO	C/C	7	5
XP1TE/XP20RO	C/C	1	3
XP4RO/XP20RO	C/C	3	1
XP25RO/XP11BE	A/B	61	50
XP25RO/XP4RO	A/C	60	54
XP25RO/XP3NE	A/D	51	40
XP11BE/XP3NE	B/D	53	35

a) The complementation-index is the percentage of the points in the scatter diagrams beyond both XP-95% probability limits; points with only one coordinate beyond the limits were weighted 0.5.

2.2.4 Discussion

In all 9 excision repair-deficient XP strains included in this study, UDS caused by 4NQO was less than that observed in the normal human control cells. With XP2RO (group E) the difference was marginal, but in the other strains the level of UDS was depressed considerably. In general, the degree to which UDS after 4NQO was reduced in the different XP strains followed the same pattern as was seen for the repair of UV damage, but as a rule 4NQO gave a 2-11% higher level of residual UDS. Only with one of the group A strains, XP8LO, the reverse situation was found, whereas in case of XP2RO the difference was much larger (+43%). The similarity in the depression of the UDS level after UV and 4NQO indicates that the same excision repair systems are involved in the removal of most of the damages caused by these two agents. The differences in residual repair levels may be ascribed to the fact that 4NQO induces a mixture of lesions in DNA, whereas after UV the pyrimidine dimers dominate. Probably the extent to which repair is reduced in the XP cells is not the same for each of the different lesions caused by 4NQO. This is also indicated by the observation of Ikenaga et al. (1977) that the labile 4NQO-guanine adduct did disappear from the DNA of certain XP cells, be it at a reduced rate, in contrast to the stable 4NQO-purine adducts

whose removal appeared to be completely blocked. Furthermore, Regan and Setlow (1973) showed that lesions produced by 4NQO can be classified according to the repair modes of their removal as subjected to short- or long-patch repair; it was shown that in XP cells the long-patch mode was affected, whereas the short-patch mode was present.

It appears plausible, therefore, that the stable 4NQO-purine adducts are removed via a pathway giving long repair patches, which is also responsible for the removal of pyrimidine dimers. Consequently, the residual repair level for this type of lesions will correspond to the reduced level of repair of UV-induced lesions in the same XP strain. The labile 4NQO-guanine adducts, on the other hand, supposedly are removed via a short-patch repair pathway, which is blocked to a lesser extent in the XP cells and which gives a considerably higher contribution to total UDS after 4NQO than after UV. This may explain the higher residual UDS usually found for 4NQO.

It will be difficult, however, to explain the high residual UDS after 4NQO observed in XP2RO cells, which is not markedly depressed in comparison with the controls, along these lines. A different explanation might be that one gene product, viz. the one being defective in this E group strain, is only involved in the repair of UV lesions. It is also possible that the affected product in the E group has a reduced affinity for UV lesions, but a normal affinity for 4NQO-induced DNA damage. A handicap in the interpretation of the UDS level in this strain after UV is the not understood dose-dependency of this phenomenon. De Weerd-Kastelein et al. (1974) obtained an increase of this level to 75% of normal by increasing the UV dose to 100 $J.m^{-2}$. Evidently the repair behaviour of this strain is not easily interpreted.

Our results indicate that repair pathways of UV and 4NQO lesions have steps in common, since 8 of the 9 XP strains tested showed a strong analogy in the depression of the UDS level after treatment with either agent. This strongly suggests that the gene products responsible for the repair deficiency in the groups A, B, C, and D are of equal importance for the repair of the majority of the 4NQO damages.

For the interpretation of the complementation results the nature of the defect and the mechanism of complementation must be considered. The exact nature of the primary defect in each of the XP complementation groups, which is due to a gene mutation, is unknown. The mutation can have its effect at the regulatory level and the affected product can be involved in the regulation of transcription or translation of genes involved in repair, or the defect is at the structural level and the affected product is an enzyme or protein involved in a repair pathway, either in the recognition of the lesions or in the incision process (Zelle and Lohman, 1979: paper II of the appendix). The structure of the defective factor itself is unknown. It can act as a single unit, or it can be a monomer of a multimeric structure composed either of identical or different subunits.

The most simple interpretation of the presence of complementation is that different genes are involved in the repair deficiency of xeroderma pigmentosum in cells of different complementation groups. In binuclear heterokaryons derived from XP cells from different complementation groups each nucleus is able to provide the other nucleus with a normal substitute of the gene product that the other lacks or possesses in a malfunctional state. As a result of this, each nucleus will have the complete set of normal gene products available to attain the normal level of repair (intergenic complementation).

Interallelic or intragenic complementation might also occur. Then the gene that is affected in either of the two complementing strains is the same, but the mutation causing the defect is different. Moreover, repair should depend on a multimeric structure comprising at least two gene products as subunits. In the XP cells of one strain the affected subunits are then identical, resulting in an inactive multimer, whereas in the heterokaryons two classes of subunits will be present with different alterations; then a hybrid enzyme might be formed, which is still not identical to the normal products, but which may have some activity. In such a situation incomplete complementation towards the repair of certain DNA lesions might be expected. It can be visualized that this altered multimeric product acts differently on UV- and 4NQO-induced lesions. The hybrid gene product might still be able to fully restore the cells capability to repair UV lesions but is less efficient in the excision of 4NQO-induced damage, or vica versa. Consequently, the complementation pattern of XP cells with regard to either lesion may not always coincide. For this reason, it may be as meaningful to search for complementation with respect to 4NQO-repair among XP strains from the same complementation group as it is to search for non-complementation between members of different groups.

In order to discriminate between the various possibilities we have included in our study a number of XP strains from the same complementation groups, with different responses to UV. Especially with the chosen A group strains the residual level of UDS (after UV) varies strongly, since XP8LO was included which has an exceptionally high level for this group (De Weerd-Kastelein et al., 1976). For the C group XP4RO and XP1TE were chosen because these 2 strains differ greatly in the induction of sister-chromatid exchanges by UV (De Weerd-Kastelein et al., 1977). These differences within one complementation group might indicate that the repair deficiency is caused by a differently affected gene product in the different strains, which was thought to increase the chance of finding deviations in the behaviour towards 4NQO lesions, e.g. complementation within the group, which, however, was not observed.

The fusion experiments show that in the 10 combinations studied, viz. 3 within group A, 3 within group C and 4 of the mixed type, the same pattern of complementation was observed after exposure to 4NQO and UV. Although not all possible combinations could be tested, none

of the results obtained sofar indicates a deviation from the behaviour expected when only intergenic complementation occurs.

2.3 Kinetics of complementation in xeroderma pigmentosum[a]

2.3.1 Introduction

Each of the seven complementation groups of xeroderma pigmentosum might correspond to the defectiveness of one particular step or function of a complex repair system in human cells, although no specific defect has been attributed to any of these complementation groups, nor has evidence been presented that the different groups correspond to defects in different genes. In this respect it would be of great interest if properties of the gene product that functions abnormally in a specific complementation group could be found, which would enable the characterization of that group. Giannelli and Pawsey (1974) observed the existence of rapid and slow complementing varieties of XP gene products in their study of complementation of the repair defect in XP fibroblasts after cell fusion with normal human skin fibroblasts. The rapidly complementing XP strain belongs to complementation group A and the slowly complementing strain to group C (Pawsey et al., 1979). We investigated whether the rate at which the repair capacity is restored after cell fusion is a specific property of an XP complementation group.

2.3.2 Materials and methods

Cell strains

The primary human fibroblast strains used, are shown in Table III. Cells were cultured in Ham's F10 medium (Flow, U.K.) supplemented with 10% foetal calf serum. Cells from confluent cultures were used for cell fusion.

UV-irradiation

Cells were seeded in plastic tissue culture dishes (diameter: 60 mm or 35 mm). The drained cultures were irradiated with 254 nm UV from a single low-pressure mercury, germicidal lamp (Philips TUV lamp) at a dose of 10 $J.m^{-2}$ after washing with a bicarbonate-buffered salt solution (solA: 0.13 M NaCl, 2.7 mM KCl, 14 mM $NaHCO_3$ and 10 mM D-glucose).

a) This paper by S. Matsukuma, B. Zelle and D. Bootsma is submitted for publication in Experimental Cell Research.

TABLE III
CELL STRAINS USED

Strain	Nature	UV-induced UDS (% of normal)	
MAR	control	-	
XP25RO	XP group A	0-5	(Kleijer et al., 1973; Lehmann et al., 1977)
XP1BI	XP group A	6	(this thesis: chapter 2.2)
XP4RO	XP group C	10-15	(De Weerd-Kastelein et al., 1972; Lehmann et al., 1977)
XP20RO	XP group C	10	(Kleijer et al., 1973)

Cell fusion

UV-inactivated sendai virus at a titer of 400 haemagglutinating units per ml was used for fusing the cells. Cells of 2 strains were mixed in equal proportion and suspended in ice-chilled Hanks balanced salt solution without glucose (0.14 M NaCl, 5.4 mM KCl, 3.6 mM $CaCl_2$, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4 and 4.2 mM $NaHCO_3$). After 2 washes, the cells were suspended in 1 ml of this solution containing sendai virus and incubated for 15 min at 37°C. The cells were then suspended in culture medium containing 10% foetal calf serum; this moment was considered as the start of the fusion. For the experiments with UV-irradiated cells fused with unirradiated cells, cultures of XP cells in plastic dishes (diameter: 60 mm) at confluent stage were irradiated with UV, incubated for 3 h and processed for fusion.

Unscheduled DNA synthesis

Fused cells were seeded and allowed to flatten on glass cover slips placed in plastic dishes (diameter: 35 mm), which were incubated at 37°C in humidified air with 5% CO_2. Cultures that had been UV-irradiated after fusion were incubated in medium with (^{3}H)TdR (10 µCi/ml; 2 Ci/mmol; The Radiochemical Centre, U.K.) for 1 h before (to label the cells in S-phase) and for 2 h after irradiation. When UV-irradiated cells had been fused with unirradiated cells, the cultures were incubated in medium with (^{3}H)TdR (10 µCi/ml) for 2 h immediately after fusion and chased in medium with 30 µM nonradioactive thymidine and 30 µM cytidine for 18 h, after which period the cultures were fixed. In the long-term UDS experiments the cultures were incubated in medium with (^{3}H)TdR (1 µCi/ml) for 20 h after fusion. At the end of the incubation the cultures were washed, fixed with Bouins fixative and washed with demineralized water and 70% ethanol.

Autoradiography

The cover slip preparations were mounted onto glass microscope slides and processed for autoradiography. Kodak AR10 stripping film was used and the preparations were exposed for 10 days at 4^0C. After development and fixation of the preparations, the cells were stained with Giemsa's solution and covered with a glass cover slip. The grains over the nuclei were counted. Cells in S-phase, which had more than 100 grains over the nucleus, were excluded from counting. These cells were rare in our preparations since we used cells from confluent cultures and because all labelling procedures were done within 24 h after trypsinization of these cultures.

2.3.3 Results and conclusions

UDS in XP cells UV-irradiated at 4 h after fusion with normal fibroblasts

Restoration of DNA repair in XP nuclei in heterokaryons shortly after fusion was investigated by determination of UDS. Cells of the XP strains XP25RO, XP1BI (both of group A), XP4RO and XP20RO (both of group C), were fused with cells of a strain of normal human fibroblasts (MAR) by inactivated sendai virus. Four hours after fusion the cultures were irradiated with 10 $J.m^{-2}$ of UV and immediately incubated with (^{3}H)TdR for 2 h. In the autoradiograms of the binucleated cells in these cultures, 3 different types of labelled nuclei were expected to be found, viz. nuclei in homokaryons of normal fibroblasts with a normal grain number, nuclei in homokaryons of XP cells with a reduced grain number, and nuclei in heterokaryons of normal and XP cells with the normal grain number over one nucleus and, presumably, a grain number greater than the reduced one, representing the restored level of repair, over the other. Probably this restored level of UDS will vary, depending upon the XP strain involved and on the time elapsed after fusion (Giannelli and Pawsey, 1974).

The grains over the 2 nuclei of binucleated cells in the autoradiograms were counted and each pair of grain numbers was plotted in a cartesian diagram in such a way that the greater number was plotted on the abscissa and the smaller one along the ordinate (Fig. 2). The distribution of dots for the cultures of XP25RO fused with normal cells (Fig. 2A) was similar to that of the normal controls, the cultures of normal fibroblasts fused with themselves (Fig. 2E), except for the dots near the origin which represent the residual UDS of homokaryons of XP25RO cells. The distribution for the fusion of XP1BI with normal cells appeared to be more dispersed, i.e. no clear separation in 2 groups (Fig. 2B) was seen. The results for the fusions of XP4RO and XP20RO with normal cells were much more dispersed and showed an almost uniform distribution over the diagrams (Fig. 2C-D).

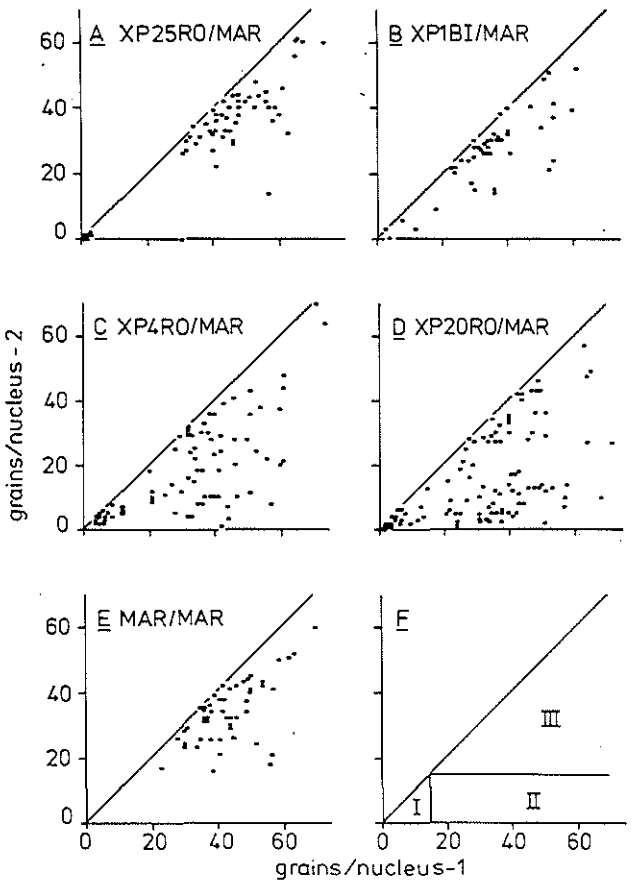

Fig. 2. Unscheduled DNA synthesis in dikaryons, UV-irradiated at 4 h after fusion, determined over 2 h after irradiation. After autoradiography the grains over both nuclei were counted. Each dot represents one binucleated cell, the grain number of the heavier labelled nucleus is plotted along the abscissa, the smaller value along the ordinate. Fusions of the following XP cells with normal human fibroblasts (MAR), A: XP25RO, B: XP1BI, C: XP4RO and D: XP20RO. Panel E: control fusion of MAR/MAR. For explanation of panel F: see text.

In the diagram for the fusion of XP20RO cells with normal fibroblasts (Fig. 2D), the dots can be thought to be distributed over 3 regions, i.e. a region that contains the dots for homokaryons of normal cells (compare Fig. 2E), a region near the origin where we expect the dots for homokaryons of XP20RO cells, and a region where the dots are thought to represent heterokaryons of normal and XP cells, when the nuclei of the latter show an intermediate level of repair. These regions can be selected as is shown in Fig. 2F on the

basis of an arbitrary criterium, viz. that a value of more than 15 grains per nucleus indicates a normal level of repair (the mean of control cells, 38.9 grains/nucleus, minus about two times the standard deviation): (I) the region for cells with no more than 15 grains over either nucleus, (II) the regions for cells with no more than 15 grains over only one of the 2 nuclei, and (III) the region for cells with more than 15 grains over each of the 2 nuclei. For each of the experiments shown in Fig. 2 the number of cells giving results that fell in each of these regions were counted and expressed as percentages of the total population (Table IV). The fraction of the cells found in region II gives an indication of the extent to which complementation has occured. This fraction was the smallest for the fusion of XP25RO with normal cells and the greatest for the fusions with XP20RO. This might indicate that after fusion the repair defect in XP25RO nuclei is restored much faster than that in XP20RO nuclei. (When equal numbers of the two cell types have participated in the fusion and no complementation occurs, about 50% of the binucleated cells are expected in region II, and 25% in each of the regions I and III). A low percentage in region II is also seen for the fusions with XP1BI, the second representative of group A, again pointing at a rapid complementation, but since the fraction in region I is abnormally low for these fusion products this result should be interpreted with caution. A much higher percentage in region II is shown by the fusions with XP4RO, the other strain of group C. In general, therefore, the results are compatible with a conception that the repair defect in XP cells belonging to group A is complemented rapidly after fusion (almost completely within 4 h), whereas cells of group C show a much slower recovery of their repair capacities.

TABLE IV
LABELLING PATTERN OF THE NUCLEI IN DIKARYONS, UV-IRRADIATED AT 4 h AFTER FUSION

UDS over the first 2 h after irradiation was determined.

Strains	Labelling pattern[a]			Number of cells counted
	I	II	III	
	a,b $\leq$ 15	a $\leq$ 15 < b	a,b > 15	
XP25RO/MAR	19	3	78	63
XP1BI/MAR	9	9	83	47
XP4RO/MAR	20	27	53	79
XP20RO/MAR	29	37	34	120
MAR/MAR	0	0	100	51

a) Points in the indicated region of Fig. 2 in % of the total population.

UDS in UV-irradiated XP cells immediately after fusion with normal fibroblasts

The two XP strains showing the largest differences in the rate of complementation were also compared for the degree of recovery of repair immediately after fusion. In these studies we fused UV-irradiated XP25RO (group A) and XP20RO (group C) cells with unirradiated normal cells. Immediately thereafter the cultures were incubated in medium with (^{3}H)TdR for 2 h, then they were chased for 18 h with nonradioactive thymidine and cytidine to have the cells sufficiently flattened at the time of fixation, and finally they were processed for autoradiography. In these preparations the homokaryons of normal cells will not produce grains resulting from UDS, because they have not been damaged by UV. The homokaryons of XP cells will show a small number of grains above their nuclei, in accordance with their residual UDS. In heterokaryons of XP and normal cells, however, only the XP nuclei will have grains above background, in amounts that will depend on the degree to which repair is restored. Bi- and trinucleated cells containing both labelled and unlabelled nuclei were scored; these were supposed to be heterokaryons.

The results shown in Fig. 2 and Table IV already indicate the fast restoration of UDS in heterokaryons with XP25RO. This behaviour was also observed in the experiments presented in this section: already in the first 2 h after fusion a considerable complementation of the repair system in XP25RO nuclei had occured. The average number of grains over the labelled nuclei in heterokaryons after fusion of UV-irradiated XP25RO with unirradiated normal cells was 30 (Fig. 3; results of binuclear cells), which is relatively high since the average grain number over the nuclei of UV-irradiated normal cells that were processed under the same conditions amounted to 38. On the other hand, the average number of grains over labelled nuclei in heterokaryons after fusion of XP20RO with normal cells was as low as the residual UDS level of XP20RO itself, which is not unexpected in view of the much slower restoration of repair observed in the preceding experiments.

The effect of the ratio of normal nuclei to XP nuclei in heterokaryons

If we assume that the restoration of the repair capacity of XP nuclei in heterokaryons is brought about by diffusable factors coded by the nuclei of normal cells, an influence of the number of normal nuclei per XP nucleus on the extent of restoration might be expected. To investigate the effect of the ratio of normal to XP nuclei on the repair capacity of XP nuclei in heterokaryons, the numbers of grains over the labelled nuclei were also averaged separately for trikaryons with 2 labelled nuclei, dikaryons with 1 labelled nucleus and trikaryons with 1 labelled nucleus, and compared with each other. In both XP25RO and XP20RO cells fused with normal cells, a significant gradual rise of the average grain number as function of the fraction

of normal nuclei in heterokaryons was obtained (Fig. 3). This observation indicates that the degree of repair restoration is correlated with the amount of normal "XP factor" brought into the XP nuclei after fusion.

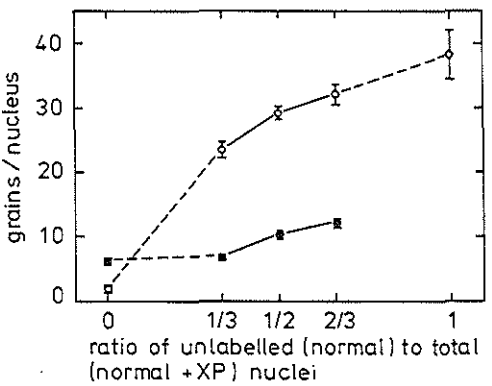

Fig. 3. Unscheduled DNA synthesis of UV irradiated XP25RO (○—○) or XP20RO (●—●) cells that were fused with unirradiated normal human cells. UDS was determined by autoradiography over 2 h immediately after fusion, for the labelled (=XP) nuclei in either bi- or trinucleated cells. UDS was plotted as a function of the ratio of normal to total nuclei. The points at ratio zero represent UDS of the unfused, irradiated XP strains and the point at ratio = 1 represents the UDS of the normal control cells. Bars indicate standard error of the mean (S.E.M.).

Rate of repair restoration in XP nuclei after fusion

The results presented sofar indicated a very rapid, almost immediate complementation after fusion of the repair defect in XP25RO and a much slower process when XP20RO cells were involved. To measure the rate of recovery of the repair system in both strains, at different times after fusion the UDS induced by UV was determined. In these studies XP20RO cells fused with XP25RO instead of normal cells were used to be able to discriminate between heterokaryons and homokaryons, which is problematic when the repair proficient homokaryons of normal cells have to be distinguished from (almost) fully complemented heterokaryons. The cultures were irradiated at various times after fusion and incubated with (^{3}H)TdR for 2 h immediately after irradiation. In these cultures, homokaryons will produce few grains representing residual UDS, while in heterokaryons the XP25RO nuclei probably will produce the grain number of normal cells since in these nuclei repair is restored immediately after fusion with normal cells. The XP20RO nuclei in the heterokaryons, on

the other hand, were expected to show a low level of UDS immediately after fusion, easily distinguishable from that of XP25RO, which would gradually increase with the period elapsed after fusion.

In the autoradiograms of cultures that were irradiated at 2, 4 or 6 h after fusion, binucleated cells were of 2 types, one with a small number of grains over both nuclei representing the residual UDS level, and the other type with many more grains over one nucleus than over the other. Binucleated cells with 15 or less grains over either nucleus were excluded from counting, because they were considered to be homokaryons. Dikaryons containing 1 nucleus with 16 or more grains, were assumed to be heterokaryons. Grains above the nuclei of these cells were counted, and the results are shown in Fig. 4. In the cells irradiated 2, 4 or 6 h after fusion, the average for the nuclei with many grains was (almost) as high as 30, with little influence of the period between fusion and the onset of labelling after irradiation. The average grain number counted over the nuclei with less grains in these presumed heterokaryons varied around a value of 10, with a small increase as function of the time between fusion and irradiation.

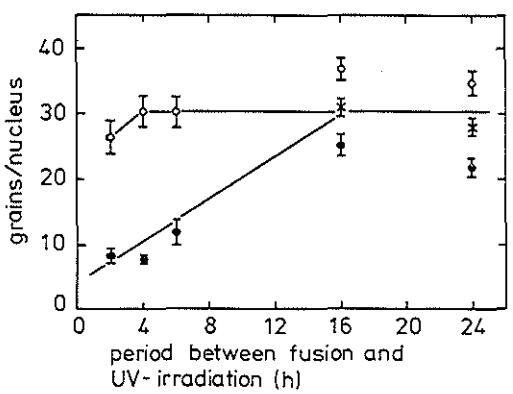

Fig. 4. Unscheduled DNA synthesis of dikaryons, obtained by fusion of XP25RO with XP20RO cells, UV-irradiated after the period indicated on the abscissa and cultured in the presence of (^{3}H)thymidine immediately thereafter for 2 h. The grains over the nuclei of binucleated cells with at least 1 nucleus with more than 15 grains were counted. The grain numbers counted over the heavier labelled nuclei were averaged (o—o), and the same was done with the lower values of all the pairs of data (●—●). The crosses (×) at 16 and 24 h indicate the average of all nuclei counted. Bars indicate S.E.M.

In cultures that were irradiated at 16 or 24 h after fusion, the number of grains over both nuclei in binucleated cells was either as small as to represent the residual UDS level or as high as to represent the normal UDS level. Binucleated cells of the first type

were assumed to be homokaryons and were excluded from counting. Binucleated cells of the second type were assumed to be heterokaryons and the grains over both nuclei were counted. When the grain numbers were averaged separately for the heavier and lighter labelled partner-nuclei, as was done in the experiments described above, after 16 h values of about 37 and 25, respectively, were obtained; after 24 h the values were 35 and 22 (see Fig. 4). We should be cautious, however, in the interpretation of the differences between the 2 values found for each of these 2 periods after fusion. On the one hand they might indicate a real difference in repair capacity between XP25RO and the XP20RO nuclei; in that case, in the XP20RO nucleus complementation is not yet complete, not even after 24 h. On the other hand, the differences may very well be statistical artefacts due to the manipulation: when repair is restored completely after 16 h, the two nuclei in a heterokaryon will have become undistinguishable with regard to UDS. In that situation, with our method, we randomly select pairs of numbers out of a single distribution of grain numbers, whereafter the greater and the smaller number of each pair is treated as belonging to 2 separate populations, which will also yield 2 different averaged values. Statistically, the latter procedure will result in a difference of $2 \times \pi^{-\frac{1}{2}} \times$ standard deviation of the total population of grain numbers (see addendum). This difference is 14.4 for the results at 16 h and 14.5 at 24 h, almost equal to the differences found (12 and 13, respectively). The average grain numbers of all nuclei of heterodikaryons in the cultures irradiated at 16 h or 24 h after fusion were 30.8 and 28.1, respectively. These are nearly the same as the values obtained for the nuclei regarded to be from XP25RO in the cultures that were irradiated at 2, 4 or 6 h after fusion (Fig. 4). It would be a plausible interpretation, therefore, that repair in XP20RO nuclei is restored slowly after fusion with XP25RO, reaching the normal level after about 16 h.

Repair in XP heterokaryons during 20 h after fusion

From the results presented so far it is apparent that the repair capacity of XP25RO is restored to normal immediately after fusion with normal or with XP20RO cells, while XP20RO nuclei need about 16 h to reach the normal level of repair after fusion with XP25RO. This difference between XP25RO and XP20RO cells in the rate of restoration of the repair capacity was expected to be measurable also when UDS was determined, cumulatively, over the whole period after fusion in which the repair of XP20RO nuclei reaches its normal level.

UV-irradiated XP25RO or XP20RO fibroblasts were fused with unirradiated normal fibroblasts and incubated in medium with (^{3}H)TdR (1 µCi/ml) for 20 h after the fusion. In the autoradiograms of these cultures dikaryons with one nucleus having a background labelling and the other a substantial labelling, were selected, because we assumed that these binucleated cells were heterokaryons derived from UV-irradiated XP and unirradiated normal cells. The

grains over a number of nuclei with the substantial labelling were counted. The resulting frequency distributions of grains are presented in Fig. 5. In the experiments with XP25RO the average number of grains over the XP nuclei amounted to 44.6, which was almost twice as high as the value found with XP20RO, 27.0. The difference between these values is in good agreement with the previous results, because the amount of repair performed by nuclei from XP20RO is expected to be about 65% of that performed by XP25RO nuclei, when it is assumed that the repair capacity of XP20RO increases linearly from residual to normal in 16 h after fusion with complementing cells (according to the line drawn in Fig. 4).

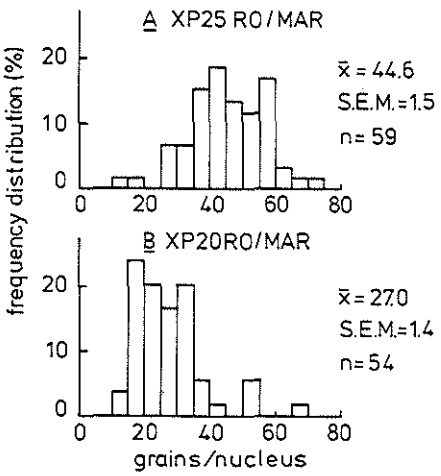

Fig. 5. Frequency distributions of grains counted over the UDS-performing nuclei in binucleated cells obtained by fusion of UV-irradiated XP cells with unirradiated normal human cells (MAR). Dikaryons were counted with only one labelled nucleus after a labelling period of 20 h starting immediately after fusion. A: UV-irradiated XP25RO; B: UV-irradiated XP20RO.

Analogous experiments were performed in which the normal cells were replaced by XP cells of a different complementation group. UV-irradiated XP25RO cells were fused with either unirradiated XP4RO or XP20RO cells, and irradiated XP20RO cells were fused with either unirradiated XP25RO or XP1BI cells. These cultures were labelled for 20 h and subsequently processed for autoradiography; results are given in Table V. The average numbers of grains over the labelled nuclei in dikaryons after the fusions using irradiated XP25RO were statistically not different from the value obtained after the fusion with normal cells. Similarly, the average grain numbers after the fusions using irradiated XP20RO coincided with the value obtained after the fusion with normal cells.

TABLE V
UNSCHEDULED DNA SYNTHESIS OVER 20 h AFTER FUSION IN UV-IRRADIATED NUCLEI IN BINUCLEATED HETEROKARYONS

UV-irradiated XP cells were fused with the indicated unirradiated cells and then UDS over the next 20 h was determined.

Unirradiated strain	UV-irradiated partner			
	XP25RO	XP1BI	XP4RO	XP20RO
XP25RO	-	-	32.5 ± 2.1[a]	27.3 ± 0.6
XP1BI	-	-	33.4 ± 1.1	27.7 ± 1.7
XP4RO	45.0 ± 2.2	37.8 ± 2.3	-	-
XP20RO	40.6 ± 3.5	32.0 ± 1.6	-	-
MAR	44.6 ± 1.5	-	-	27.0 ± 1.4

a) mean ± S.E.M.
The average background value ± S.E.M. is 3.8 ± 0.5.

In the same way irradiated XP1BI and XP4RO cells were examined for the amount of UDS performed during 20 h after fusion with unirradiated XP cells (Table V). The average of the number of grains over the labelled nuclei after fusion of irradiated XP1BI cells with either XP4RO or XP20RO was significantly lower than that obtained in the experiments with irradiated XP25RO cells, although these 2 strains, XP25RO and XP1BI, both belong to complementation group A. UV-irradiated XP4RO cells yielded results which were very similar to those of the experiments with irradiated XP1BI cells; consequently the values were not as low as the ones obtained with irradiated XP20RO cells, although XP4RO and XP20RO belong to the same complementation group C.

For each of the UV-irradiated XP strains the grains counted over all labelled nuclei were averaged, irrespective whether the unirradiated partner cells were from a complementing XP-group or were normal cells, and these averages were expressed as percentage of the normal control value (Table VI). When we assume a linear increase of UDS in irradiated XP nuclei as a function of time after fusion, until it reaches the normal level, and then remains constant, we obtain Fig. 6 with aid of Table VI. This was done by fitting the areas under the graphs to the ratios of UDS performed during 20 h after fusion relative to normal control cells. The graphs for XP25RO and XP20RO are similar to those in Fig. 4, which followed experimental points.

TABLE VI
UNSCHEDULED DNA SYNTHESIS OVER 20 h AFTER FUSION IN UV-IRRADIATED XP NUCLEI IN BINUCLEATED HETEROKARYONS IN COMPARISON TO IRRADIATED NORMAL CONTROL CELLS

UV-irradiated XP cells were fused either with normal cells or with XP cells of a complementing group. UDS in control cells (MAR) was measured without fusion.

Irradiated strain	Grains/nucleus	Ratio to normal cells[a]
XP25RO	44.2 ± 1.2[b]	1.03
XP1BI	34.4 ± 1.5	0.78
XP4RO	33.1 ± 1.0	0.74
XP20RO	27.2 ± 0.5	0.59
MAR	43.2 ± 1.2	1

a) After background correction.
b) mean ± S.E.M., not corrected for background.

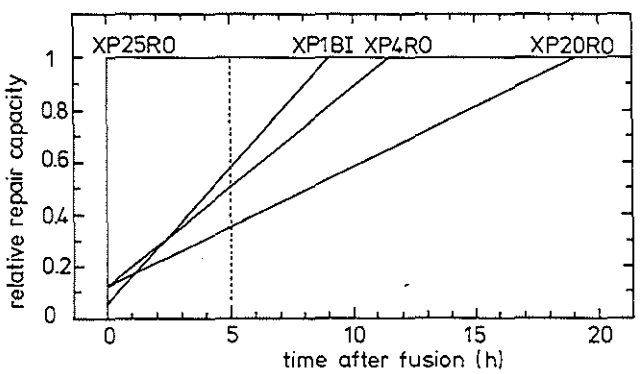

Fig. 6. Schematic presentation of the rate at which the capacity to perform UDS is restored in nuclei of XP25RO, XP1BI, XP4RO and XP20RO cells after fusion with normal cells or with XP cells from a different complementation group. The relative repair capacity is the ratio of the autoradiographically determined UDS level of an XP nucleus in a binucleated heterokaryon to the UDS measured in normal cells.

In Fig. 6 we see that XP25RO reaches the normal level immediately after fusion and that XP20RO needs about 19 h to reach this level. The periods for XP1BI and XP4RO were 9 and 11 h, respectively. Fig. 6 also shows that the UDS in UV-irradiated XP25RO, XP1BI, XP4RO and XP20RO nuclei performed between 4 and 6 h after fusion with normal

cells should be about 100, 59, 51 and 34%, respectively, of the UDS in normal cells (see dotted line in Fig. 6). These predictions can be compared with the results of Fig. 2 which refer to the situation 4-6 h after fusion and which, therefore, should yield the same values. In the experiment with XP25RO (Fig. 1A) the average grain number of the cells corresponding to regions II and III, 43, was about the same as that of the control cells, 39, which means that the complementation was 100%. When we assume that the fraction of heterokaryons of the dikaryons that resulted in points in the regions II and III of Fig. 2A-D is 2/3, then the fraction of XP nuclei of all nuclei in these dikaryons should be 1/3. Now the average grain number of these XP nuclei can be calculated from the average grain numbers of the cells in the regions II and III, which should be the weighted average of the grain numbers of the XP nuclei and those of the nuclei of the normal cells fused with themselves. When these values were expressed as percentages of the normal grain numbers, the results for XP1BI, XP4RO and XP20RO were 58, 46 and 28%, respectively. Apparently, the model depicted in Fig. 6 fits very well with our experiments. From the results shown in Fig. 4, a mechanism of complementation resulting in a fairly steep, possibly sigmoid increase in repair capacity after a certain period could not be excluded. In view of the good agreement between the percentages calculated from the experiments of Fig. 2 and the theoretical values taken from Fig. 6, such an explanation appears to be unlikely. A more asymptotic approach of the normal value than that shown in Fig. 6 remains well possible, however.

2.3.4 Discussion

The 2 XP strains XP25RO and XP20RO, of complementation group A and C, respectively, differ greatly in the rate at which their repair capacity is restored after fusion with normal cells or with complementing XP cells. Also Giannelli and Pawsey (1974) observed rapidly and slowly complementing varieties of XP. The nuclei of one of their XP strains reached the normal level of repair within 6 h after fusion, whereas the nuclei of another strain remained at a low level during this period. XP25RO could be of the first type and XP20RO of the second. These 2 strains belong to different complementation groups, and it is of interest to know whether the rate of complementation, i.e. the rate at which the repair capacity in XP nuclei is restored after fusion, is a specific property of a complementation group. Our original results obtained with two different XP strains of group A and two belonging to group C at 4 h after fusion with normal cells, did to some extent support such a conception. But an analysis of the UDS performed over a period of 20 h after fusion by these 4 strains fused with various complementing partner-cells revealed considerable differences between the 2 strains of the same complementation group in the rate at which the repair defect was complemented. For XP25RO the repair capacity was restored

within 2 h, whereas the other strain of group A, XP1BI needed about 9 h. In this respect XP1BI was rather similar to strain XP4RO, belonging to group C, which reached normal repair some 11 h after fusion, substantially faster than the other strain of this group, XP20RO (16-20 h). It is evident from these results that the rate at which the repair capacity of XP nuclei is restored after fusion with complementing cells can vary from strain to strain also within the same complementation group and thus is not a specific property characterizing a group.

Presumably the nature of the malfunctional product in a specific XP strain, which we call "XP factor", determines the rate of complementation. A fast recovery of repair is expected when this factor is completely absent in a specific XP strain and when it is abundantly present in the cytoplasm of the complementing cell. The same applies when an XP strain has a malfunctional XP factor that has a relatively low affinity for its substrate or is loosely associated with the (inactive) complex of which it forms a part, then it can be rapidly replaced by the normal product. A slow complementation will occur when the defective XP factor has a high affinity for its substrate, so there is competition between the normal product and the XP factor. A slow rate of complementation is also foreseen when the XP factor is tightly bound in a multimeric structure. Then the normal multimer must be reconstituted, and this might need time. So, even if we assume that the same gene product is defective in XP strains belonging to the same complementation group, different rates of complementation are not totally unexpected.

The rate of complementation can also be determined by the availability of the normal product. Giannelli and Pawsey (1976) observed that complementation in a strain with a slow rate could be blocked by inhibition of protein synthesis, whereas this was ineffective with a strain showing a fast rate of complementation. The authors assume that the normal product that should replace the defective factor in this strain is irreversibly attached to an acceptor in the normal nucleus and cannot return to the cytoplasm, so complementation can only occur when the normal product is translated from its mRNA in the cytoplasm. According to such a mechanism, a low rate of complementation would be a characteristic property of at least one complementation group. Our results indicate that group A does not fall in this category; the factor in this group must be of the well available, rapidly diffusing type.

2.3.5 Addendum

Calculation of the average difference between 2 samples taken from one single normal distribution

When the difference in repair replication between 2 nuclei of a binucleated heterokaryon is only due to statistical variation, our

method of attributing the higher value to the nucleus of the repair-proficient cell and the lower one to the nucleus being restored in its repair capacity will correspond with taking 2 samples at a time from one single population and then putting together all high and all low values to be treated as 2 different populations. The expected outcome of such an exercise can be calculated as follows.

The expectation or mean of a stochastic variable x is defined as:

$$Ex = \int_{-\infty}^{\infty} x.f(x)dx$$

When x has a normal distribution with mean m and standard deviation σ, f(x) is defined as:

$$f(x) = \exp(-(x-m)^2/(2.\sigma^2))/(\sigma.(2\pi)^{\frac{1}{2}})$$

The expectation of the difference between the pairs $(x_{1,i}, x_{2,i})$ of paired samples from a normal distribution is again normally distributed: when $z = x_1 - x_2$, then the mean value of z will be 0 and $\sigma_z = \sigma_x.\sqrt{2}$. The expectation of the absolute value of z can be calculated as follows:

$$E(|z|) = \int_{-\infty}^{\infty} |z|.(\exp(-(z/2\sigma_x)^2)/(2\sigma_x.\pi^{\frac{1}{2}}) = 2.\pi^{-\frac{1}{2}}.\sigma_x.$$

Thus when pairs of samples are randomly taken from a normal distribution with standard deviation σ, the mean value of the absolute differences of these pairs will be $2.\pi^{-\frac{1}{2}}.\sigma$.

CHAPTER 3

BIOCHEMICAL ANALYSIS OF DEFECTIVE DNA REPAIR IN XERODERMA PIGMENTOSUM

3.1 Introduction

The genetic heterogeneity of XP, reflected by the identification of seven complementation groups, indicates that different mutations give rise to a repair-deficient phenotype. The repair defect is usually demonstrated by a reduced level of UV-induced unscheduled DNA synthesis (UDS). The disadvantage of this technique is that it does not give information on the nature of the lesion(s) whose excision results in UDS.

The predominant UV-lesion is the pyrimidine dimer. By following its removal from the DNA of UV-irradiated cells, either by autoradiographic analysis of chromatograms (Setlow et al., 1969) or by the use of specific UV-endonucleases (UV-endo assay) (Paterson et al., 1973), it was shown that the reduction of repair synthesis in XP cells of complementation groups A and C was caused by a reduced excision of pyrimidine dimers. In chapter 3.2 and paper II of the appendix a comparative study using the UV-endo assay, of all complementation groups is presented. These experiments suggest that excision-deficient XP cells lack a functional dimer-specific UV-endonuclease, which is needed to make an incision in the lesion-containing region of the DNA. An alternative explanation could be a defect in one of the enzymes that act in one of the subsequent steps of excision repair (excision, repair synthesis and ligation), when these enzymes together will form an enzyme complex in which the enzymes function in a co-ordinated way. A defect in one of the enzymes could then block the action of the complex at the first lesion, which will be manifested as a deficiency in the first step of the repair pathway.

Evidence that the defect is at the level of the incision step of the repair process was presented by Tanaka and coworkers (1975). They permeabilized XP strains from complementation groups A, B, C, D and E with UV-inactivated sendai virus, and exposed the cells to a dimer-specific incision enzyme from bacteriophage T4 (T4-endonuclease V). It was shown that these cells restored the level of UV-induced UDS to normal values. Consequently, T4-endonuclease V is active on chromatin and once incision is made the subsequent steps can be performed. In extracts of human fibroblasts and Hela cells an endonuclease activity is present that preferentially incises UV-irradiated DNA (Bacchetti et al., 1972; Duker and Teebor, 1977). It was shown, however, that this activity is present to a similar degree in XP cells. Evidence for the possibility that XP cells are defective in products that facilitate the endonucleolytic step have been presented by Mortelmans et al. (1976). They demonstrated excision of dimers from isolated UV-irradiated, bacterial DNA by sonicates of XP

cells from groups A, C and D. Cell free sonicates made of XP cells from complementation group A were unable to excise dimers from their own chromatin. This was shown to reflect a deficiency in enzyme activity rather than an abnormality of the XP DNA, as sonicates of normal human cells could excise dimers both from isolated bacterial DNA and chromatin from their own and from XP group A cells. Interpretation of these observations is complicated by the results of subsequent experiments with the same system on XP group D cells and on XP variants (Friedberg et al., 1976). Sonicates of XP group A cells were defective in the excision of dimers from their own chromatin, whereas sonicates of group D cells were able to perform excision on their own chromatin, and variants were unable to do so. The conflicting results of these *in vitro* experiments have not yet been explained satisfactory.

As stated in the former chapter (section 2.2.1), XP cells are not only impaired in the repair of UV-induced DNA lesions, but also in the repair of DNA damage induced by a variety of other agents. This deficiency in the repair of a large number of different lesions indicates that the defect in XP is probably not in enzymes that recognize specific damages, but involves more general functions, such as recognition of structural deviations of the superhelical structure of the DNA molecule.

3.2 Removal of UV-endonuclease-susceptible sites in the seven complementation groups of xeroderma pigmentosum A through G

Paterson et al. (1973) have studied the removal of sites, susceptible to a bacterial UV-endonuclease in excision repair-deficient XP strains belonging to complementation groups A and C. These strains appeared to be defective in the removal of these sites from their DNA. The question arose whether the defect in each complementation group results in a defective removal of UV-endonuclease-sensitive sites. In this section experiments are discussed on XP strains of each of the 7 existing complementation groups. Details of these experiments are presented in paper II of the appendix.

The disappearance of pyrimidine dimers from the DNA of UV-irradiated cells can directly be measured by application of the radiochromatographic assay developed by Carrier and Setlow (1971). To this end cells are preincubated in the presence of (^{3}H)TdR, subsequently UV-irradiated and then allowed to repair their DNA during a post-irradiation incubation period. The DNA is isolated and hydrolyzed to obtain the free bases and the irradiation products, including the pyrimidine dimers. The hydrolysate is chromatographed in 2 dimensions to separate the monomeric thymine bases from the thymine-containing photoproducts. The percentage of the total radioactivity present as photoproducts indicates the number of dimers that was present in the DNA just before hydrolyzation. A reduction of

this percentage as function of post-irradiation incubation time is indicative of the *in vivo* removal of the lesions. A drawback of this assay is the use of high doses of UV to obtain dimers in a detectable amount.

Reliable determinations of the number of dimers per unit length of DNA in cells irradiated with low doses of UV has been enabled by the development of enzymatic techniques. Evidence for the presence in the bacterium *Micrococcus luteus* of enzymes that produce incisions in the DNA exposed to UV was obtained by Strauss (1962) and Rörsch and coworkers (1964). Using an extract from *M. luteus* the disappearance of dimers from the DNA of UV-irradiated *Bacillus subtilis* cells (Strauss et al., 1966) and human cells (Paterson et al., 1973) could be measured. The outline of this procedure is shown in Fig. 1. Diploid fibroblasts whose DNA is labelled with (^{3}H)TdR are irradiated with UV. The DNA is isolated, purified and duplicate samples are incubated, one with and the other without an extract of *M. luteus*, containing UV-endonucleases. The 2 samples are sedimented separately in alkaline sucrose gradients to measure the weight-average molecular weight. These values are used to calculate the number of UV-endonuclease-induced single-strand breaks, which equals the number of pyrimidine dimers (Setlow et al., 1975; see also: chapter 4).

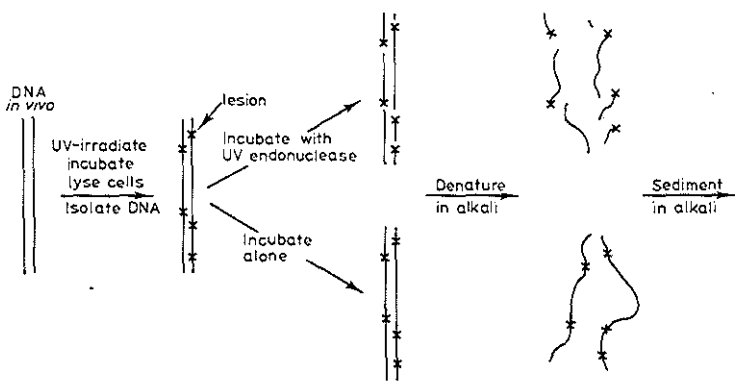

Fig. 1. Scheme of the use of UV-endonuclease from *Micrococcus luteus* to measure the number of pyrimidine dimers in (^{3}H)-labelled DNA of mammalian cells irradiated *in vivo*.

This technique has been used to study the disappearance of dimers from strains from all known XP complementation groups and a XP variant

cell strain. Their repair capacity was compared with normal human cells (Zelle and Lohman, 1979: paper II of the appendix). Excision-deficient XP cell strains of all complementation groups appeared to have a reduced capacity to remove dimers from their DNA after a relative low dose of UV (2.9 $J.m^{-2}$). The biochemical defect in the excision repair pathway in all XP complementation groups appears to be located in the steps preceding incision of the lesions or in the incision step itself. In general, this reduced capacity was in agreement with another repair parameter, the reduction in repair DNA synthesis measured autoradiographically. XP23OS showed to be an exception and more dimers were removed from its DNA than was expected based on its low level of repair DNA synthesis (Zelle and Lohman, 1979: paper II of the appendix). This cell strain will be discussed in more detail in the next section.

3.3 Repair of ultraviolet radiation damage in xeroderma pigmentosum cell strains of groups E and F[a]

3.3.1 Introduction

The repair capacity of XP strains, relative to normal cells, can be estimated in various ways. Usually the different methods agree rather well in the residual level of repair found, which generally is in fair agreement with related properties such as UV sensitivity. With 2 strains, however, viz. XP2RO and XP23OS, distinct discrepancies have been observed in this respect. The nature of these discrepancies is the subject of the investigations discussed in this section.

XP2RO is a primary fibroblast strain, the only representative of complementation group E (De Weerd-Kastelein et al., 1974; Kraemer et al., 1975). With regard to survival after UV-irradiation this strain is the most resistant among the analyzed XP strains; it is only slightly more sensitive than normal human control cells (Lehmann et al., 1977; Andrews et al., 1978). As for the induction of sister-chromatid exchanges by UV this strain is indistinguishable from normal human control cells (De Weerd-Kastelein et al., 1977). The repair DNA synthesis induced by UV in XP2RO, however, reached only 50% of the level observed in normal human cells. This is true for both unscheduled DNA synthesis measured by autoradiography and repair replication determined by isopycnic centrifugation of DNA (Kleijer et al., 1973).

The level of unscheduled DNA synthesis is determined by the rate of excision and the patch size. Decreased levels of repair synthesis are usually attributed to reduced numbers of lesions that are removed

a) This paper by B. Zelle and P.H.M. Lohman is submitted for publication in Mutation Research.

from the DNA. When this applies to XP2RO, this strain, in comparison with normal cells, would remove only half the number of damages in the same period of time. Alternatively, a reduced repair synthesis may mean that not the number of lesions that are repaired is decreased, but that the repair process has changed in such a manner that the removal of one lesion involves less DNA synthesis, i.e. shorter repair patches are formed. The latter mechanism might explain the discrepancy between the (apparently) reduced repair capacity of XP2RO and the relatively high survival level after UV exposure. To test whether the second explanation might apply we compared UV-irradiated XP2RO cells and normal human cells with regard to the number of repair sites and to the size of the repair patches. The number of sites was obtained by using the UV-endo assay. A simultaneous determination of the number of repair sites and their size was obtained by the BUdR-photolysis technique (Regan et al., 1971; Regan and Setlow, 1974).

XP230S is a primary fibroblast strain classified into group F (Arase et al., 1979). This strain is peculiar in so far that it combines a low level of unscheduled DNA synthesis (UDS), i.e. about 10% of the normal value, with an intermediate repair capacity as judged from survival after UV and from host-cell reactivation of UV-irradiated herpes simplex virus (Arase et al., 1979). As we have reported recently (Zelle and Lohman, 1979: paper II of the appendix) also the ability of this strain to remove pyrimidine dimers from its DNA is not strongly reduced (80% of normal human cells, 24 h after an UV dose of 2.9 $J.m^{-2}$). To investigate the nature of these differences we studied the kinetics of UDS in XP230S cells over the first 24 h after UV-irradiation and compared this with the results from the assay measuring the removal of UV-endonuclease-susceptible sites.

3.3.2 Materials and methods

Cell strains and tissue culture

Normal human primary fibroblast strains AH and HAN were derived from 2 healthy human volunteers. The primary fibroblast strains XP2RO and XP230S were derived from patients with xeroderma pigmentosum, and were classified as complementation groups E and F, respectively (De Weerd-Kastelein et al., 1974; Kraemer et al., 1975; Arase et al., 1979). Culture conditions were as described in chapter 2, section 2.2.2.

UV-irradiation

Irradiation was performed as described in the former chapter, section 2.2.2.

Unscheduled DNA synthesis

The cells were seeded in small tissue culture dishes (Greiner, diameter: 3.5 cm) containing a sterile glass coverslip (18x18 mm). After 2 days of incubation the cultures were labelled with (^{3}H)TdR (10 µCi/ml; 19 Ci/mmol) for 1 h before UV-irradiation, to label cells in S-phase, and for 2 h after irradiation. Subsequently the cells were washed twice with PBS, fixed with Bouins fixative, washed with water and dried. The coverslips were mounted on microscope slides and covered with photographic emulsion by dipping into Ilford K2 nuclear research emulsion (diluted at 1:1 (v/v) with distilled water) at 45^{0}C. The preparations were stored in the dark for 4 days at 4^{0}C and then developed, fixed, washed and stained with either hematoxylin and eosin (XP2RO experiments) or May-Grünwalds modified solution and Giemsa's solution (XP23OS experiments). In the resulting autoradiograms the grains over the nuclei of a number of non-S-phase cells were counted. These cells could be distinguished because typical S-phase cells had too many grains over the nucleus to be counted.

In the studies of UDS-kinetics the cells were incubated in medium containing (^{3}H)TdR for a 2 h period, starting at different times after UV-irradiation. No prelabelling for S-phase discrimination could be used, as this would be senseless in experiments with a time lag between irradiation and the onset of the incubation in the radioactive medium. In order to suppress the fraction of cells in S-phase the medium was replaced on the day following inocculation of the cells by medium containing only 1% foetal calf serum. Irradiation was done after 2 days of incubation in this medium. Moreover, 1 h prior to the determination of UDS the cells were incubated in medium with 4 mM hydroxyurea (HU) and the same concentration of HU was also present during incubation with (^{3}H)TdR.

Excision of UV-endonuclease-susceptible sites

The procedure used to determine the number of sites, susceptible to UV-endonuclease of *M. luteus*, was as developed by Paterson et al. (1973) but modified in order to increase the sensitivity by isolating DNA with a high molecular weight as was described in detail recently (Zelle and Lohman, 1979: paper II of the appendix).

BUdR-photolysis technique

The procedure used was essentially the one developed by Regan and Setlow (Regan et al., 1971; Regan and Setlow, 1974). Per cell strain 2 tissue culture dishes (Greiner, diameter: 9 cm) were seeded with approx. 5x10^5 cells each. After 2 days of incubation the cells were labelled for 24-48 h with either (^{3}H)TdR (1 µCi/ml; 19 Ci/mmol) or (^{14}C)TdR (1 µCi/ml; 61 mCi/mmol). About 4 h before irradiation the radioactive medium was replaced by normal medium and 30 min prior to irradiation the (^{3}H)-labelled cultures were incubated in medium

supplemented with 3% foetal calf serum, 0.1 mM BUdR and 2 mM HU, whereas the (^{14}C)-labelled cultures were incubated in medium containing 3% foetal calf serum, 0.1 mM TdR and 2 mM HU. After irradiation with 254 nm UV at a dose of 2.9 J.m^{-2} the cultures were incubated for 20 h in the same medium as during the 30 min prior to irradiation. Subsequently the cells were washed, trypsinized, washed with PBS and counted; 2.5x10^5 (^{3}H)TdR and BUdR labelled cells were suspended with the same number of (^{14}C)TdR labelled cells in 2.5 ml of PBS. A volume of 2.1 ml of this suspension was brought into a glass cuvette to be irradiated with 313 nm UV. The light source was a 1000 W XeHg lamp (Hannovia), operated at 950 W. Power supply, lamp housing, collimator, filter and monochromator were supplied by Oriel (USA). The light is collimated on the entrance slit of a grating-monochromator after passage through a cooled water filter to absorb infrared. The entrance and exit slits have a width of 3.2 mm resulting in a half-maximum bandwidth of 20 nm. The light leaving the monochromator passes a filter made of the lid of a 6 cm Greiner tissue culture dish, which absorbs the radiation with a wavelength below 290 nm, and a shutter and finally reaches the cuvette, placed in a water-cooled cuvette holder, with a dose-rate of about 45 W.m^{-2}. The dose-rate was determined in each experiment by the actinometrical procedure of Parker and Hatchard (Calvert and Pitts, 1966); the actinometrical solution was illuminated in the same cuvette as used for the irradiation of the cells.

At different times during irradiation of the cells, the light path was shut off, the cells were resuspended with a pasteur pipette and a 50 µl sample, containing 10^4 cells, was taken and irradiation continued. The first sample was taken at the onset of the irradiation. Each sample was sandwiched between 2 layers of 75 µl of lysis solution of 1 M NaOH and 0.01 M EDTA on top of a high-salt alkaline sucrose gradient (5-20% w/v, 2 M NaCl, 0.01 M EDTA and 0.33 M NaOH). After a lysis and denaturation period of 60 min the tubes with the gradients were centrifuged at 30 000 rpm in a Spinco SW60Ti rotor for 150 min at 20^{0}C. Subsequently the size distribution of the DNA was determined in the same manner as in the UV-endonuclease experiments.

When the repaired regions are distributed over the DNA in a random fashion and when also their sizes have a normal distribution, with a BUdR content proportional to the length of the repaired gap, the number of measurable single strand breaks in the DNA induced by irradiation with 313 nm UV will be described by the following equation (Regan and Setlow, 1974):

$$Bss = N.(1-\exp(-n.\sigma.F)),$$

where Bss is the number of measurable single strand breaks per 10^6 mol. weight of DNA, i.e. the number of repair-patches containing at least 1 break due to the 313 nm UV irradiation (the small fragments resulting from multiple hits within one repair-patch are not scored). N is the average number of repaired sites per 10^6 mol. weight of DNA, n the average number of BUdR residues incorporated per repair region, σ the BUdR cross-section for 313 nm break induction in m^2.J^{-1} and F

the dose of 313 nm radiation in $J.m^{-2}$. In an experiment with cells in which the thymidine nucleotides of the (^3H)-labelled DNA were fully substituted by BUdR, it was determined that with our irradiation conditions the value for σ is 0.505×10^{-6} $m^2.J^{-1}$; the assumption was made that the percentage of thymidine nucleotides in the DNA of mammalian cells is 30%.

3.3.3 Results

XP2RO CELLS (E group)

Unscheduled DNA synthesis

XP2RO cells were compared with two normal human fibroblast strains AH and HAN. One hour before and 2 h after irradiation with 254 nm UV at a dose of 5 $J.m^{-2}$ the cells were cultured in a medium with (^3H)TdR. Subsequently the extent of unscheduled DNA synthesis was determined by autoradiography for which the grains over 40 non-S-phase nuclei were counted (Table I). According to this criterium the XP2RO cells only show half the repair activity of normal cells.

TABLE I
UNSCHEDULED DNA SYNTHESIS IN HUMAN PRIMARY FIBROBLASTS DURING 2 h AFTER IRRADIATION WITH 254 nm UV (5 $J.m^{-2}$)

Strain	Grain counts per nucleus (mean ± S.E.M.; n=40)	
	unirradiated cells	irradiated cells
AH	8.8 ± 0.7	94.0 ± 3.5
HAN	7.2 ± 0.4	96.0 ± 2.0
XP2RO	10.5 ± 1.1	54.0 ± 1.9

Removal of UV-endonuclease-susceptible sites

Treatment of UV-irradiated DNA with a crude extract of *M. luteus* allows the determination of the number of UV-endonuclease-sensitive sites introduced in the DNA by the radiation. On application of this assay to XP2RO, HAN and AH cells an identical linear relationship between the number of sites and the UV dose was observed (doses 1.2, 2.9, 11.6 and 23.2 $J.m^{-2}$; see Fig. 2). The number of sites induced per $J.m^{-2}$ of 254 nm UV radiation is 0.04 per 10^6 mol. weight of DNA. The removal of UV-endonuclease-sensitive sites was measured by determination of the remaining sites during a post-irradiation incubation period, at intervals of 8 h. Three different doses were used and the removal was followed over a period of 32 h (Fig. 3). At all doses less sites were removed in XP2RO than in the normal human

cell strains. After the lowest UV dose (2.9 J.m^{-2}) the reduction in the amount of repaired sites sensitive to UV-endonuclease, in comparison with normal cells, was 63% (Fig. 3A). At higher doses the removal of UV-endonuclease-sensitive sites in XP2RO is even more reduced, compared to normal human fibroblasts (Fig. 3B and 3C).

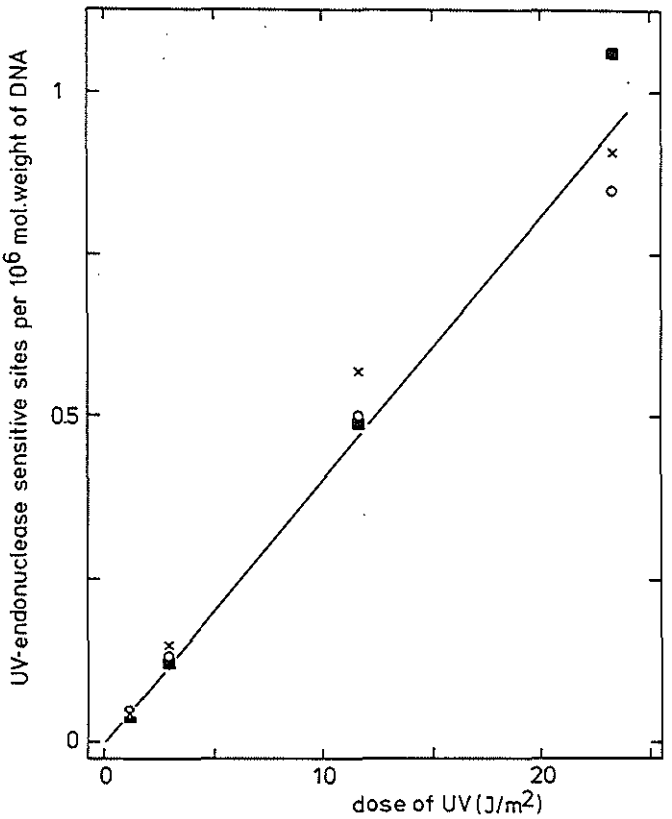

Fig. 2. The induction of UV-endonuclease-susceptible sites in the DNA of primary human fibroblasts by irradiation with 254 nm UV.
○: XP2RO; ×: AH; ■: HAN.

The size and the amount of repair gaps

The number and size of the repair gaps were determined simultaneously by the BUdR-photolysis method. To this end XP2RO and AH cells irradiated with 2.9 J.m^{-2} of 254 nm UV were incubated for 20 h in a medium containing BUdR. Then the cells were irradiated with various doses of 313 nm UV for the induction of breaks in the repair patches and the number of breaks was determined. The results of 2 independent experiments are shown in Fig. 4. To the combined data

for each strain a curve was fitted by means of a least squares method on the basis of the theoretical relationship described in Materials and methods (section 3.3.2). The parameters of this curve yielded the most probable values for N and n, the number and BUdR-content of the repair sites, respectively. The latter value was converted into the size of the repair sites by division through the assumed TdR content (30%) of normal mammalian DNA. These values are given in Table II, together with the values for the numbers of repaired UV-endonuclease-susceptible sites after an identical 254 nm UV dose and repair period, which were taken from experiments shown in Fig. 3A. These results clearly indicate that there is a substantial reduction in the number of sites repaired in the XP2RO fibroblasts, which is about the same for the 2 methods, whereas the size of the repair regions is not markedly different.

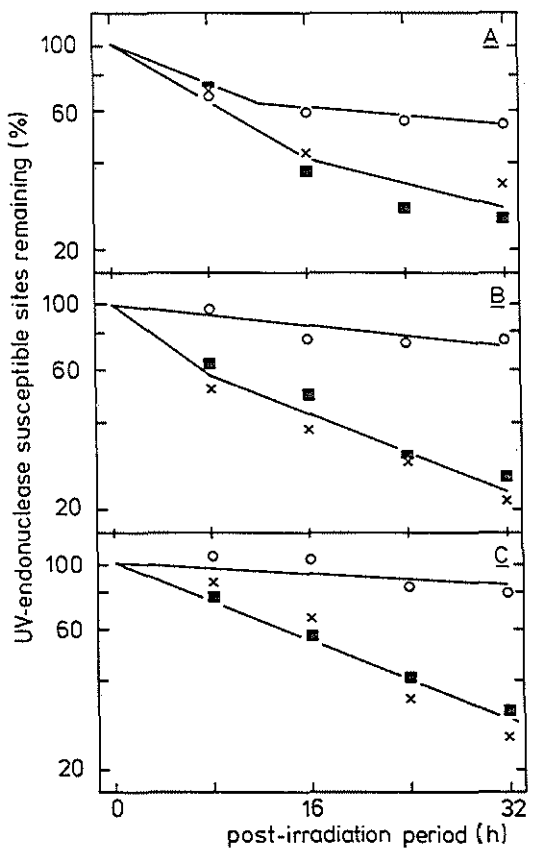

Fig. 3. Disappearance of UV-endonuclease-susceptible sites from the DNA of primary human fibroblasts as a function of time after irradiation with different doses of 254 nm UV. Panels A,B and C: 2.9, 11.6 and 23.2 $J.m^{-2}$, respectively.
o: XP2RO; x: AH; ■: HAN.

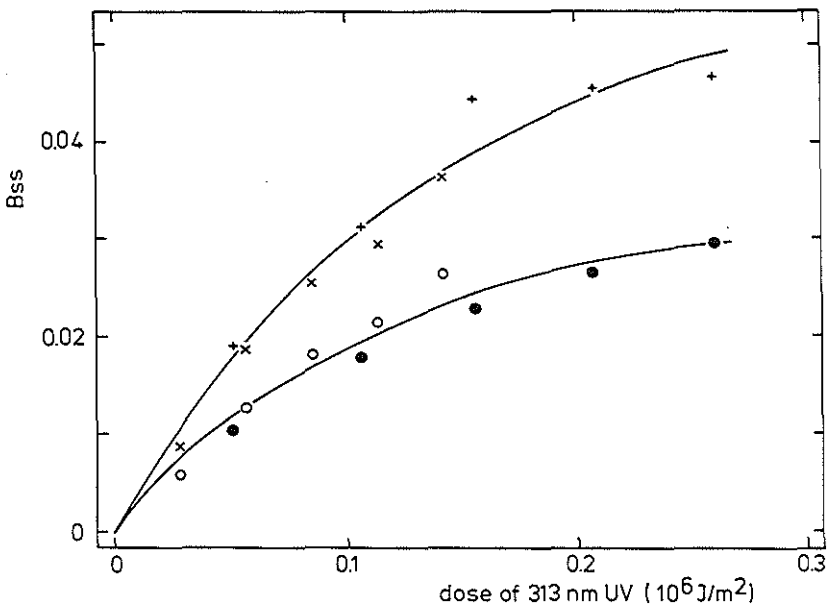

Fig. 4. The induction of breaks by irradiation with 313 nm UV in DNA with BUdR-containing regions. Primary human fibroblasts were irradiated with 254 nm UV (2.9 J.m^{-2}) and allowed a 20 h repair period in a medium with BUdR. Subsequently the cells were irradiated with various doses of 313 nm UV which induces breaks in the BUdR-containing regions. Bss: number of repair-sites with one or more single-strand breaks per 10^6 mol. weight of DNA. See text for further details.
○,●: XP2RO; ×,+: AH.

TABLE II
THE NUMBER AND SIZE OF REPAIRED SITES IN THE DNA OF HUMAN PRIMARY FIBROBLASTS AFTER IRRADIATION WITH 254 nm UV (2.9 J.m^{-2}) FOLLOWED BY A 20 h REPAIR PERIOD

Two methods were used to study the removal of lesions from the DNA of UV-irradiated cells. The BUdR-photolysis method yields both the number of all repaired sites and their patch size, whereas the UV-endo method measures the removal of one specific lesion, the pyrimidine dimer.

Strain	BUdR-photolysis method		UV-endo method
	Number of nucleotides per repair region	Number of repaired sites per 10^6 m.w.	Number of repaired sites per 10^6 m.w.
AH	48	0.058	0.063
XP2RO	56	0.033	0.040

XP230S cells (F group)

Unscheduled DNA synthesis

Unscheduled DNA synthesis was determined in XP230S cells and normal human fibroblasts of strain AH after irradiation with doses of 254 nm UV ranging from 1.0 to 5.1 J.m^{-2}, over a repair period of 2 h (Fig. 5). In AH cells the number of grains per nucleus increased with dose, while in XP230S cells a rather constant number of grains over the nuclei was observed in the different preparations. As a consequence, the relative repair capacity of XP230S is dose dependent and decreases from about 20% at 1 J.m^{-2} to approx. 8% after 5.1 J.m^{-2}. This level of residual repair synthesis is in good agreement with the results of Arase et al. (1979).

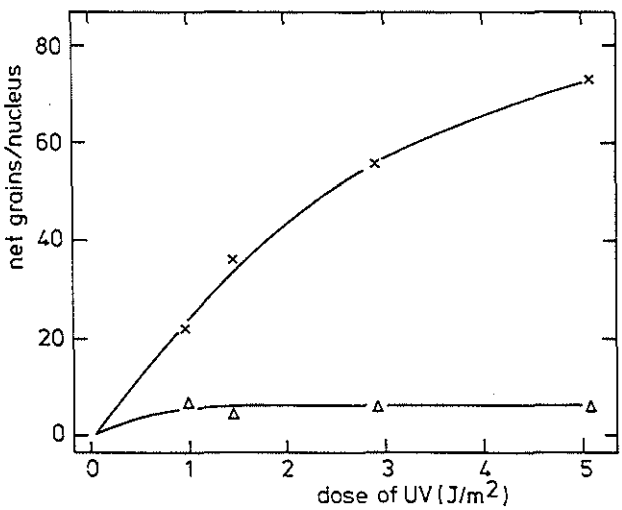

Fig. 5. Unscheduled DNA synthesis, during the first 2 h after UV-irradiation, determined by autoradiography, as function of 254 nm UV dose and corrected for the number of grains over the nuclei of unirradiated cells.
Δ: XP230S, background level: 1.40 ± 0.13 (SEM); ×: AH, background level: 1.76 ± 0.18 (SEM). n=50.

Kinetics of unscheduled DNA synthesis

The low level of UDS in XP230S appeared to conflict with the rather high capacity to remove pyrimidine dimers (Zelle and Lohman, 1979: paper II of the appendix). The two results were obtained under different conditions, however, since UDS was measured over the first

few hours after irradiation, whereas a reliable determination of the removal of UV-endonuclease-sensitive sites requires a repair period of at least 8 h. For this reason the duration of the repair period in the UDS studies was extended to include the period suitable for measurements of dimer removal, and the kinetics of the two processes were compared. Because the reduction in UDS was most pronounced after the high UV doses (see Fig. 5), a dose of 5.1 $J.m^{-2}$ was used for the UDS studies, which were done with XP230S and AH cells. UDS was measured over consecutive 2 h periods for the first 24 h after irradiation. The results are presented in Fig. 6. Normal AH cells showed a rapid repair synthesis during the first few hours after irradiation, but after about 8 h a slower rate was observed. The XP230S cells, on the other hand, started with a low level of UDS immediately after irradiation, and the rate remained constant. Consequently the relative UDS level of XP230S cells increased as function of the post-irradiation incubation period, being 12.5% over the first 2 h, 24% over the first 8 h and 45% over the whole 24 h period. The rate of UDS in AH cells became constant after about 8 h

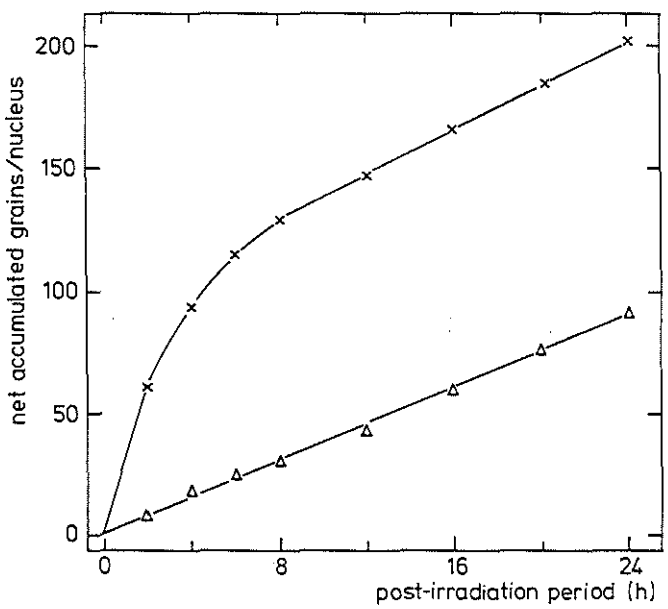

Fig. 6. Kinetics of unscheduled DNA synthesis. Repair synthesis is measured over consecutive 2 h periods following irradiation with a 254 nm UV dose of 5.1 $J.m^{-2}$. Each point represents the accumulated number of grains per nucleus, corrected for the grains over the nuclei of unirradiated cells. The increases over the intervals 8-10, 12-14, 16-18 and 20-22 h were interpolated from the results found for the preceding and following 2 h period.
Δ: XP230S, background level: 3.06 ± 0.22 (SEM); ×: AH, background level: 1.40 ± 0.17 (SEM). n=50.

and thereafter was almost equal to that in XP230S. In AH cells after 4.8 h UDS had reached half of the value observed over the 24 h period, which is in good agreement with the value of 4.5 h published by Ehmann et al. (1978) for cultured human cells.

Comparison of UDS with the removal of UV-endonuclease-susceptible sites

The results of the kinetic study on UDS suggested that repair replication in XP230S might show a close correspondence with the removal of UV-endonuclease-susceptible sites than was originally thought, provided the two phenomena are studied after the same irradiation dose and over an identical repair period. This was checked by a determination of the removal of UV-endonuclease-susceptible sites from the DNA in XP230S and in AH cells which were treated identically to the cells used for the UDS study. The UV dose was 5.1 $J.m^{-2}$, inducing 0.14 sites per 10^6 mol. weight of DNA in both strains, and the number of sites not removed was determined after 12 and 24 h. The results (Fig. 7) showed a distinct difference between the two strains, with a relative level of repair in XP230S of about 28% after 12 h and 44% after 24 h.

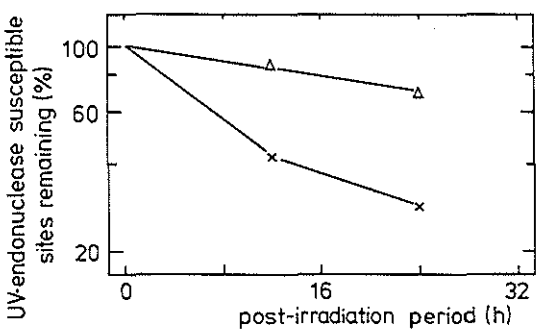

Fig. 7. Disappearance of UV-endonuclease-susceptible sites from the DNA of primary human fibroblasts as a function of the time after irradiation with a dose of 254 nm UV of 5.1 $J.m^{-2}$.
Δ: XP230S; ×: AH.

3.3.4 Discussion

The results obtained by the determination of the disappearance of sites susceptible to UV-endonuclease from *M. luteus*, indicate that the repair defect in XP2RO cells leads to a reduction of the fraction of the sites that are repaired per unit time in comparison with normal

human cells. The BUdR-photolysis technique yields simultaneously the total number of repair regions and their average size. The difference between the two methods is that the former specifically determines the number of repaired pyrimidine dimers (Patrick and Harm, 1973), whereas the latter shows all sites that are removed by excision repair. The fact that the two methods show a good correspondance in the measured number of repair sites, indicates that the predominant UV-induced lesion removed by excision repair is the pyrimidine dimer, both in normal human and in XP2RO cells. Both methods demonstrate once more the reduced capacity of XP2RO cells to repair this lesion. Furthermore, the BUdR-photolysis technique shows that the size of the repaired regions in XP2RO is not different from that in normal cells. These two sets of results together disprove the hypothesis that the apparent discrepancy between a clearly reduced capacity for repair replication and an only slightly increased UV-sensitivity could be due to a smaller size of the repaired patches.

The relation between the residual repair level of UV-endonuclease-sensitive sites and the level of repair replication in XP2RO cells is not clear. Our results on the reduction in site removal in this strain after a low dose of UV (Table II) agree well with the residual level of UDS after about the same UV dose found by us (Table I) and by Kleijer et al. (1973). After higher UV doses (12 and 23 $J.m^{-2}$) the repair of UV-endonuclease-susceptible sites in XP2RO is more reduced in comparison with normal human cells than after the low dose of 2.9 $J.m^{-2}$. This is in contrast with the observations made by Kleijer et al. (1973) on UDS after doses ranging from 10 to 100 $J.m^{-2}$, who found that the reduction in repair activity became smaller at higher doses. The experiments are not strictly comparable, however, since these measurements were carried out over a rather short period of 3 h following UV-irradiation, whereas the removal of UV-endonuclease-susceptible sites was determined after periods of at least 8 h. This difference may be very relevant, as is shown by our UDS experiments with XP230S. More experiments will be needed to clarify the repair properties of XP2RO and their relation to other phenomena such as the only slightly affected UV-survival and the induction of sister-chromatid exchanges.

The low level of UDS in XP230S as observed by Arase et al. (1979) was confirmed by our experiments. A certain dose-dependency was seen which could not be determined very accurately, however, due to the small numbers of grains in the XP strain. The residual UDS activity dropped from about 20% after 1 $J.m^{-2}$ to about 10% after 5 $J.m^{-2}$. So, here a clear discrepancy appeared to exist with the data reported for the removal of UV-endonuclease-susceptible sites in XP230S, viz. a residual activity of about 80% (Zelle and Lohman, 1979: paper II of the appendix). Our study of the time-course of UDS over a 24 h period after UV-irradiation has now revealed the reason for this apparent discrepancy. Owing to a difference between normal human cells and XP230S with regard to the kinetics of repair replication, the relative amount of UDS shown by the XP cells after 5 $J.m^{-2}$ of 254 nm UV

increases from 12.5% over the first 2 h to 45% when measured over 24 h after irradiation. In the corresponding experiment checking the repair of UV-endonuclease-susceptible sites under identical experimental conditions a complete agreement was obtained with the relative UDS level over the same periods: over the first 12 h removal of sites was approx. 28% of normal (UDS 30%) and over 24 h a level of 44% was obtained. Consequently, also for XP230S the various methods to measure the residual repair capacity appear to give similar results provided the determinations apply to comparable experimental conditions. This is further illustrated by the results of a study on the time-course of the disappearance of UV-endonuclease-susceptible sites in XP230S (Zelle and Lohman, 1979: paper II of the appendix), which was performed after a lower dose of UV (2.9 $J.m^{-2}$). Also in these experiments an increase in the relative repair capacity was observed, from roughly 50% after 8 h up to about 80% over 32 h. This agrees with our observation obtained with UDS, that the apparent residual capacity of XP230S to repair UV lesions increases when the UV dose is lowered.

In summary, the intermediate level of repair in XP230S as indicated by UV-survival, host-cell reactivation and removal of UV-endonuclease-susceptible sites can fully be reconciled with the results obtained in studies of the repair replication in these cells. These observations clearly indicate that the variation with the UV dose and the strong influence of the repair period on the apparent degree of repair deficiency have to be taken into account when an XP strain is biochemically characterized. In the case of XP230S, the fraction of UV lesions repaired in comparison to normal cells increases with time. Many of the biological effects, such as lethality, the induction of mutations or sister-chromatid exchanges, probably depend on the amount of lesions still unrepaired at the moment the DNA replication during S-phase starts.

For kinetic studies on repair replication, UDS is a more suitable criterium than removal of UV-endonuclease-susceptible sites, since the latter process cannot be accurately studied over the initial periods of repair. Our comparison of the kinetics of UDS in normal human cells and XP230S has revealed a remarkable difference. It is tempting to speculate on the cause of the biphasic pattern of UDS observed with normal cells and the constant repair rate in XP230S cells, which is practically the same as the rate after 8 h in AH cells. This observation suggests the existence of two types of UV lesions, both recognized by UV-endonuclease (so both being pyrimidine dimers) which are repaired in human cells by two different repair systems only one of which is operative in XP230S.

CHAPTER 4

COMPARISON OF THE EFFECTS OF FAR- AND NEAR-UV ON MAMMALIAN CELLS[a]

4.1 Introduction

The action of ultraviolet radiation (UV) on mammalian cells in culture is usually studied with the use of low-pressure mercury lamps ("germicidal lamps") as the laboratory source of "far-UV". The emitted radiation is fairly monochromatic and most of the radiant energy is emitted at a wavelength of 254 nm. Because of the strong absorbance of DNA at this wavelength, lesions are readily formed, the cyclobutyl pyrimidine dimer being the predominant photoproduct (Setlow, 1968). For this reason the germicidal lamps are excellent tools for the rather specific induction of pyrimidine dimers.

The relevance of results obtained from studies using these lamps in understanding the effects of the UV region of sunlight can be doubted, however, as wavelengths below 290 nm normally do not reach the earths surface. Several investigators have shown that differences exist between the biological effects of far-UV and of UV of longer wavelengths ("near-UV"), with regard to survival and mutation induction (Todd et al., 1968; Coohill et al., 1977; Hsie et al., 1977; Jacobson et al., 1978; Rothman and Setlow, 1979). In these studies, UV-doses have been compared on the basis of emitted or absorbed energy, whereas the relation between the biological effects and the number of pyrimidine dimers has been given little attention.

The clinical symptoms of XP are caused by the near-UV region of the spectrum of sunlight. Therefore, insight in the effects of UV radiation of different wavelengths at the molecular and cellular level is required to understand the clinical features of XP and the interaction of sunlight and the human skin in general. As a first approach we used chinese hamster ovary (CHO) cells, which are easy to work with in survival and mutation experiments. We have compared the induction of dimers by far-UV and near-UV sources, and their removal by excision repair, and we have correlated the cytotoxicity and the mutagenicity of the different types of UV with the number of dimers induced by the radiation. In these studies three forms of radiation were compared, viz. the normal 254 nm far-UV and the radiation emitted by a "Sun Lamp" filtered such that either UV <290 nm or <310 nm was removed. Dimers were measured by using the UV-endo assay.

a) This paper by B. Zelle, R.J. Reynolds, M.J. Kottenhagen, A. Schuite and P.H.M. Lohman is submitted for publication in Mutation Research.

4.2 Materials and methods

Cell culture

Monolayer cultures of chinese hamster ovary (CHO) cells, kindly donated by Drs. R.E. Meyn and R.M. Humphrey, were grown in Ham's F10 medium, supplemented with 15% newborn calf serum (or with 15% foetal calf serum in the experiments for measuring the removal of UV-endonuclease-susceptible sites, 30 µM hypoxanthine, 1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The absence of mycoplasma infections was periodically checked by the method of Peden (1975). Cells to be used for experiments, were seeded in tissue culture dishes and incubated at 37°C in a humidified 5% CO_2-atmosphere.

Radiation sources

Far-UV radiation, predominantly at 254 nm, was provided by a single 15 W, low-pressure mercury, germicidal lamp (Philips TUV lamp). The incident dose rate of the 254 nm radiation was 0.39 $W.m^{-2}$ as determined by chemical actinometry according to the procedure of Parker and Hatchard (Calvert and Pitts, 1966); the value was corrected for the contribution of radiation of longer wavelengths on the basis of the relative intensities given by the manufacturer.

Near-UV radiation was provided by two 20 W, "Sunlight" fluorescent lamps (Westinghouse FS20) at a distance of 32 cm from the samples. In all experiments near-UV radiation was filtered, either through the plastic lids of 6 cm diameter, tissue culture dishes (Greiner; the lids were all from the same batch, transmission curves vary from batch to batch!) to remove wavelengths below 290 nm, or through a sheet of Melinex film (ICI, type 442, thickness 23 µm) to remove wavelengths below 310 nm. The incident dose rate of the two types of radiation was respectively 2.5 and 2.2 $W.m^{-2}$, as determined by a calibrated thermopile (Eppley Laboratories, USA). The resulting spectra are presented in Fig. 1. The spectra were calculated as the product of the filter transmission spectra (also shown in the figure) and the emission spectrum of the "Sun Lamp" (taken from the Westinghouse technical bulletin).

Assay of UV-endonuclease-susceptible sites (UV-endo sites)

To label cellular DNA, $2x10^5$ CHO cells were seeded on 6 cm tissue culture dishes (Greiner), grown in nonradioactive medium for 24 h and then in medium containing 0.25 µCi/ml (^{3}H)TdR (17 Ci/mmol) for 24 h. Prior to irradiation the cells were washed free of medium with 2 ml of phospate-buffered saline (PBS), consisting of 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.7 mM KCl, and then covered with 2 ml of this solution. Irradiations were done from above and through the PBS layer. After irradiation cells to receive further incubations were

covered with fresh, nonradioactive medium and incubated at 37°C. At different times thereafter, up to 32 h, the cells still in monolayer were rinsed twice with PBS and stored at -70°C prior to the DNA isolation, necessary for the determination of the UV-endo sites.

UV-endo sites were assayed by a procedure similar to that of Paterson et al. (1973) which recently was described in detail (Zelle and Lohman, 1979: paper II of the appendix). Briefly, DNA purified from CHO cell lysates by phenol extraction and dialyzed against the endonuclease reaction buffer was divided into two fractions and incubated with or without UV-endonuclease from *M. luteus*. The numbers of UV-endo sites were calculated from the resulting DNA molecular weight distributions as determined by sedimentation through calibrated alkaline sucrose gradients.

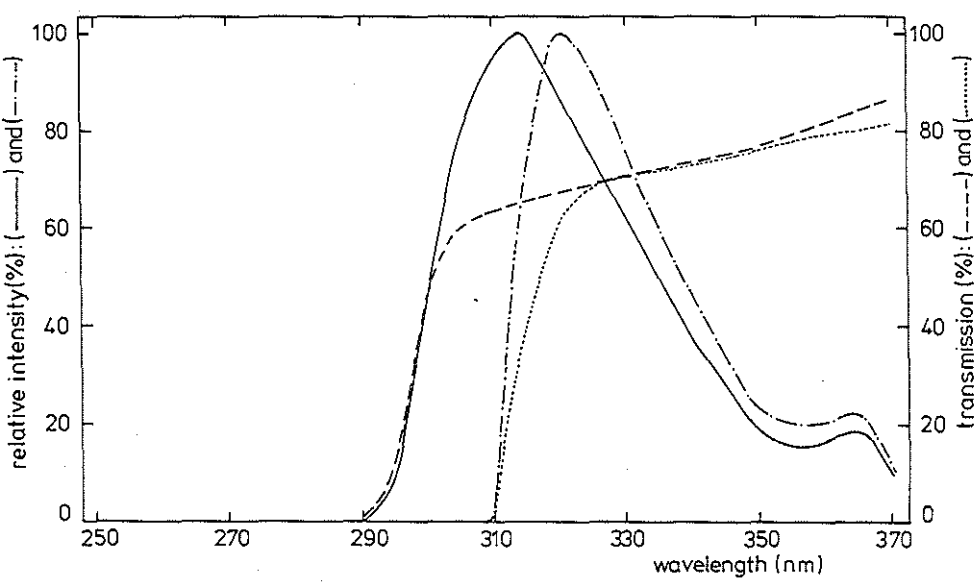

Fig. 1. Emission spectra of the fluorescent Sunlamp (Westinghouse FS20) filtered either by the lid of a tissue culture dish (Greiner, 6 cm diameter) (——) or ICI Melinex type 442 film (23 µm thickness) (— · —), and transmission spectra of the filters used (– – – and ·····, respectively). The emission spectra were calculted from the transmission spectra measured with a Beckman Acta III spectrophotometer, and the lamp's unfiltered emission spectrum as given by the manufacturer.

In vitro photoreactivation of dimers

The photoreactivating enzyme (PRE) used was purified from the actinomycete *Streptomyces griseus* and a generous gift from Dr. A.P.M. Eker (Eker and Fichtinger-Schepman, 1975). DNA was extracted from either irradiated or unirradiated cells identical to the procedure followed for UV-endonuclease-sensitive site analysis up to the dialysis step. The aqueous layer containing the DNA obtained after phenol extraction was dialyzed overnight against 0.01 M NaCl, 0.01 M KH_2PO_4/K_2HPO_4 at pH 7.0 and 4^0C.

The reaction mixture contained 125 µl DNA solution, 7.5 µl tRNA (dissolved at 1 mg/ml), 100 µl PRE-buffer (125 µl when PRE was not to be added) consisting of 0.04 M NaCl, 0.1% (w/v) bovine serum albumine, 5 mM 2-mercaptoethanol, 0.01 m KH_2PO_4/K_2HPO_4 at pH 7.0. The samples to which PRE was to be added received 25 µl of PRE solution made by diluting stock-PRE 1:12 in PRE buffer. Before this addition all samples were prewarmed to 37^0C. The samples to be incubated for 0 min were then put in the dark and in ice, and thereafter the enzyme was added to all samples meant to receive it. The samples with longer incubation periods were incubated at 37^0C, with or without PRE and with or without exposure to white fluorescent light, filtered through windowglass with a thickness of 8 mm, for 2.5 to 5 min. In the experiments with far-UV irradiated DNA the PRE was added already before the prewarming period at 37^0C and exposure time to the white fluorescent light was shortened to 1 or 2.5 min.

The reaction was stopped by putting the samples in the dark and in ice. DNA was reextracted with phenol and processed as described for the determination of UV-endonuclease-susceptible sites.

Chromatographic analysis of thymine-containing dimers

CHO cells were incubated for 3 days in medium containing (^{3}H)TdR (1 µCi/ml; 17 Ci/mmol). The cells were thoroughly washed with PBS and exposed to far-UV or to near-UV >290 nm. DNA was isolated according to the procedure of the assay for UV-endo sites, and was precipitated by adding 1.5 ml of icecold 20% trichloroacetic acid (TCA) to 1 ml of DNA solution. The precipitate was centrifuged at 3000 g and dried. Hydrolysis and chromatography were done as described by Carrier and Setlow (1971). The amount of thymine-containing dimers present in each sample was expressed as a percentage of the total detected (^{3}H)-activity on the chromatogram.

DNA repair replication

Three days before irradiation about 10^5 cells were seeded onto culture dishes (Greiner; diameter: 6 cm) in 3 ml F10 medium and incubated at 37^0C. Two hours before irradiation, the medium was replaced by 3 ml fresh medium containing 5-bromodeoxyuridine (BUdR; 6.5 µM) and 5-fluorodeoxyuridine (FUdR; 1 µM). FUdR inhibits the

conversion of deoxyuridine 5'-monophosphate to deoxythymidine 5'-monophosphate. Subsequently the cells were washed twice with PBS and UV irradiations were done through a thin layer of PBS (2 ml/plate) covering the cells. The PBS was replaced by 3 ml medium containing 4 mM hydroxyurea, 6.5 μM BUdR, 1 μM FUdR and (^{3}H)TdR (10 μCi/ml; 17 Ci/mmol). The cells were incubated in this medium for 3 h at 37^{0}C. Then the cells were incubated for 1 h in the same medium but without the radioactive DNA precursor ("chase"). After completion of the whole procedure the medium was removed from the dishes and the cells were frozen at -70^{0}C, until the DNA was isolated and analyzed by sodium iodide density gradient centrifugation by a modified method of Lohman et al. (1973). In this method two isopycnic bandings of DNA were performed on guidance of ethidium bromide present in the gradients, which forms a fluorescent complex with DNA.

After the first run in NaI gradients the DNA band, having the normal buoyant density of chinese hamster DNA, was collected and mixed with a known amount of (^{14}C)-labelled *M. luteus* DNA. This mixture was rebanded in a second run. (The *M. luteus* DNA, which has a higher density than the normal chinese hamster DNA, served as an internal standard in the second NaI gradient for determining the absolute amount of DNA in each gradient.)

After the second centrifugation the gradient was scanned directly in a fluorimeter for measuring the fluoresence of both the chinese hamster and the *M. luteus* DNA. After scanning of the tube the gradient was fractioned into about 16 fractions of approx. 0.5 ml each, to which was added 0.5 ml of a solution containing calfthymus DNA (200 μg/ml) and 2 ml 10% (w/v) TCA in 0.01 M sodium pyrophosphate. The fractions were kept in ice for about 2.5 h and subsequently filtered through glass fiber filters type GF/C (Whatman). The filters were washed with cold 5% (w/v) TCA and with cold ethanol, dried and the radioactivity was measured as described by Lohman et al. (1973). The procedures of these authors were also followed for the expression of the results as desintegrations per minute per microgram of parental DNA.

Survival determinations

Cells were trypsinized, counted in a haemocytometer, serially diluted and plated on 6 cm dishes. The dilutions were chosen so as to result in 100-300 clones per plate; per experiment and per dose 4 plates were used. The dishes were allowed to stand for 4 h at 37^{0}C to permit attachment of the cells to the surface of the plate. Then the medium was removed, the plates were rinsed twice with PBS and irradiated through a thin layer of PBS (2 ml/plate). Following irradiation fresh medium was added and the cells were incubated for 7 days, after which period the clones were scored.

Mutation induction

Mutation to 6-thioguanine (TG) resistance was detected as described by Van Zeeland and Simons (1976). Following irradiation with either far-UV or near-UV (>290 nm or >310 nm) cells were allowed expression times of 8 days prior to being replated in selective medium which contained TG (5 µg/ml). Per experiment and per UV dose ten 9 cm dishes were inoculated after the expression period, each with 10^5 cells. Resistant colonies were counted after 7 days of growth in the selective medium. Simultaneously the plating efficiencies were determined by inoculating four 6 cm dishes with each 250 cells which were incubated in non-selective medium.

4.3 Results

Induction of UV-endonuclease-susceptible sites

Typical dose response curves for the induction of UV-endonuclease-susceptible sites (UV-endo sites) by the three types of UV are presented in Fig. 2. Each point represents the average of 2 or 3 independent determinations; standard deviations are denoted by vertical bars. In all three cases the number of UV-induced sites increased linearly with the dose, i.e. with the exposure time.

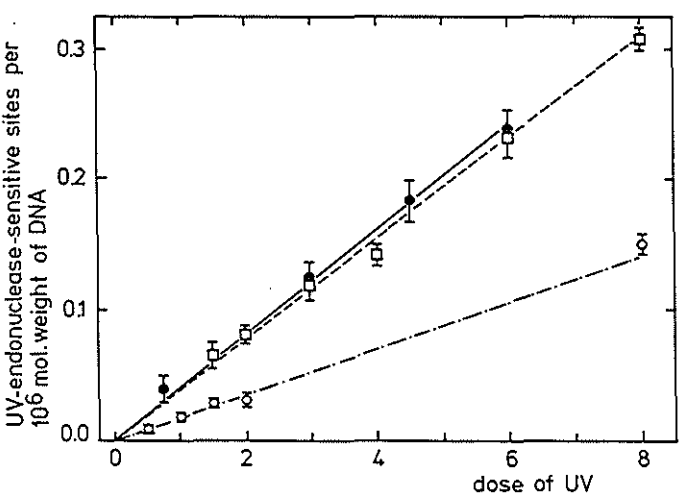

Fig. 2. Induction of UV-endonuclease-sensitive sites in the DNA of CHO cells by irradiation with far- and near-UV. The UV dose is given in arbitrary units, proportional to the duration of the irradiation.
●—●: 254 nm UV; 1 unit = 4 s of irradiation. □– –□: >290 nm UV; 1 unit = 1 min. ○–·–○: >310 nm UV; 1 unit = 100 min.

Near-UV induced the sites much less efficiently than 254 nm UV and induction of similar numbers of sites therefore required considerably longer exposure times, viz. 15 fold longer for near-UV >290 nm and 3800 fold longer for near-UV >310 nm.

For a proper comparison of the efficiencies with which the UV-endo sites are induced, the differences in exposure time have to be corrected for the differences in dose rate. The rates were 0.39, 2.5 and 2.2 $W.m^{-2}$, respectively, for 254 nm, >290 nm and >310 nm UV. The incident energy dependence for the induction of UV-endo sites in CHO cells calculated from the data amounts to 0.026 sites per 10^6 mol. weight of DNA induced per $J.m^{-2}$ for 254 nm UV, 0.27×10^{-3} for >290 nm UV and 1.2×10^{-6} for >310 nm UV. Thus per unit of incident energy the far-UV source was 96 fold more efficient in the induction of UV-endonuclease-susceptible sites than the near-UV >290 nm source and 22 000 fold more efficient than the near-UV >310 nm source.

UV-endonuclease substrate specificity

For the interpretation of the results obtained with the *M. luteus* endonucleases it appeared of paramount importance to establish whether this method really exclusively measures pyrimidine dimers or that also other lesions in UV-irradiated DNA are assayed.

To investigate the substrate specificity of the *M. luteus* UV-endonuclease the susceptibility of UV-endo sites to *in vitro* photoreactivation was examined. CHO cells grown in the presence of (^{3}H)TdR were exposed either to near- or to far-UV for durations calculated to induce comparable numbers of UV-endo sites. A far-UV exposure time of 12 s, corresponding to 4.7 $J.m^{-2}$, was used to induce approx. 0.12 UV-endo sites per 10^6 mol. weight of DNA. A near-UV >290 nm exposure time of 180 s and a near-UV >310 nm exposure time of 800 min was used to induce a similar number of sites. DNA extracted from the irradiated cells was exposed to photoreactivating light in the presence of photoreactivating enzyme (PRE) purified from *Streptomyces griseus* (Eker and Fichtinger-Schepman, 1975), reextracted with phenol and assayed for the presence of UV-endo sites.

More than 90% of the UV-endo sites induced either by far-UV, near-UV >290 nm or near-UV >310 nm were photoreactivable (Fig. 3). In the three experiments depicted in Fig. 3 the rate of photoreactivation appears to be higher with the far-UV irradiated DNA (note differences in the scales of the abscissa). This is due to the fact that far-UV irradiated DNA was preincubated with PRE. In later experiments with near-UV irradiated DNA this preincubation was omitted because it was feared that preincubation might lead to a partial inactivation of PRE, although control experiments comparing the two methods gave identical results. Incubation of the DNA with PRE in the dark or exposure of the DNA to photoreactivating light in the absence of PRE had no effect on the numbers of UV-endo sites detected.

Since cyclobutyl pyrimidine dimers are the only UV-induced lesions known to be susceptible to enzymatic photoreactivation (Wulff

and Rupert, 1962; Cook, 1967; Setlow, 1967) these experiments indicate that most, if not all UV-endo sites induced either by far-UV, near-UV >290 nm or near-UV >310 nm are equivalent to pyrimidine dimers in DNA. Recently evidence has been presented for photoreactivation of non-dimer photoproducts in *E. coli* cells (Harm, 1978), but it was not shown that this photoreactivation resulted from an enzymatic reaction.

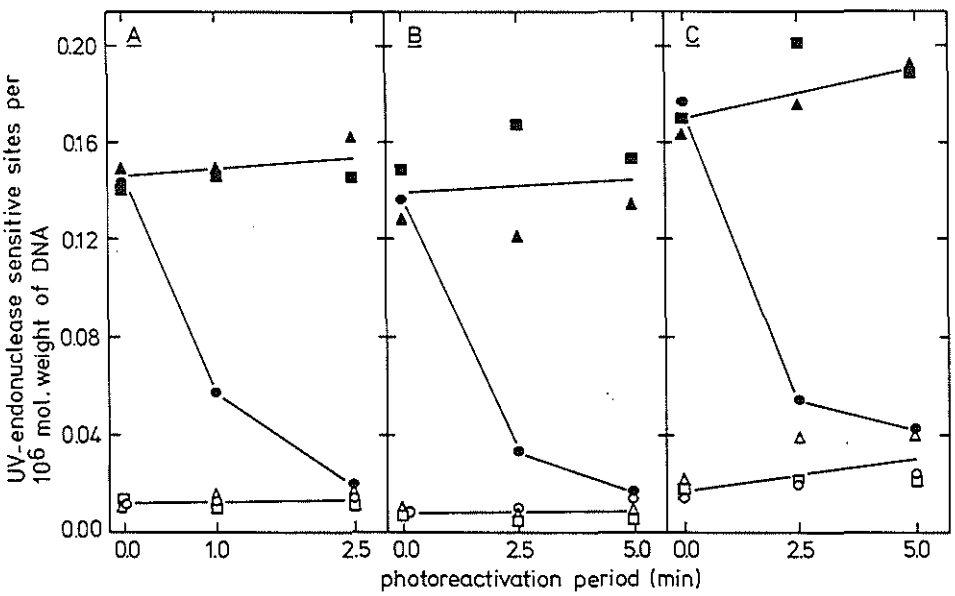

Fig. 3. Enzymatic photoreactivation of UV-endonuclease-sensitive sites *in vitro*. Phenol-extracted DNA was from unirradiated, far-UV-irradiated (panel A), near-UV >290 nm-irradiated (panel B) or near-UV >310 nm-irradiated (panel C) CHO cells. The DNA was then incubated at 37°C with or without photoreactivating enzyme (PRE) and with or without exposure to white fluorescent light (PRL) prior to extraction with phenol and UV-endonuclease-sensitive site determination.
Unirradiated cells: △,□,○; irradiated cells: ▲,■,●.
+PRE,-PRL (△,▲); -PRE,+PRL (□,■); +PRE,+PRL (○,●).
UV doses were 4.7 J.m^{-2}, 470 J.m^{-2} and 106 kJ.m^{-2}, respectively, for the three types of radiation.

Close examination of Fig. 3 will reveal that small numbers of UV-endo sites were found even in the unirradiated DNA. UV-endo sites were found in unirradiated DNA only when radioactively (^{3}H) labelled DNA was held at -70°C for at least several days prior to extraction and analysis for sites. This "nonspecific" endonucleolytic activity was probably the result of γ-endonuclease activity present in crude extracts of *M. luteus* (Paterson and Setlow, 1972) which probably

attacks lesions produced by radioisotopic decay in the (^{3}H)-labelled DNA during storage at -70^{0}C (W.L. Carrier, personal communication). For the purpose of quantitating UV-endo sites, unirradiated samples were run with all experiments and all other data were corrected for the numbers of "nonspecific" sites detected.

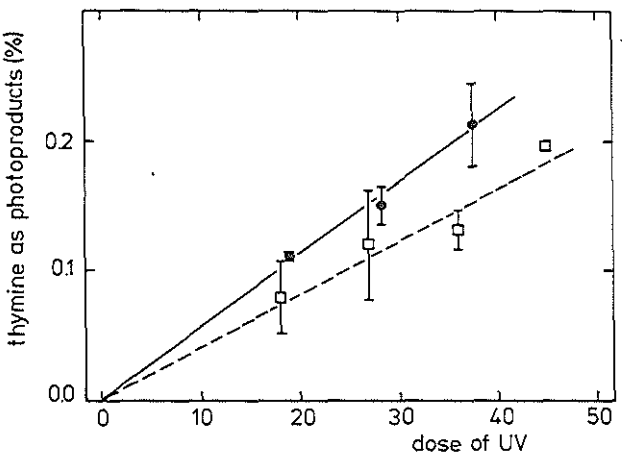

Fig. 4. Induction of thymine-containing photoproducts, identified as pyrimidine dimers by chromatography, in the DNA of CHO cells by irradiation with far-UV and near-UV. The UV dose is given in arbitrary units, proportional to the duration of the irradiation.
●—●: 254 nm UV; 1 unit = 4 s of irradiation. □—□: >290 nm UV; 1 unit = 1 min.
Each point is the average of 2 (far-UV) or 3 (near-UV) experiments (error bars represent the standard deviations), with the exception of the result after 45 min of near-UV, which was determined once.

The photoreactivation experiments clearly showed that the sites detected by the UV-endonuclease preparation used are equivalent to pyrimidine dimers for at least 90%. The possibility remained, however, that the enzyme does not recognize all dimers, but only a fraction. Since it was essential for our studies to be certain that all pyrimidine dimers were detected, we checked this possibility by comparing the number of UV-endo sites induced in the DNA per unit energy with the number of dimers that could be detected chromatographically. For the chromatographic analysis CHO cells were grown in the presence of (^{3}H)TdR and were exposed either to far-UV at doses ranging from 29 to 59 J.m^{-2} or to near-UV >290 nm at doses ranging from 2.6 to 6.8 kJ.m^{-2}. DNA was isolated, hydrolyzed and subjected to chromatography. The results were expressed as the fraction of the thymine molecules that were found to be incorporated

in thymine-containing dimers. Fig. 4 shows the linear relationship between the incorporation of thymine in these photoproducts and UV-dose. The fraction of thymine-molecules converted to thymine-containing dimers is 36×10^{-6} per $J.m^{-2}$ for far-UV and 0.27×10^{-6} per $J.m^{-2}$ for near-UV >290 nm.

The ratio of the 3 types of dimers, viz. thymine-thymine (T-T), thymine-cytosine (T-C) and cytosine-cytosine (C-C), in the DNA of UV-irradiated mammalian cells differs from that in the DNA of UV-irradiated bacteria. This can be due to the difference in substrate, being naked DNA in bacteria and chromatin in mammalian cells, but also to differences in GC/AT-ratio (Rothman and Setlow, 1979). The ratio T-T/T-C/C-C in the DNA of Hela cells is 60/26/14 after far-UV and 43.5/43.5/13 after near-UV (W.L. Carrier, personal communication). From these ratios it can be calculated that 1 thymine molecule found in a photoproduct represents 0.685 dimer after far-UV and 0.766 dimer after near-UV.

In mammalian DNA approximately 30% of the nucleotides is thymine, so 10^6 mol. weight of DNA contains 976 thymines. On the basis of these data we calculate that per 10^6 mol. weight of DNA in mammalian cells far-UV will induce 24×10^{-3} pyrimidine dimers per $J.m^{-2}$, and near-UV >290 nm 0.20×10^{-3}. These values compare very well with the experimentally determined numbers of UV-endo sites induced per 10^6 mol. weight, which were 26×10^{-3} and 0.27×10^{-3}, respectively. The conclusion appears warranted, therefore, that the UV-endo sites detected with our procedures are fully representative for the pyrimidine dimers present in the DNA.

Postirradiation disappearance of UV-endonuclease-sensitive sites

CHO cells are capable of excision repair of pyrimidine dimers (Meyn et al., 1974). For the intended comparison of the biological effects of the three types of UV, it was of importance to know whether dimers induced by far- or by near-UV are repaired with equal efficiency, or that differences in additionally induced lesions affect the repair system. This was investigated by examination of the decrease of the number of UV-endo sites during a postirradiation incubation of the cells at 37^0C. Experiments with far- and near-UV were performed simultaneously and doses were chosen so that comparisons could be made following the induction of similar numbers of UV-endo sites.

For these studies 2 doses of UV were used, the lower one inducing about 0.03 sites per 10^6 mol. weight of DNA, and the higher one 2.5 times as many. For 254 nm UV the doses were 1.2 and 2.9 $J.m^{-2}$, respectively, and for >290 nm UV 96 and 240 $J.m^{-2}$. For >310 nm UV, however, only the lower dose could be given (26 $kJ.m^{-2}$), since an irradiation inducing 0.075 sites per 10^6 mol. weight of DNA would have required an exposition of about 8 h.

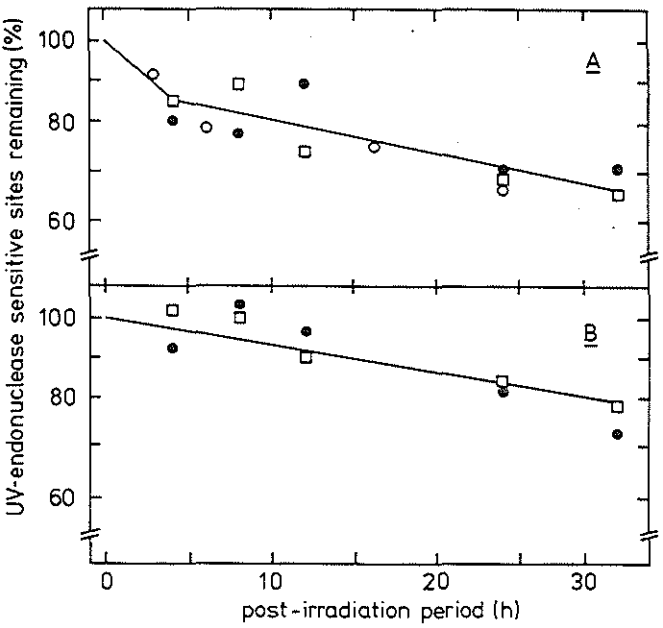

Fig. 5. Disappearance of UV-endonuclease-sensitive sites in CHO cells during incubation after irradiation with far-UV (●), near-UV >290 nm (□) or near-UV >310 nm (○). Far-UV doses were 1.2 (panel A) and 2.9 (panel B) $J.m^{-2}$, and near-UV exposures were chosen so as to result in the induction of comparable numbers of UV-endo sites. Near-UV >290 nm doses were 96 and 240 $J.m^{-2}$ in panels A and B, respectively, and the near-UV >310 nm dose was 26 $kJ.m^{-2}$ (panel A). Each point is the average of 3 independent determinations.

The results are shown in Fig. 5A (lower doses) and Fig. 5B (higher doses). In all cases a time-dependent reduction in the number of detectable sites was found. The time-course and the extent of this reduction were the same, irrespective whether far- or near-UV was used, when a comparable number of sites was induced. After the lower dose 30% fewer sites were detected 24 h after the irradiation, whereas after the higher dose a decrease of about 20% was observed over this period. The extent of dimer removal is in agreement with results obtained by Meyn et al. (1974) in the same cell line after 254 nm UV irradiation.

DNA repair replication

As a second criterium that could be used to detect possible differences in repair of lesions induced by either far- or near-UV, DNA repair replication was measured. For these determinations the incorporation of (^{3}H)TdR in non-replicating DNA during the first 3 h

after irradiation was assayed, with a method using sodium iodide density gradient centrifugation. Various UV doses were given that ranged between 0 and 20 $J.m^{-2}$ of far-UV and 0 and 2700 $J.m^{-2}$ of near-UV >290 nm; in both cases the numbers of UV-endo sites induced per 10^6 mol. weight of DNA ranged between 0 and 0.5. Irradiations with near-UV >310 nm were not included in these experiments as the exposure times would have had to be extremely long.

The results of duplicate experiments are shown in Fig. 6. From this we can conclude that there is no clear difference in repair replication in CHO cells irradiated with either far- or near-UV >290 nm.

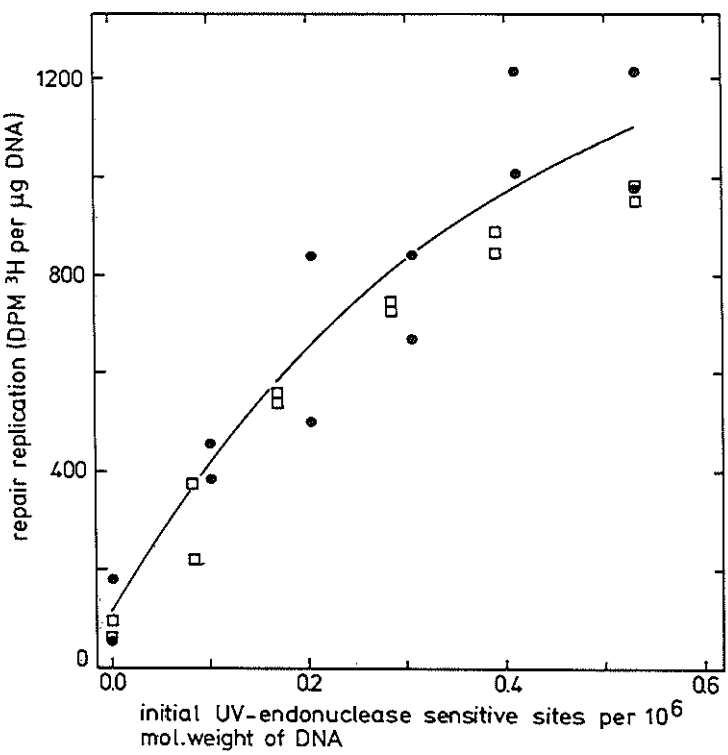

Fig. 6. DNA repair synthesis induced in CHO cells by far-UV (●) or near-UV >290 nm (□), expressed as function of the number of UV-endonuclease-sensitive sites induced. Repair synthesis is expressed as (^{3}H)thymidine incorporation in DNA.

Cytotoxicity of far- and near-UV radiations

To determine the relative cytotoxicities of far- and near-UV the colony-forming ability of CHO cells was examined after various radiation exposures. Fig. 7 shows the results for far-UV and near-UV >290 nm. In Fig. 7A these results are presented as functions of the incident energies. Clearly, per unit of incident energy far-UV is much more cytotoxic. Although not visible due to the scale used, also the far-UV survival curve is characterized by a well defined shoulder region at low doses and by an exponential decline at higher doses; when plotted on appropriate scales, the two curves are rather similar in shape. For both curves a D_{37} as well as a D_0 can be given. For far-UV the D_{37} (the dose killing 63% of the cells) amounts to 10 $J.m^{-2}$; the D_0 (the additional dose required to lower survival by 63% in the exponential section of the curve) is 2.4 $J.m^{-2}$. The values for near-UV >290 nm are 700 and 130 $J.m^{-2}$, respectively.

In Fig. 7B the colony-forming abilities of far-UV and near-UV >290 nm irradiated cells are replotted as functions of the numbers of UV-endo sites induced. When compared in this way CHO cells are more sensitive to near-UV >290 nm radiation than to far-UV. Expressed in UV-endo sites per 10^6 mol. weight of DNA the D_{37} and D_0 values are for far-UV 0.26 and 0.062, for near-UV >290 nm 0.19 and 0.035, respectively.

Also survival after irradiation with >310 nm UV was examined. For practical reasons irradiation periods had to be limited to maximally 200 min, so only the survival range between 100 and 70% could be studied. By extrapolation the D_{37} was calculated to be as high as 46 $kJ.m^{-2}$. The results did not allow the determination of D_0. Fig. 8 shows the relation between survival and the number of UV-endo sites measured in these preparations. For comparison the corresponding parts of the curves in Fig. 7B have been included. It is obvious that the >310 nm UV - although much less lethal per unit dose of energy - is by far the most harmful on the basis of cell killing per induced dimer site. When expressed in this manner the extrapolated D_{37} value is 0.055 UV-endo sites per 10^6 mol. weight of DNA.

Since the experiments with >310 nm UV required irradiation periods up to more than 3 h, as compared to 1 or 10 min for the other 2 UV sources, the possible occurrence of artefacts had to be considered. For that reason a number of control experiments was performed. With each survival experiment it was checked whether 200 min incubation with PBS itself did influence the plating efficiency of the cells in comparison to an incubation of 10 min or less; the plating efficiencies were equal and ranged from 70-90% among the various experiments. It was also checked whether the combination of irradiation and long incubation in PBS did influence survival, for example by having an effect on the cell cycle. To this end cells were irradiated with a dose of >290 nm UV that under normal conditions resulted in 24% survival. For this dose a 6.5 min

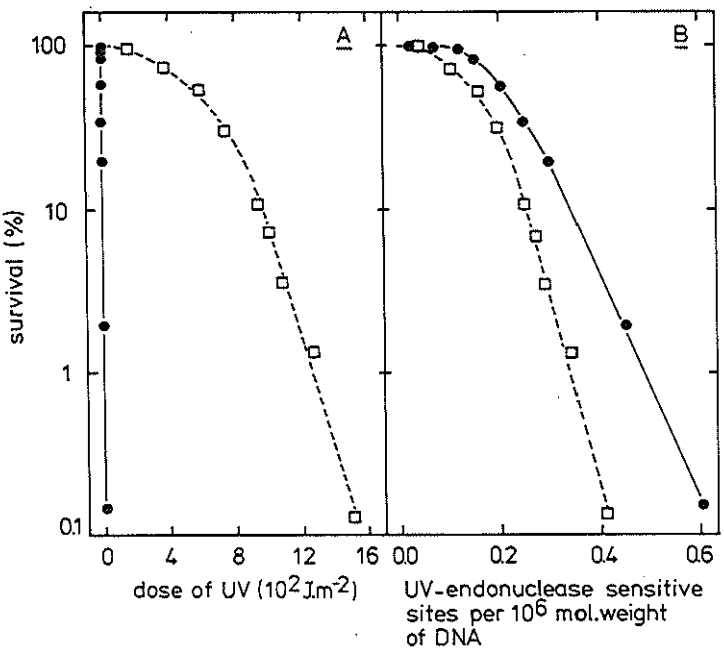

Fig. 7. Survival of CHO cells irradiated with far-UV (●) or near-UV >290 nm (□), expressed as function of UV dose (panel A) or of number of UV-endo sites induced (panel B). Each point is the average of 3 or more experiments.

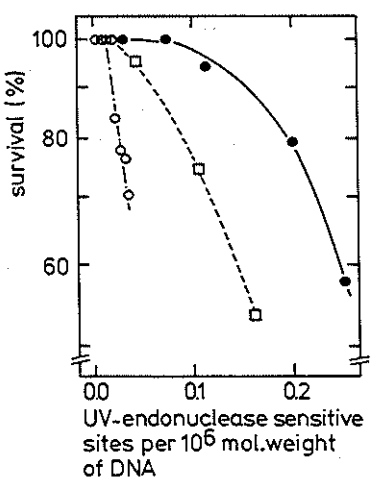

Fig. 8. Survival of CHO cells irradiated with far-UV (●), near-UV >290 nm (□) or near-UV >310 nm (○), expressed as function of number of UV-endo sites induced. Each point is the average of 3 or more experiments.

irradiation period was required. Various set-ups were tested: after irradiation cells were immediately incubated with medium, or they were incubated with PBS for 150 min and then with medium; they were first incubated with PBS for 150 min and then irradiated, or they were given half of the UV dose before a 150 min incubation with PBS and the second half thereafter. In all cases the same survival was obtained.

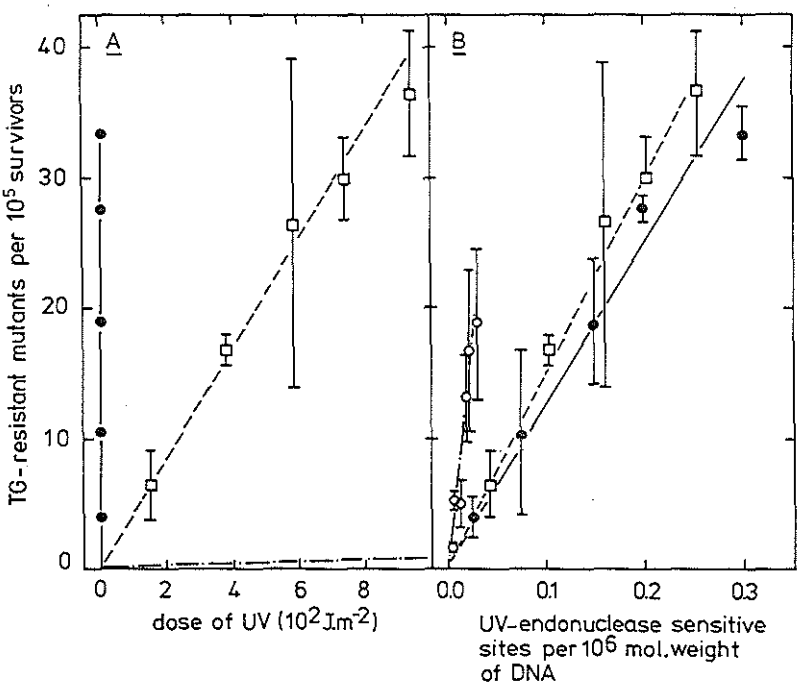

Fig. 9. Mutation of CHO cells to TG resistancy by irradiation with far-UV (●—●), near-UV >290 nm (□--□) or near-UV >310 nm (○-·-○). The mutation frequency, corrected for the number of spontaneous mutations determined with each experiment, expressed as function of dose (panel A) or number of UV-endo sites induced (panel B). The points represent the averages of at least 3 independent experiments; standard deviations are denoted by error bars. The 6 experimental points obtained with >310 nm UV fell outside the abscissa-scale of panel A.

Mutation induction by far- and near-UV radiations

To examine the relative mutagenicities of far- and near-UV radiations, the abilities of these radiations to induce resistance to 6-thioguanine (TG) in CHO cells were studied. Normal CHO cells are sensitive to TG and resistance has been shown to arise through forward

mutations in the structural gene for hypoxanthine-guanine phosphoribosyltransferase (HPRTase) (Van Zeeland and Simons, 1976; O'Neill et al., 1977). To allow complete expression of the mutations, our procedure included an expression time of 8 days as suggested by Van Zeeland and Simons (1976) before the cells were incubated in medium with TG. The numbers of TG-resistant clones were corrected for the corresponding plating efficiencies.

In Fig. 9A the induced mutation frequencies have been plotted as functions of the total incident energies. All three radiation sources induce mutations in proportion to dose, the slopes of the dose response curves corresponding to 3.3, 0.042 and 0.95×10^{-3} mutations per 10^5 surviving cells per $J.m^{-2}$ for far-UV, near-UV >290 nm and near-UV >310 nm, respectively. Thus as was found for the loss of colony-forming ability per unit of incident energy, far-UV is much more effective than near-UV in the induction of mutations.

In Fig. 9B the mutation frequencies have been replotted as functions of the number of UV-endo sites induced. The numbers of mutations per 10^5 surviving cells expected to occur when 1 site per 10^6 mol. weight of DNA is induced, are 130, 150 and 800 for far-UV, near-UV >290 nm and near-UV >310 nm, respectively. Per UV-endo site induced, near-UV >290 nm is at least as mutagenic as far-UV, whereas near-UV >310 nm is markedly more mutagenic.

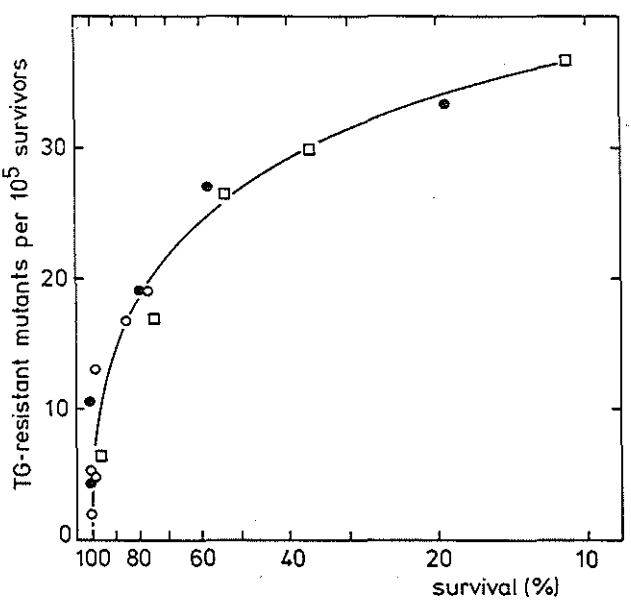

Fig. 10. Mutation induction to TG resistancy as a function of survival of CHO cells for far-UV (●), near-UV >290 nm (□) and near-UV >310 nm (○).

The survival and mutation induction results of Figs. 7, 8 and 9 are combined in Fig. 10, where the number of induced mutants is plotted as function of survival. From this graph it is clear that no distinct differences exist in the relations between mutagenicity and cytotoxicity of the 3 sources of UV.

A compilation of the various effects determined for the three types of UV is given in Table I.

TABLE I
COMPARISON OF THE BIOCHEMICAL AND BIOLOGICAL EFFECTS IN CHO CELLS OF 3 UV SOURCES

Property	(unit)	254 nm UV	>290 nm UV	>310 nm UV
ESS[a]	(per $J.m^{-2}$)	26×10^{-3}	0.27×10^{-3}	1.2×10^{-6}
dimers[b]	(per $J.m^{-2}$)	24×10^{-3}	0.20×10^{-3}	n.d.[c]
D_{37}	($J.m^{-2}$)	10	700	46×10^{3}
D_{37}	(ESS)	0.26	0.19	0.055
D_0	($J.m^{-2}$)	2.4	130	n.d.
D_0	(ESS)	0.062	0.035	n.d.
IMF[d]	(per $J.m^{-2}$)	3.3	0.042	0.95×10^{-3}
IMF	(per ESS)	130	150	790

a) ESS = UV-endo sites induced per 10^6 mol. weight of DNA.
b) induced per 10^6 mol. weight of DNA.
c) n.d. = not determined.
d) IMF = induced mutation frequency, i.e. number of TG-resistant mutants induced per 10^5 survivors.

4.4 Discussion

In this study we have investigated the induction in DNA of CHO cells of the predominant UV lesion, the cyclobutyl pyrimidine dimer, by far- and near-UV sources. As near-UV sources we used a "Sun Lamp" which emitted light that was filtered either by the lids of tissue culture dishes that absorb all radiation below 290 nm or by Melinex (polyethylene terephthalate) film that absorbs all radiation below 310 nm.

All 3 types of radiations induced *M. luteus* UV-endonuclease-sensitive sites in direct proportionality to the dose. These sites could be removed from the DNA for more than 90% by treatment with a purified photoreactivating enzyme *in vitro*. This strongly suggests that the sites recognized by the *M. luteus* extract are the pyrimidine dimers induced by UV. It must, however, be realized that the specificity of photolyases or photoreactivating enzymes towards the monomerization of cyclobutyl pyrimidine dimers is not definitively proven; so far no other DNA photoproducts have shown to be

enzymatically photoreactivable (see for a review: Setlow, 1967). On the other hand, supporting evidence that UV-endo sites are equivalent to pyrimidine dimers has now been obtained by a comparison with the chromatographically determined numbers of dimers (see also: Setlow et al., 1975). We feel rather confident therefore that the UV-endo sites measured in our experiments represent true pyrimidine dimers.

The remaining of a small fraction of the induced endo-sites after several minutes of treatment with photoreactivating light in the presence of DNA-photolyase is in agreement with results of Cook and Worthy (1972) and Childs et al. (1978), that about 10% of the initially induced pyrimidine dimers (measured chromatographically or as T4-UV-endo sites, respectively) could not be photoreactivated.

Dimer induction has been reported for wavelengths up to 365 nm (Tyrell, 1973; Webb et al., 1976), but as in these experiments broad spectrum UV sources were used from which the 365 nm radiation was selected by use of a monochromator and filters that cut off below 310 nm, it might be possible that the dimers measured were induced by wavelengths just above the cut-off wavelength of 310 nm. Nevertheless, these results agree with our finding that dimers can be induced by wavelengths above 310 nm.

The enzymatic dimer assay has the great advantage that it is sufficiently sensitive to allow the determination of dimers induced by non-lethal doses of UV, and even to follow their postirradiation removal, whereas chromatographic methods of dimer analysis after an acidic hydrolysis of DNA are rather insensitive and need higher doses. We were able to study the *in vivo* removal of dimers after doses that would allow more than 70% of the cells to survive. It is known that UV induces a variety of lesions in addition to the dimers, of which have been identified cytosine hydrate, thymine-thymine adducts and their dehydration product (Murphy, 1975), single-strand breaks, alkali-labile bonds (Elkind and Han, 1978), the spore-photoproduct 5-thyminyl-5,6-dihydrothymine (Varghese, 1970), and DNA-protein cross-links (Smith, 1962). Furthermore it has been shown that DNA of cells irradiated with near-UV during incubation in medium can be damaged by photoproducts from components of the medium, e.g. tryptophan, riboflavine and tyrosine (Nixon and Wang, 1977). In our experiments we have tried to prevent these damages by thoroughly washing the cells with and irradiation while incubated in phosphate-buffered saline. But the other photoproducts mentioned above will also have occured in our preparations. It appears likely that most of these photoproducts have different action spectra. It is to be expected that the ratios between the various products formed, including pyrimidine dimers, vary with the wavelength of the UV with which the cells are irradiated. Consequently, for each of the 3 types of UV studied, a differently composed set of lesions will be induced. It would not have been suprising, therefore, if this had affected the repair processes in such a manner that the rate of dimer removal had been found to depend on the wavelength of irradiation. However, the time courses for the disappearance of dimer sites in far- or near-UV

irradiated cells were indistinguishable from each other when comparable numbers of sites had been induced. Evidently, the other UV-induced lesions do not interfere with the removal of dimers.

As a second approach to study the repair of lesions induced in the DNA by the various UV sources we determined DNA repair replication. This technique measures the incorporation of DNA precursors in parental DNA as the result of excision repair processes during the 3 h postirradation incubation period studied. The amount of repair replication depends both on the number of excised photoproducts and on the size of the repair patches. It must be emphasized that lesions involved in repair processes that give short patches, e.g. single-strand breaks, will not be detected by this assay unless they form a substantial part of the total population of lesions being repaired. However, also when repair replication was used as the criterium, there appeared to be no difference in the response of CHO cells whether irradiated by far-UV or near-UV >290 nm if the same number of dimers was initially induced. So no evidence could be obtained for non-dimer lesions being involved in repair pathways, although it must be assumed that these lesions are induced at different ratios to dimers by UV of different wavelengths. The occurrence of repair of these lesions by processes not involving resynthesis of DNA is, of course, not excluded by this result.

Neither of the biochemical assays used did reveal differences in excision repair of the DNA of CHO cells, damaged by irradiation with either far- or near-UV, when comparabe amount of dimers were induced.

With respect to the biological effects of the radiation used we compared their cytotoxicity and mutagenicity. Survival studies with mammalian cells in culture after irradiation with UV of different wavelengths have been reported (Todd et al., 1968; Hsie et al., 1977; Elkind et al., 1978; Jacobson et al., 1978), but comparisons were made only on the basis of UV dose and the shape of the survival curves. In most cases similarities between the survival curves were observed, but Elkind et al. (1978) noticed differences when they compared the effects of germicidal and Sun-lamps. We have used the initial number of UV-endonuclease-sensitive sites induced by far-UV, near-UV >290 nm and near-UV >310 nm to compare the cytotoxicity of these types of radiation. The results show that on this basis clear differences exist, with the lethality per dimer being the lowest after far-UV and the highest after near-UV >310 nm. The conclusion must be that dimers are not the only lethal lesions induced by near-UV radiation. This is in agreement with the observations of Elkind et al. (1978).

The mutagenic effects of the three types of radiation used varied greatly with the dose, 254 nm UV being far more mutagenic per unit of incident energy than near-UV. But when the mutation frequency is compared on the basis of an equal number of induced dimers, far-UV has the lowest mutagenic effect and >310 nm UV the strongest, the same order as with the cytotoxicity. Per induced dimer near-UV >310 nm was 5-6 times more mutagenic than the other two radiations. This stresses the probability that lesions other than pyrimidine dimers play a

significant role in the biological effects of near-ultraviolet radiations.

When mutation frequency is expressed as function of survival the 3 types of radiation are comparable, indicating that the different lesions that contribute to the lethality are equally important for the induction of mutations.

Our results demonstrate that wavelength regions of UV that are emitted by our natural source of UV, the sun, but also by the various types of solaria presently enjoying a still increasing popularity, induce damages in cellular DNA that are both cytotoxic and mutagenic. Although relatively high doses of these radiations are needed for the induction of a substantial amount of damage, the effects should not be neglected in view of the rather widespread exposition. Little is known about the specific nature of the harmful DNA lesions caused by UV of long wavelengths. They may or may not be identical to (some of) the lesions induced, next to the pyrimidine dimers, by far-UV. For the understanding of the biological effects of the UV sources to which humans are normally exposed, and especially for the understanding of the relationship between DNA damage and the clinical symptoms observed in XP patients, it is necessary to use also near-UV sources in experiments with human cells. Our data indicate that with far-UV the possibly also induced "near-UV lesions" are totally overshadowed with regard to their deleterious biological effects by the much more effectively induced pyrimidine dimers.

SUMMARY

An important feature of living cells is their capacity to maintain the integrity of their hereditary material, the DNA. DNA can be damaged by a variety of physical and chemical agents, among which ultraviolet radiation (UV), ionizing radiation and chemical carcinogens as 4-nitroquinoline-1-oxide (4NQO). Most organisms, including man, are able to repair damaged DNA. The DNA repair processes play a very important role, which is demonstrated by the inherited skin disease xeroderma pigmentosum (XP). This disease is manifested primarily by cellular atrophy, pigmentation abnormalities and malignancies in the sun-exposed areas of the skin. Some of the affected individuals also have neurological complications. Cells derived from most XP patients are abnormally sensitive to UV, which is ascribed to the fact that these cells show a reduced unscheduled DNA synthesis after UV-irradiation and appear to be deficient in the excision of pyrimidine dimers from their DNA. Cultured cells from another group of XP patients show a normal excision repair, but are thought to have a defect in a process associated with the circumvention of lesions present in the DNA during replication (postreplication repair). These are designated as XP variants.

The clinical heterogeneity observed in this disease suggested the possibility of differences in the nature of the genetic defects in different patients (genetic heterogeneity). Genetic heterogeneity can be studied with the aid of complementation analysis, by fusion of cells derived from different patients. In several combinations the presence of the genomes of two patients in a hybrid binuclear cell resulted in a normal level of DNA repair, suggesting that these patients have a mutation at different sites in the genome. Excision repair-defective XP cell strains have now been classified in 7 complementation groups.

The investigations described in this thesis were performed to study the genetic heterogeneity of excision repair-deficient XP strains (chapter 2) and the biochemical defects in their repair processes (chapter 3). In most studies described in the literature a far-UV source is used, while the UV sources encountered in practice emit near-UV. To investigate the relevance of far-UV studies for the understanding of the effects of near-UV, the sensitivity for different wavelengths of UV was studied with an established chinese hamster cell line (chapter 4).

In our studies on genetic classification of XP patients we investigated the cells of a patient from Great Britain (XP2BI). Clinically this patient was of interest because of the absence of skin tumours, and progressive mental deteroriation started at the age of 11. Cultured cells derived from this patient showed complementation after fusion with XP strains from the six already existing complementation groups (chapter 2.1; appendix: paper I). This study revealed the existence of a seventh complementation group G. The

repair deficiency of this strain was indicated by its reduced ability to survive UV-irradiation and was apparent from its reduced repair synthesis as measured by autoradiography (UDS), its reduced removal of UV-induced damages recognized by a specific UV-endonuclease and the low capacity to perform host-cell reactivation of UV-irradiated viral SV40 DNA.

XP cells are not only extremely sensitive to UV, but also to a variety of chemical carcinogens, including 4NQO. Complementation analysis with XP strains from 4 different complementation groups with respect to the repair of 4NQO-induced DNA damage, revealed that the classification of the strains into complementation groups with respect to 4NQO-induced repair coincides with the classification based on the repair of UV damage (chapter 2.2). The gene products being defective in these XP complementation groups appeared to have a similar effect on the repair of different types of DNA lesions, revealing that these gene products operate in steps in the DNA repair process which do not show a lesion specificity.

We studied the kinetics of complementation in fusions involving A and C group XP cells to get information on the mechanism of complementation. The rate of restoration of the repair defect in XP cells shortly after fusion with normal human fibroblasts or with XP fibroblasts of the other group was investigated (chapter 2.3). One strain of group A showed a rapid recovery of repair, reaching the normal level in 2 h after fusion, but a strain of group C showed a slower rate of repair restoration, which took about 16 h to reach the normal level. Two other strains, one of complementation group A and the other of group C showed intermediate rates of recovery. The variation in the rate of complementation, even within the same complementation group, revealed that this rate is not a property characteristic for a specific group.

The biochemical nature of the repair defect in XP was investigated by using a sensitive assay for the detection of pyrimidine dimers, the predominant lesions induced in the DNA by UV (chapter 3.2; appendix: paper II). Seven XP strains chosen from the 7 complementation groups A through G and an XP variant were compared with normal human fibroblasts. The XP variant showed a normal level of dimer removal, whereas 6 of the other XP strains had a greatly reduced capacity to remove this DNA damage, in agreement with the level of repair DNA synthesis (UDS) in these cells. The F group strain XP23OS, however, was found to remove more dimers from its DNA than was expected from the low level of UDS in this strain.

This F group strain was studied in more detail, in order to explain the discrepancy between the rate of dimer removal and the level of UDS. In the same study also group E cells (XP2RO) were included to investigate the rate of dimer removal in relation with its rather normal sensitivity in terms of cell survival (chapter 3.3). When UDS was followed over a longer period than the two or three hours normally used in UDS analysis, it appeared that in XP23OS cells the rate of UDS remained constant, while in normal control cells this rate

was initially higher but lowered after 8 h. Consequently the residual level of UDS was low, approx. 13%, when measured over the first 2 h after irradiation, but increased to 45% over 24 h. In XP2RO cells repair DNA synthesis measured by autoradiography (UDS) was about 50% of the value found in normal human cells. The removal of UV-endonuclease-sensitive sites proceeds in XP2RO cells at a reduced rate. By having BUdR incorporated in the repaired regions, followed by the induction of breaks in these patches by 313 nm UV, it was shown that the reduced repair synthesis is not caused by a shorter length of the repair regions in XP2rO, but is solely due to a reduction in the number of sites removed by excision repair. An explanation for the discrepancy between the reduced capacity to repair damaged DNA and the normal sensitivity to UV as measured in cell survival experiments could not be given.

The common laboratory source of UV used in DNA repair studies, is a lamp that emits ultraviolet radiation with a most effective wavelength at 254 nm. This lamp is an excellent tool for many studies of DNA repair, as it produces predominantly pyrimidine dimers. It can be doubted, however, whether the results obtained with these lamps have any relevance to biological effects produced by sunlight or artificial light sources to which human being are exposed in practice. Therefore, insight in the effects of UV radiation of different wavelengths at the molecular and cellular level is required to understand the clinical features of XP and the interaction of sunlight with the human skin in general. In a first approach of this problem we choose chinese hamster ovary (CHO) cells, which are easily handled in survival and mutation experiments. The cytotoxicity and mutagenicity of far-UV radiation emitted by a low-pressure mercury lamp ("germicidal lamp") was compared with those of near-UV radiation, emitted by a fluorescent lamp with a continuous spectrum (Westinghouse "Sun Lamp") of which only the radiation with wavelengths greater than 290 nm or greater than 310 nm was transmitted to the cells (chapter 4). The radiation effects were compared on basis of an equal number of pyrimidine dimers, the predominant lesion induced in DNA by far-UV, for the induction of which much more energy is needed with near-UV than with 254 nm radiation. The number of induced dimers was determined by a biochemical method detecting UV-endonuclease-susceptible sites. The equivalence of these sites with pyrimidine dimers was shown, qualitatively and quantitatively, in studies using enzymic photoreactivation *in vitro* and chromatographic analysis of dimers. On the basis of induced dimers more cells are killed by >310 nm UV than by >290 nm UV; both forms of irradiation are more lethal than 254 nm UV. Moreover, 5-6x as many mutants are induced per dimer by >310 nm UV than by >290 nm UV; the latter appeared approximately equally mutagenic as 254 nm UV. The differences in lethality and mutagenicity are not caused by differences in repair of dimers: cells with an equal number of dimers induced by either 254 nm or near-UV showed the same removal of UV-endonuclease-sensitive sites, as well as an identical amount of repair replication. The results

indicate that near-UV induces, besides pyrimidine dimers, other lesions which appear of high biological significance. The nature of these lesions and their effects on human cells will be most adequately studied with near-UV sources. Such studies will have practical importance for the understanding of the relation between clinical symptoms of XP patients and the associated defect in DNA repair.

SAMENVATTING

Een belangrijke eigenschap van levende cellen is hun vermogen om het genetisch materiaal, het desoxyribonucleinezuur (DNA), dat de informatie draagt voor al hun verrichtingen, in ongeschonden staat te houden. DNA kan beschadigd worden door een groot aantal verschillende fysische en chemische agentia, zoals ultraviolette straling (UV), ioniserende straling en chemische, meestal kankerverwekkende stoffen als 4-nitrochinoline-1-oxide (4NQO). De cellen van de meeste levende wezens, ook die van de mens, kunnen schades die in het DNA zijn aangebracht, herstellen. Het belang van dit DNA herstel is gebleken in het onderzoek naar de oorzaken van de erfelijke ziekte xeroderma pigmentosum (XP). Het belangrijkste symptoom van deze zeldzame ziekte is de grote gevoeligheid van de huid voor zonlicht. Dit komt tot uiting in afsterving van cellen, wat leidt tot een droge huid (xeroderma), pigmentatie afwijkingen (pigmentosum) en zeer vaak tumoren in de aan zonlicht blootgestelde delen van de huid. Sommige XP patiënten hebben ook meer of minder ernstige neurologische afwijkingen. In 1968 ontdekte Cleaver, dat in kweek gebrachte cellen van deze patiënten in vergelijking met die van gezonde mensen na blootstelling aan ultraviolette straling (UV) een verminderd herstel van het DNA te zien gaven. Deze XP cellen zijn ook veel gevoeliger voor UV, wat blijkt uit een verminderde overleving na bestraling met UV. De voornaamste schade die hierbij een rol speelt is het pyrimidine dimeer. Normale cellen zijn in staat deze schade te verwijderen door middel van een herstelmechanisme dat excisieherstel wordt genoemd. Hierbij wordt de dimeer eruit geknipt en vervangen door nieuw DNA, waarbij de volgorde van de basen in de oude staat wordt terug gebracht. XP cellen bleken deze dimeren er niet of minder goed uit te knippen dan normale cellen. Het is echter gebleken, dat niet bij alle XP patiënten een defect in dit excisieherstel proces aanwezig is. De gevoeligheid voor zonlicht wordt hier waarschijnlijk veroorzaakt door een defect in een proces, dat een rol speelt bij het passeren van DNA schade tijdens de replicatie van het DNA, voorafgaand aan de celdeling. Men noemt dit proces postreplicatie herstel. Deze groep patiënten staat bekend onder de naam XP varianten.

De heterogeniteit van deze ziekte blijkt op klinisch niveau uit een variatie in de aard van de symptomen bij de verschillende patiënten, terwijl het op cellulair niveau tot uiting komt in verschillen in activiteit van de herstelprocessen. Deze heterogeniteit zou kunnen wijzen op verschillen in de genetische achtergrond van de hersteldefecten in verschillende XP patiënten. Deze genetische heterogeniteit kan onderzocht worden door middel van een zogenaamde complementatie analyse, waarbij de genetische eigenschappen van cellen, afkomstig van twee patiënten, worden samengebracht in één hybride, tweekernige cel (heterokaryon). Door De Weerd-Kastelein en medewerkers werd in 1972 aangetoond, dat de aanwezigheid van het erfelijk materiaal (de genomen) van twee

patiënten in één heterokaryon kon leiden tot een normaal DNA herstel van de hybride cel. Dit werd verklaard door aan te nemen dat deze patiënten niet in hetzelfde gen, betrokken bij het herstel, gemuteerd zijn. In dat geval beschikt het partnergenoom over het normale gen, dat bij de ander gemuteerd is. De genproducten van deze normale genen zorgen voor een normaal herstel van het DNA in deze heterokaryon (complementatie). Op deze wijze kunnen de verschillende XP cellijnen worden ingedeeld in zogenaamde complementatie groepen. Tussen cellijnen van één groep treedt geen complementatie op. Thans worden er 7 complementatie groepen, in XP met een defect in het excisieherstel, onderscheiden.

Het doel van het onderzoek dat in dit proefschrift wordt beschreven, is het inzicht te vergroten in de achtergronden van de hersteldefecten in het syndroom xeroderma pigmentosum. In hoofdstuk 2 wordt onderzoek beschreven, gericht op de genetische heterogeniteit, terwijl in hoofdstuk 3 de biochemische achtergrond van defecten in het DNA herstel aan de orde komt. In dit en in het meeste in de literatuur beschreven onderzoek op dit gebied werd als bron voor ultraviolette straling een lamp gebruikt, die UV uitstraalt met een golflengte van voornamelijk 254 nm ("far-UV"). Deze golflengte komt echter niet voor in het zonlicht dat de aarde bereikt. Dit licht bevat UV met golflengtes groter dan 290 nm ("near-UV"). Aangezien de meeste symptomen bij XP samenhangen met bestraling door zonlicht, is het van belang de effecten van "far-UV" en "near-UV" te vergelijken. In een eerste benadering werd dit uitgevoerd met een chinese hamster cellijn. Dit onderzoek is beschreven in hoofdstuk 4.

In ons onderzoek naar de genetische heterogeniteit van XP patiënten werd een complementatie analyse uitgevoerd met cellen afkomstig van een patiënte uit Engeland (XP2BI). Klinisch is deze patiënte interessant, omdat zij tot nu toe (17 jaar) geen tumoren heeft gekregen, terwijl vanaf haar elfde jaar progressieve mentale retardatie optradt. Gekweekte cellen van deze patiënte complementeerden na fusie cellen van alle zes tot dan toe beschreven XP complementatie groepen (hoofdstuk 2.1; appendix: artikel I). Dit betekende het bestaan van een zevende complementatie groep; deze groep werd aangeduid met de letter G. Een indicatie voor een defect in een herstelproces voor UV-schade was, dat deze cellijn veel minder goed in staat is UV bestraling te overleven. Op cellulair niveau bleek, dat cellen van deze patiënte, na bestaling met UV, gestoord zijn in het verwijderen van pyrimidine dimeren uit het DNA.

XP cellen zijn niet alleen extreem gevoelig voor ultraviolette straling, maar ook voor een aantal chemische, kankerverwekkende stoffen, zoals 4NQO. Onderzocht werd of het patroon van complementatie van XP cellijnen na UV bestraling eveneens gold voor blootstelling aan 4NQO. Een identiek complementatie patroon werd inderdaad gevonden (hoofdstuk 2.2). Blijkbaar speelt het verschil in type schade geen rol bij het optreden van complementatie in het heterokaryon. Wanneer voor deze complementatie samenwerking vereist is tussen genproducten van beide oudercellen, dan heeft het gevormde actieve product normale

affiniteit tot beide typen schades. Dit ondersteunt de hypothese dat de mutaties in verschillende genen zijn gelokaliseerd.

Het mechanisme van complementatie werd eveneens bestudeerd in fusies waarin XP cellen uit de A en de C groep betrokken waren. Hiertoe werd de kinetiek van het complementatie proces onderzocht. De snelheid, waarmee het herstel weer optreedt in de XP kern na fusie van de XP cel met een normale mensencel of met een XP cel van een complementerende groep, werd bepaald (hoofdstuk 2.3). Eén lijn van groep A vertoonde een zodanige snelle terugkeer van het herstelvermogen, dat het normale niveau binnen 2 uur na fusie bereikt werd, terwijl een lijn van groep C dit niveau veel langzamer bereikte en er minstens 16 uur voor nodig had. Twee andere cellijnen, één van complementatie groep A en de ander van groep C, bleken het normale niveau met tussenliggende snelheden te bereiken. De variatie in de snelheid van complementatie, zelfs binnen eenzelfde complementatie groep, betekent dat deze snelheid geen eigenschap is, die karakteristiek is voor een bepaalde groep.

Het biochemische karakter van de hersteldefecten in XP cellijnen werd onderzocht met behulp van een gevoelige methode voor de bepaling van pyrimidine dimeren (UV-endo techniek) (hoofdstuk 3.2; appendix: artikel II). Zeven XP lijnen gekozen uit de 7 complementatie groepen A tot en met G en één XP variant werden vergeleken met normale mensencellen. De XP variant verwijderde dimeren uit het DNA even snel als normale cellen, terwijl 6 van de XP lijnen deze schades veel minder efficiënt konden verwijderen. Het dimeer-herstel vermogen van elk van deze lijnen was in overeenstemming met het niveau van herstel DNA synthese, zoals dit met autoradiografische technieken wordt gemeten ("unscheduled DNA synthesis", UDS). De F groep lijn XP230S verwijderde echter meer dimeren uit het DNA dan op grond van het geringe niveau van UDS verwacht werd.

Deze F groep cellijn werd nader bestudeerd, teneinde de discrepantie te verklaren tussen de snelheid van dimeerherstel en het niveau van UDS (hoofdstuk 3.3). Wanneer UDS werd gevolgd over een langere periode dan de twee tot drie uren welke gewoonlijk in UDS analyses gebruikt wordt, bleek dat in XP230S cellen de UDS snelheid constant bleef, terwijl deze in normale mensencellen aanvankelijk hoger was, maar na 8 uren verminderde. Daardoor was het restniveau van UDS laag, ongeveer 13%, wanneer dat werd gemeten over de eerste 2 uren na bestraling, maar dit nam toe tot 45% over 24 uren. In hetzelfde onderzoek werden ook E groep cellen (XP2RO) betrokken, om de snelheid van verwijdering van dimeren te bestuderen in samenhang met de in geringe mate verminderde gevoeligheid, gemeten in overlevingsexperimenten na bestraling. In XP2RO cellen was de herstel DNA synthese ongeveer 50% van de UDS in normale cellen. De vrijwel normale gevoeligheid van deze cellen zou verklaard kunnen worden door aan te nemen dat, in vergelijking met normale cellen, evenveel pyrimidine dimeren worden verwijderd, echter met een kortere lengte van het uitgeknipte en weer ingevulde stuk DNA (herstelgebied). Voor het meten van de omvang van het herstelgebied is gebruik gemaakt van

de inbouw van 5-bromodeoxyuridine (BUdR), in plaats van thymidine, bij de herstel DNA synthese. De aanwezigheid van BUdR veroorzaakt bij blootstelling van de cellen aan UV met een golflengte van 313 nm breuken in het DNA, die op zich een maat vormen voor het aantal en de grootte van de herstelgebieden. Gevonden werd, dat de verminderde herstel DNA synthese niet veroorzaakt wordt door een kortere lengte van de herstelgebieden, maar alleen door een vermindering van het aantal schades dat door excisie herstel verwijderd wordt. Deze conclusie werd bevestigd door de waarneming, met behulp van de UV-endo techniek, dat de verwijdering van pyrimidine dimeren in deze cellen minder efficiënt plaats vindt dan in normale cellen. Een verklaring voor de discrepantie tussen het verminderde vermogen beschadigd DNA te herstellen en de geringe UV gevoeligheid, zoals die tot uiting komt in overlevingsexperimenten, kon niet gegeven worden.

In vergelijkend onderzoek naar de effecten van "far-UV" en "near-UV" werden chinese hamstercellen bestraald met UV met een golflengte van 254 nm ("far-UV") of met "near-UV" dat zodanig gefilterd werd, dat of UV met golflengtes groter dan 290 nm of groter dan 310 nm werd doorgelaten. Door "far-UV" worden efficiënt pyrimidine dimeren geinduceerd, terwijl de frequentie van dit type schade bij langere golflengtes afneemt. Het vermogen van de straling cellen te doden en het vermogen mutaties aan te brengen werden onderzocht door deze parameters te betrekken op het aantal geinduceerde pyrimidine dimeren (hoofdstuk 4). Het aantal geinduceerde dimeren werd bepaald met de UV-endo techniek. Eerst werd aangetoond dat deze techniek specifiek is voor het aantonen van pyrimidine dimeren. Uitgaande van een gelijk aantal geinduceerde dimeren was de gevoeligheid van deze cellen in overlevingsexperimenten het grootst bij blootstelling aan UV met golflengtes groter dan 310 nm. Deze gevoeligheid nam af bij blootstelling aan UV met golflengtes groter dan 290 nm en is het geringst bij blootstelling aan "far-UV". Bovendien werden er per dimeer 5 tot 6 maal zoveel mutaties veroorzaakt door UV met golflengtes groter dan 310 nm dan door UV met golflengtes groter dan 290 nm, terwijl dit laatste type UV evenveel mutaties veroorzaakt als "far-UV". De genoemde verschillen werden niet veroorzaakt door een verschil in het herstel van dimeren. Pyrimidine dimeren werden ongeacht het type UV, waarmee de cellen bestraald waren, met dezelfde snelheid verwijderd, terwijl ook evenveel herstel DNA synthese gemeten werd. Deze resultaten wijzen erop dat "near-UV", naast pyrimidine dimeren, andere DNA schades veroorzaakt die van belang zijn voor de overleving en voor de inductie van mutaties. Hiermee is de noodzaak aangetoond, dat voor het begrijpen van de relatie tussen de klinische verschijnselen die worden waargenomen bij XP patiënten en de ermee verband houdende DNA herstel defecten, onderzoek met "near-UV" bronnen noodzakelijk is.

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CURRICULUM VITAE

In 1949 werd Bauke Zelle te Leeuwarden geboren. Het eindexamen HBS-B werd in 1968 afgelegd aan de Chr. H.B.S. en M.M.S. te Leeuwarden. De militaire dienstplicht werd vervuld van januari 1969 tot en met april 1970. In september 1970 werd aangevangen met de studie in de scheikundige technologie aan de Technische Hogeschool te Delft. Het kandidaatsexamen werd in december 1973 afgelegd. In 1974 werd hem de Unilever Chemieprijs 1974 voor de TH Delft toegekend. Het afstudeeronderzoek werd verricht in het Laboratorium voor Biochemie en Biofysica, en stond onder leiding van Prof. W. Berends en Ir. A.T. Makaliwy. Tijdens deze periode was hij als student-assistent aan het Laboratorium verbonden. Het ingenieursexamen werd in juni 1975 afgelegd.

In augustus 1975 trad hij in dienst van het Medisch Biologisch Laboratorium TNO, waar onder leiding van Dr.Ir. P.H.M. Lohman een aanvang werd gemaakt met het in dit proefschrift beschreven onderzoek. Dit dienstverband eindigde op 31 december 1978. Afronding van het onderzoek werd mogelijk gemaakt door een tijdelijke aanstelling bij de Erasmus Universiteit te Rotterdam, als medewerker van de vakgroep Celbiologie en Genetica.

Een door de Organisatie voor Zuiver Wetenschappelijk Onderzoek verleend stipendium stelt hem in staat vanaf februari 1980 een jaar onderzoek te verrichten in het Laboratory of Radiobiology van Prof.Dr. J.E. Cleaver, University of California, San Fransisco, U.S.A.

NAWOORD

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A SEVENTH COMPLEMENTATION GROUP IN EXCISION-DEFICIENT XERODERMA PIGMENTOSUM

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Summary

Cells from a xeroderma pigmentosum patient XP2BI who has reached 17 years of age with no keratoses or skin tumours constitute a new, 7th complementation group G. These cells exhibit a low residual level of excision repair, 2% of normal after a UV dose of 5 J/m^2 and an impairment of post-replication repair characteristic of excision-defective XPs. They are also sensitive to the lethal effects of UV and defective in host-cell reactivation of UV-irradiated SV40 DNA.

Two classes of xeroderma pigmentosum (XP) patients have been recognized based on the response of their cells to ultraviolet light in DNA-repair test systems. One class, subdivided into 6 complementation groups [12,13,8,4] comprises patients whose cells are defective in the excision of pyrimidine dimers. The second so called XP-variant class, is characterized by cells with a defect in the ability to convert newly synthesized DNA from low to high molecular weight after UV irradiation [10].

In our search for new DNA-repair mutants we investigated the cells of an XP patient, recently described by Cheesbrough and Kinmont [6], XP2BI. By complementation analysis this patient is now assigned to a new complementation group (group G). In this paper the results of DNA-repair analysis of XP2BI cells at the molecular and cellular level and of cell fusion are described.

Materials and methods

Cell strains and culture conditions

XP2BI is a strain originating from a skin biopsy of a 17-year-old girl suffering from XP [6]. In this patient photosensitivity of the skin was noticed at the age of 3 months. There is marked freckling, telangiectasia, pigmentation and atrophy in the exposed areas of the skin but no keratoses or skin tumors. Mentally she has deteriorated since the age of 11 years. She is small, with bird-like facies. Her age at the time of biopsy was 16 years.

The other XP strains and the control cell strains are described in Table 1. Cells were cultured in F10 medium supplemented with 15% foetal calf serum (FKS) and antibiotics. The survival experiments were performed in Eagle's MEM + 15% newborn calf serum, the host-cell reactivation experiments in Eagle's MEM + 15% FKS.

Cell survival

Cell-survival experiments were carried out as described earlier [5], with some modifications. The cells used for the feeder layer were irradiated with 5 krad of cobalt-60 γ-radiation. The colonies were fixed and stained with methylene blue after 16 days of growth in Eagle's minimum essential medium, supplemented with 15% newborn calf serum and antibiotics and with no medium changes.

Host-cell reactivation of UV irradiated SV40 DNA

SV40, clone 307L, was used to infect CV-1 cells at a multiplicity of infection of 0.01 PFU/cell. Viral DNA was extracted from infected cells by the Hirt procedure [7] and purified by ethanol precipitation and two cycles of banding in CsCl-equilibrium gradients (density 1.56 g/cm^3) containing ethidium bro-

TABLE 1

FIBROBLAST STRAINS USED IN THIS STUDY

Cell strain	Origin	Complementation group	UDS level (% of control)	Ref.
XP25RO	Exc.-def.XP	A	0— 5	8
XP11BE	Exc.-def.XP	B	5	8
XP4RO	Exc.-def.XP	C	10—15	8
XP5BE	Exc.-def.XP	D	25—55	8
XP2RO	Exc.-def.XP	E	40—60	13
XP23OS	Exc.-def.XP	F	10	4
XP30RO	XP variant	—	88	9
XP7TA	XP variant	—	99	9
XP5SE	XP variant [a]	—	85	11a
CV1	normal monkey	—	—	—
VH-8	normal human	—	100	—
C1RO	normal human	—	100	—
C3RO	normal human	—	100	—
C5RO	normal human	—	100	—
C7RO	normal human	—	100	—
AH	normal human	—	100	—
C2BI	normal human	—	100	—

[a] The XP5SE strain has previously been assigned to complementation group E [11a]. A reinvestigation showed that this strain is an XP variant and not an E group strain.

mide at 200 μg/ml. Only the closed circular DNA (Form 1) present in the denser band was used. Ethidium bromide was removed from the DNA by extraction with iso-amyl alcohol and the DNA was dialysed in Tris-buffered saline (TBS).

The infectivity of SV40 DNA in human cells was assayed by determining the number of infective centres on CV-1 cells, according to the method described previously [1].

Excision-repair analysis

The extent of excision repair after UV exposure was measured autoradiographically by the incorporation of [^{3}H]thymidine (^{3}H-TdR) in cells in the G1- or G2-phase of the cell cycle (unscheduled DNA synthesis, UDS).

Cultures grown for 2 days after seeding were labelled for 1 h with 10 μCi ^{3}H-TdR per ml medium (spec. act. 2 Ci/mmol), washed and exposed to UV doses of 5, 10 or 50 J/m^2. The cultures were grown again in the ^{3}H-TdR-containing medium for 2 h and fixed. Autoradiography was performed using Kodak AR10 stripping film with an exposure time of 1 week.

Excision of pyrimidine dimers was measured by applying the UV-endonuclease technique essentially as described by Paterson et al. [11] (UV-endotest). The number of UV-endonuclease susceptible sites in the DNA after exposure to UV light was determined by treating the extracted DNA with a crude extract of *Micrococcus luteus.*

Post-replication repair

Semiconfluent cells were subcultured and 10^5 cells were seeded in 5-cm petri dishes. After 24 h they were UV irradiated with 7.0 J/m^2, incubated for a further 60 min and then pulse-labelled for 60 min with ^{3}H-TdR (33 μCi/ml, spec. act. 20 Ci/mmol) and chased for 3 h with medium containing unlabelled thymidine and deoxycytidine (10^{-5} M). Where indicated caffeine was present in all post-irradiation incubations at a concentration of 300 μg per ml. The labelled DNA was analysed by centrifugation in alkaline sucrose gradients and the weight-average molecular weights were determined [10].

Complementation test

The XP2BI cells were fused with the 6 XP cell strains representing the groups A—F, and grown on coverslips as described elsewhere [12]. After 2 days the cells were prelabelled for 1 h with ^{3}H-TdR (10 μCi/ml, spec. act. 2 Ci/mmol), washed, UV-irradiated with 10 J/m^2, labelled with ^{3}H-TdR for 2 h and fixed. Autoradiographic preparations were made with Kodak AR10 stripping film. The exposure time was 1 week. In each preparation 50 binuclear cells were counted to determine the number of grains on each of the two nuclei.

UV irradiation

In UDS and UV-endo experiments the cells were exposed to UV (predominantly 254 nm) at a dose rate of 0.7 J/m^2/sec from a Philips TUV low pressure mercury tube (15 W). Irradiation in survival experiments was performed with a Hanovia low pressure mercury lamp (dose rate: 0.8 J/m^2/sec for normal cells and 0.15 J/m^2/sec for XP2BI cells), and in host-cell reactivation experiments SV40 DNA was irradiated with 30 W Philips TUV lamp (dose rate: 5 J/m^2/sec).

Results

Survival

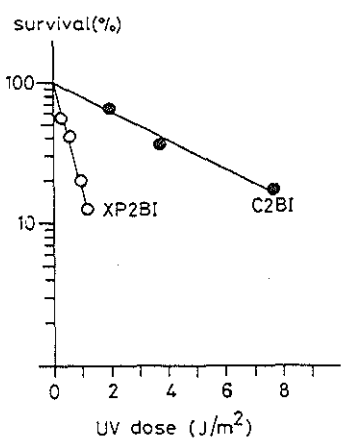
The results of survival experiments are presented in Fig. 1. XP2BI proved to be clearly sensitive to UV-light when compared to cells from an unaffected individual. The D_{37} values were 0.6 and 4.4 J/m^2 for the XP2BI and control cells respectively.

Host-cell reactivation

The repair capacity of XP2BI cells was determined in host-cell reactivation experiments using UV-irradiated SV40 circular DNA. As shown in Fig. 2 the surviving fraction of SV40 virus in XP2BI cells is lower than in control cells. The D_{37} for control VH-8 cells was 11.5×10^2 J/m^2 and for XP2BI cells 2.5×10^2 J/m^2 which corresponds to 21% of the value for normal cells.

Excision repair

UV-stimulated UDS in XP2BI cells is presented in Fig. 3. The UDS pattern of the XP2BI strain is characteristic for an excision-deficient XP strain. At 5 J/m^2 the residual activity of this strain was 2%. The defect in excision was confirmed with the UV-endo test. The number of UV-endonuclease sensitive sites induced in the DNA of XP2BI and AH cells after exposure to a dose of 2.9 J/m^2 UV was 0.09 per 10^6 daltons. The time course for the disappearance of UV-endonuclease sensitive sites during post-irradiation incubation of the cells is depicted in Fig. 4. XP2BI cells do not show detectable excision of endonuclease susceptible sites.



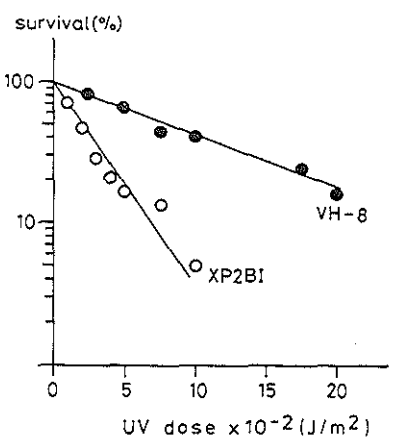
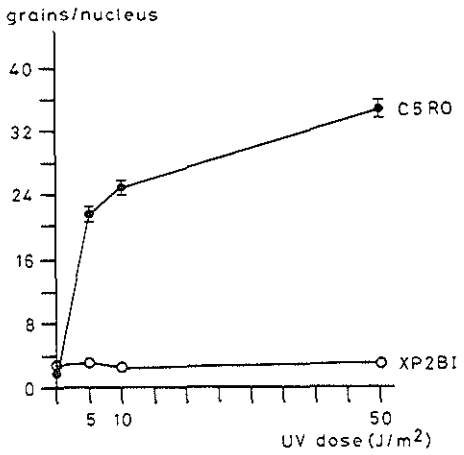


Fig. 1. Colony-forming ability (survival) of XP2BI (○) and control human cells (C2BI) (●) after UV irradiation. Cloning efficiency of XP2BI cells: 45%, and control cells 35%.

Fig. 2. Survival of UV-irradiated Form 1 SV40 DNA in XP2BI (○) and control human cells (VH-8) (●).



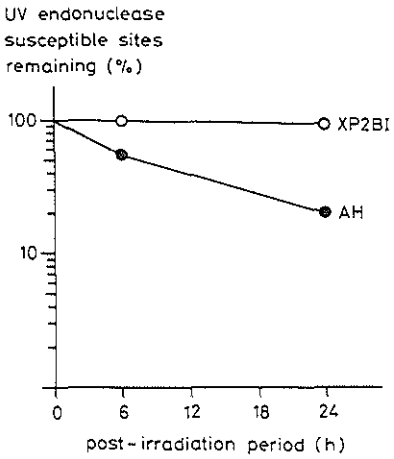


Fig. 3. UV-induced unscheduled DNA synthesis in XP2BI (○) and control human cells (C5RO) (●).

Fig. 4. Time course of disappearance of UV-endonuclease susceptible sites from the DNA of XP2BI (○) and of normal cells (AH) (●) irradiated with a dose of 2.9 J/m^2 UV light.

Post-replication repair

The weight-average molecular weight of newly synthesized DNA following UV exposure of XP2BI cells was compared with that of irradiated control, XP-variant and complementation group A cells. The results are summarized in Table 2. The molecular weights of the DNA from XP2BI cells were lower than those of control cells and higher than of XP-variant cells. This response is characteristic for excision deficient XP cells (e.g. XP25RO).

TABLE 2

POSTREPLICATION REPAIR IN DIFFERENT HUMAN CELL STRAINS

Cell strain	Classification	Weight-average molecular weight (X10^6)			
		Pulse only		Pulse + chase	
		—Caffeine	+Caffeine	—Caffeine	+Caffeine
C3RO	Normal	97	77	168 ± 20 (3) [a]	153 ± 18 (3)
C5RO	Normal	117	63	164	145
C1RO	Normal	110	80	160	150
XP30RO	Variant	35	26	94	39
XP7TA	Variant	44	23 ± 4 (3)	99 ± 7 (3)	35 ± 4 (3)
XP5SE	Variant	51	27	146 ± 6 (2)	51 ± 6 (2)
XP25RO	Group A	72 ± 9 (3)	49 ± 4 (3)	115 ± 7 (3)	75 ± 2 (3)
XP2BI	Group G	70 ± 11 (3)	49 ± 1 (3)	118 ± 5 (3)	77 ± 3 (3)

[a] Mean ± SE; numbers in parentheses: number of experiments.

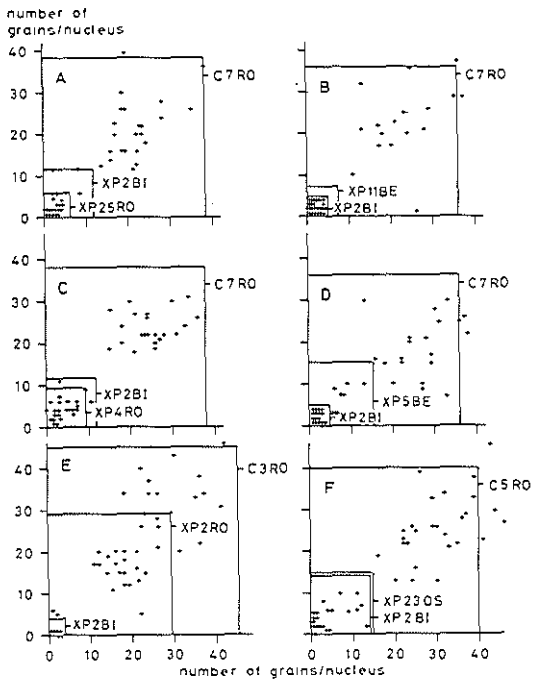


Fig. 5. Complementation analysis with the XP2BI strain. XP2BI cells were fused with XP25RO (A), XP11BE (B), XP4RO (C), XP5BE (D), XP2RO (E) and XP23OS (F) cells. Each point represents one binuclear cell, determined by the number of grains over both nuclei. The squares indicate the areas enclosing the grain numbers found over parental nuclei and nuclei of control cells with 95% tolerance limits.

Complementation test

The XP2BI cells were fused with cells of a representative strain from each of the complementation groups A, B, C, D, E and F. In the fused and UV-exposed populations the binuclear cells were chosen at random and after autoradiography the number of grains over each nucleus was counted (Fig. 5). In the 6 different fusion experiments binuclear cells were obtained showing UDS levels in the range of the normal cells. These binuclear cells indicate the occurrence of complementation after fusion of XP2BI cells with cells of all 6 complementation groups. Therefore, the XP2BI strain represents a new complementation group.

Discussion

This paper presents the evidence for a 7th complementation group amongst excision-deficient XPs. The sensitivity of the patient's skin to sunlight is reflected in the UV sensitivity of her cultured fibroblasts (XP2BI) in survival experiments. The D_{37} of 0.6 J/m^2 is comparable with the D_{37} of XP strains assigned to complementation group C [9]. These XP2BI cells appear to be

more resistant to UV than the A and D group strains derived from patients having severe neurological abnormalities [2].

In host-cell reactivation experiments with UV-exposed SV40 DNA the response of XP2BI cells is between that of the C and B group cells tested [1]. In this assay system XP2BI cells are also more resistant than A and D group cells from patients with neurological defects [1]. The properties of this strain would appear to be at variance with the hypothesis of Andrews et al. [2] who suggest a relationship between UV-sensitivity at the cellular level and neurological involvement at the clinical level. However, the rather late expression of mental abnormalities in this patient is in accordance with a relationship between UV-sensitivity and time of onset of the patients' neurological abnormalities [3]. This patient presents another example showing that low levels of UDS are compatible with the absence of tumours in the skin. A similar observation was made by Arase et al. [4] concerning the patient whose cells were assigned to the new complementation group F (XP23OS). It is not possible, however, to quantitate the extent of exposure to sunlight for these individuals.

As demonstrated by the UV-endonuclease assay system the biochemical defect in the G-group strain seems to be located in a step of the dimer-excision repair pathway preceding the excision of the dimer. If we assume that the recovery of UDS in binuclear cells is the result of intergenic complementation, the XP2BI strain represents the 7th gene, affecting a preexcision step in DNA repair and capable of mutation to an XP phenotype.

Acknowledgements

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II

Mutation Research, 62 (1979) 363—368

REPAIR OF UV-ENDONUCLEASE-SUSCEPTIBLE SITES IN THE 7 COMPLEMENTATION GROUPS OF XERODERMA PIGMENTOSUM A THROUGH G

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Summary

7 strains of human primary fibroblasts were chosen from the complementation groups A through G of xeroderma pigmentosum; these strains are UV-sensitive and deficient in excision repair of UV damage on the criterion of unscheduled DNA synthesis (UDS). They were compared with normal human fibroblasts and one xeroderma pigmentosum variant with regard to their capacity to remove pyrimidine dimers, induced in their DNA by UV at 253.7 nm. The XP variant showed a normal level of dimer removal, whereas 6 of the other XP strains had a greatly reduced capacity to remove this DNA damage, in agreement with their individual levels of UDS. Strain XP230S (complementation group F), however, only showed a 20% reduction in the removal of dimers, which is much less than expected from the low level of UDS in this strain.

Cells derived from patients with the hereditary condition xeroderma pigmentosum (XP) are abnormally sensitive to ultraviolet radiation (UV). The sensitivity is ascribed to a deficiency in the repair of damaged DNA. In most patients a defect in the process of excision repair is involved, which becomes manifest when the extent of repair synthesis of DNA after UV-irradiation is studied [3,7]. Some XP strains, however, do not show a reduced level of unscheduled DNA synthesis (UDS) when compared with normal human fibroblasts. These strains, known as XP variants, presumably have a defect in a process called post-replication repair [8]. The excision-repair-deficient XP strains have so far been classified into 7 different complementation groups on the basis of UDS studies with heterokaryons [4].

One of the techniques for measuring the repair of UV-induced DNA damage is a determination of the disappearance from the DNA of sites that are sus-

ceptible to a UV endonuclease from the bacterium *Micrococcus luteus* [11]. This enzyme recognizes the pyrimidine dimers, which are the predominant lesions in UV-irradiated DNA, and acts by nicking the strand close to the dimer. The specificity of the UV-endonuclease has been verified by studying the effect of enzymic photoreactivation [13].

At present, the application of this technique to XP cells has been reported only for a few strains, belonging to the complementation groups A and C [11], and for one variant [8]. Here we report results of a comparative study with the UV-endonuclease technique on the repair capabilities of 7 strains taken from the 7 complementation groups of excision-repair-deficient XP cells, of one XP variant and of two normal human fibroblast strains.

Materials and methods

The primary fibroblasts used are given in Table 1.

Essentially the UV-endonuclease assay according to Paterson and coworkers [11] was used, but the method was modified to obtain DNA of a higher molecular weight *. Primary fibroblasts were seeded in culture dishes (Greiner; diameter 6 cm) at a density of about 10^5 per dish, provided with 3 ml of Ham's F10 medium containing 15% (v/v) foetal calf serum and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 2 days of incubation the cells were labelled for 20 h with 0.5 μCi [^{3}H]thymidine per ml of medium (19 Ci/mmole). Then the cultures were washed twice with PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.7 mM KCl). The drained cultures were irradiated with a single Philips 15-W TUV lamp, at a dose rate of 0.39 W/m^2 of 253.7-nm radiation as determined actinometrically according to the procedure of Parker and Hatchard [5]; the value was corrected for the contribution of light of longer wavelengths on the basis of the relative intensities given by the manufacturer. Subsequently, the cells were incubated with fresh, unlabelled medium for periods from 0 to 32 h, then washed with PBS and stored at −80°C for not more than 3 days.

For the determination of the number of sites susceptible to UV endonuclease the cells were lysed at room temperature by incubation for 2–3 min with a solution of 1% (w/v) sarkosyl in 0.1 M NaCl, 0.01 M EDTA and 0.1 M Tris (pH 7.6). DNA was extracted with phenol (equilibrated against 0.1 M Tris at pH 7.6) at room temperature by gently shaking the phases in a tube at an angle of about 45° for 1 h at a frequency of 1 Hz and a horizontal amplitude of 2 cm. After centrifugation for 15 min at 10 000 rpm the top aqueous layer was transferred to a dialysis bag by a pipette with wide opening to minimize shearing of the DNA. The dialysis bags were prepared by twice dipping a 15-ml conical tube 3 cm deep into a 7% (w/v) solution of Parlodion (Mallinckrodt, U.S.A.) in a mixture of equal volumes of ethanol and ethoxyethane, followed by exposure to the air for one minute; the tubes were then immersed in water

* The described modifications herein were introduced by R.J. Reynolds during his stay as postdoctoral fellow in our laboratory.

TABLE 1

CELL STRAINS USED

Strain	Nature	UV-induced UDS (% of normal)
AH	control	—
HAN	control	—
XP5SE	XP variant [a]	70 [15]
XP25RO	XP group A	0—5 [9]
XP11BE	XP group B	3—7 [14]
XP4RO	XP group C	10—15 [9]
XP3NE	XP group D	25 [9]
XP2RO	XP group E	40—60 [9]
XP23OS	XP group F	10 [2]
XP2BI	XP group G	10—15 [b]

[a] First classified as E [15], but recently shown to be a variant [16].
[b] W. Keijzer and D. Bootsma, personal communication.

for 5 min, dipped in ethanol, and the resulting bags were pulled off and stored in water. The bags were used while floating in a standard scintillation vial filled with 20 ml of dialysis buffer (0.1 M NaCl, 0.01 M Tris and 1 mM EDTA, pH 7.6). After the bags had been loaded with the DNA samples the buffer was renewed 3 times at intervals of about 30 min, and dialysis was completed overnight at 4°C. 2 aliquots (90 μl) of each dialysed DNA preparation were provided with 10 μl tRNA (1 mg/ml; GIBCO, U.S.A.) and incubated at 37°C for 15 min, one with and the other without 10 μl of a crude extract of *Micrococcus luteus*. This extract corresponds with fraction II in the purification method of Carrier and Setlow [6]. The tRNA was added to suppress the aspecific endonucleolytic activity of the enzyme preparation. After the incubation the samples were cooled to 0°C, 110 μl 1 M NaOH with 0.01 M EDTA was added and 200 μl were layered on top of a 3.65-ml isokinetic alkaline sucrose gradient (5—20% w/v, 0.5 M NaCl; 0.01 M EDTA and 0.2 M NaOH). The gradients were centrifuged in a Spinco SW60Ti rotor at 40 000 rpm and 20°C for 80—100 min.

The subsequent procedures, involving fractionation, liquid-scintillation counting and computer analysis of the radioactivity distribution in the gradients were performed as described elsewhere [11]. They resulted in a calculation giving the number of single-strand breaks per unit length of DNA. To obtain the number of enzyme-induced single-strand breaks, the difference was taken between the number of breaks scored in the DNA aliquot treated with the UV-endonuclease preparation and the value found with the untreated sample. Then a correction was applied for the aspecific breaks caused by residual aspecific endonucleases in the Micrococcus extract; for this correction, unirradiated cells were subjected to the complete procedures. In this manner the true number of UV-induced, UV-endonuclease-susceptible sites was obtained.

As a rule the experiments were performed in duplicate; the results of corresponding studies agreed within 10%.

Results and discussion

With the UV-endonuclease method the repair of UV-induced, UV-endonuclease-susceptible sites in the 10 strains was studied after a UV dose of 2.9 J/m^2 and repair periods up to 32 h. The UV dose used induced, on average, 0.1 UV-endonuclease-sensitive sites per 10^6 molecular weight of DNA. (This number of lesions does not saturate the cellular repair system of normal human cells, as was shown by measurement of the repair synthesis of DNA [10].) Fig. 1a shows the removal of sites in normal cells; there was no difference between the two strains. The XP variant behaved identically with the normal cells (Fig. 1b), but the other XP strains showed a reduced ability for the removal of UV lesions. According to this criterion the representatives of the complementation groups A, B, C, D and G are virtually devoid of excision repair. The other two strains have a significant residual activity.

It is tempting to compare the capacity to remove UV-endonuclease-susceptible sites with the ability to perform UDS after UV irradiation. A straightforward comparison is not possible, however, because the two criteria show several differences. First, they do not relate to the same repair period. Normally, UDS is determined over the first 2—3 h after irradiation, whereas accurate measurements of the removal of UV-endonuclease-sensitive sites necessitate much longer incubation periods, usually 12—24 h. Second, the two methods have different specificities, because UDS is the result of the excision repair of

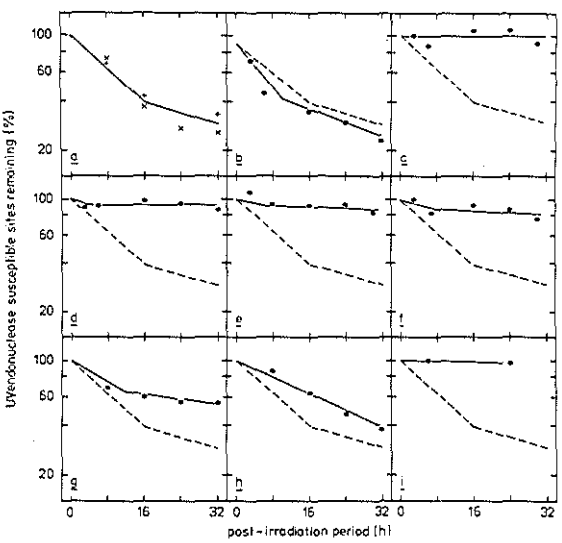

Fig. 1. The removal of UV-endonuclease-susceptible sites from the DNA of various strains of primary human fibroblasts during incubation after a dose of 253.7-nm UV of 2.9 J/m^2. a: normal cells; +, AH and X, HAN; b: XP5SE, XP variant; c: XP25RO, group A; d: XP11BE, group B; e: XP4RO, group C; f: XP3NE, group D; g: XP2RO, group E; h: XP230S group F; i: XP2BI, group G. To facilitate a comparison, in panels b—i the normal curve as shown in panel a is indicated by the dashed line.

various lesions, whereas with UV endonuclease only the removal of pyrimidine dimers is studied. A third difference is that the amount of UDS depends not only on the number of excised lesions — as in the UV-endonuclease method — but also on the length of the repair patches, which may vary with the lesions or with the cell strain.

However, one usually expects to find a certain correlation between the two phenomena, as has been shown for some XP strains [8,11]. Our results, too, show — in general — a fair correlation with the published UDS levels. For the comparison, we have related the number of sites removed in the XP strains 24 h after the UV irradiation to the corresponding results obtained with normal cells. As can be seen by comparing Fig. 1 with Table 1, the lack of excision repair found for the XP groups A, B, C, D and G agrees with the low levels of UDS in these strains. Strain XP2RO (Fig. 1g), complementation group E, has a reduction in repair activity of about 40%, which coincides with the reduction found with UDS. The representative of group F, XP230S, shows about 80% of the normal repair activity. The level of UDS in this strain, however, is only 10% of normal. It has been reported that the resistance to UV and the capacity of this strain to perform host-cell reactivation is higher than that of other XP strains with the same UDS level [2].

The approximate 50% reduction in XP2RO with regard to both site removal and UDS suggests that, in this strain, the normal excision-repair process occurs, giving normally sized repair patches, but at a reduced rate. This was shown to be true in separate experiments by direct assay of the number and average size of the gaps that are filled in by repair synthesis; the BUdR-photolysis technique [12] was used for this purpose (Zelle and Lohman, in preparation). The discrepancy observed with XP230S, however, between the level of UDS and the extent of site removal cannot be explained unequivocally at present. Two possibilities could account for these results. First, this strain may remove pyrimidine dimers with a repair process that gives shorter repair patches than the process in normal cells. Second, it is possible that the kinetics of the repair process differ from those of normal cells in such a manner that, in XP230S, site removal and gap filling proceed at a lower rate, but eventually reach the same level as in normal cells. In this case UDS is expected to be almost normal when studied for 24 h after irradiation. Investigations to check these two possibilities are in progress.

The XP strains tested, chosen from the complementation groups A—G, all showed a reduced capacity to repair UV-endonuclease-susceptible sites, i.e. pyrimidine dimers, when compared with normal human fibroblasts. In most XP strains tested this capacity corresponded with the level of unscheduled DNA synthesis as determined by autoradiography, with the exception of strain XP230S. However, the data presented here for a certain XP strain cannot be considered to indicate the level of repair for all members of the corresponding complementation group. Even though we accept that a single mutation can cause the XP-repair deficiency, and that the members of one complementation group are mutated in the same gene, the effect of different mutations may still be different. The gene product may be totally absent, or modified so as to be less active. In principle, therefore, XP strains belonging to the same complementation group may very well vary in their individual level of repair. This can

be illustrated with members of the A group: XP8LO shows more UDS than all other members studied so far [17]; moreover, with regard to survival, XP8LO and XP12BE are markedly less sensitive to UV than are other representatives of group A [1].

Acknowledgements

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