# DNA repair and the aging process



jan vijg

# DNA REPAIR AND THE AGING PROCESS



proefschrift
(DNA + ouderdom)

# STELLINGEN

1

De conclusie van Yagi et al. dat een relatief lage concentratie endogeen thymidine verantwoordelijk is voor de discrepantie tussen UDS en de verwijdering van pyrimidinedimeren in UV-bestraalde muizecellen zou sterk aan kracht hebben gewonnen indien door deze auteurs naast de gemiddelde grootte van de "herstelpatches" tevens het aantal daarvan zou zijn vermeld.

Yagi, T. et al., Mutation Res., 132 (1984) 101.

2

De door Weinfeld et al. gepostuleerde verbreking van de fosfodiesterband tussen de dimeervormende pyrimidines als initiële stap in het excisieherstelproces in UV-bestraalde humane cellen houdt geen rekening met de resultaten zoals verkregen door o.a. Zelle en Lohman voor humane cellen behorend tot xeroderma pigmentosum complementatiegroep F.

Weinfeld, M. et al., Biochemistry, 25 (1986) 2656.
Zelle, B. en P.H.M. Lohman, Mutation Res., 62 (1979) 363.

3

Bij de suggestie van Bohr et al. dat de discrepantie tussen de relatief hoge mate van overleving en het lage excisieherstelniveau van UV-bestraalde CHO cellen te wijten zou zijn aan preferentieel herstel van schade aan vitale DNA-sequenties is geen rekening gehouden met de resultaten van Mitchell et al., die aantonen dat in hetzelfde celtype thyminedimeren, gemeten m.b.v. specifieke antilichamen, snel worden verwijderd.

Bohr, V.A. et al., Cell, 40 (1985) 359.
Mitchell, D.L. et al., Biochim. Biophys. Acta, 697 (1982) 270.

4

De conclusie van Westerveld et al. een DNA-herstelgen te hebben gekloond is voorbarig.

Westerveld, A. et al., Nature, 310 (1984) 425.

5

Binnen het huidige molekulair-genetisch onderzoek wordt ten onrechte het accent gelegd op de bestudering van eiwitcoderende DNA sequenties.

6

Het nut van Drosophila als in vivo modelsysteem in de genetische toxicologie wordt beperkt door het feit dat aktief delende somatische cellen in het adulte stadium van dit organisme ontbreken.

7

Het gepostuleerde in vitro modelsysteem voor veroudering - de afname in proliferatieve aktiviteit met het aantal populatieverdubbelingen van humane fibroblasten - verliest veel van zijn waarde indien men zich realiseert dat Drosophila, een organisme zonder aktief delende somatische cellen, net als zoogdieren degeneratieve verouderingsverschijnselen vertoont en daaraan sterft.

8

Het ontbreken van een algemeen aanvaarde definitie voor het begrip "veroudering" veroorzaakt verwarring in het experimenteel gerontologisch onderzoek bij het leggen van causale verbanden en de vaststelling van criteria voor het toetsen van hypotheses.

9

Het verdient aanbeveling te onderzoeken of de staven van hoofdkantoren bij het nemen van maatregelen ter verbetering van de arbeidsproduktiviteit zichzelf soms over het hoofd hebben gezien.

10

Het op dit moment belangrijkste criterium voor het toekennen van onderzoeksubsidie, de afwezigheid van enig risico, is in flagrante tegenspraak met de aard van wetenschappelijk onderzoek.

11

De geschiedenis herhaalt zich nooit.

12

De opvatting dat een mens meer zou zijn dan een computer wordt nog slechts ondersteund door het feit dat de laatste goedkoper is.

J. Vijg,
9 april 1987.

# DNA REPAIR AND THE AGING PROCESS

## PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. J.J.M. BEENAKKER, HOOGLERAAR IN DE FACULTEIT DER WISKUNDE EN NATUURWETENSCHAPPEN, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP DONDERDAG 9 APRIL 1987 TE KLOKKE 15.15 UUR

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Aan mijn ouders
Voor Claudia en Mark

# TABLE OF CONTENTS

# CHAPTER 1

## GENERAL INTRODUCTION

### The Significance of DNA Repair for the Aging Process*

### 1.1 What is Aging?

Aging is generally considered as a deteriorative process occurring during the later stages of life and ultimately leading to death. This is rather an intuitive representation of a phenomenon not well understood, than a suitable starting point for a discussion of the nature and characteristics of the aging process. According to Webster's New World Dictionary of the American Language (second college edition, 1970), aging means "to grow old" or "to ripen or mature".

In this thesis, aging of an organism is defined as the total of all changes it undergoes from its conception to its death, including embryonal development, maturation and adulthood. This definition can be easily applied to sexually reproducing higher organisms where a conception can be identified. In this view, prokaryotes and unicellular eukaryotes might be considered to age between two exchanges of genetic material (Smith-Sonneborn, 1981). On the other hand, the decrease in proliferative capacity of somatic mammalian cells cultured in vitro (Hayflick, 1980) should not be considered as aging, although it has been suggested that in this case immortalization and/or neoplastic transformation events replace the "natural" form of genetic information exchanges (Hayflick, personal communication).

In research on aging, most attention has been focused on senescence. According to Webster's, senescence means "the process of becoming old" or "the state of being old". Here the aspect of ripening, clearly present in the meaning of aging, has disappeared. In this thesis, senescence is considered as the complex of non-reversible deteriorative changes, the effects of which become predominantly manifest during the later stages of cellular or animal lifespan. In higher animals, senescence predisposes to fatal disease. As such,

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* Parts of this work have been published previously (Vijg et al., 1985a) or are in press (Vijg and Knook, 1987a).

senescence is responsible for the natural limitation to the lifespan of individual organisms or cells. It should be emphasized that senescent (deteriorative) changes are only one aspect of the biological process of aging. This becomes clear when it is realized that even at old age adaptive "positive" changes occur; although at that time the deteriorative aspect of aging (senescence) predominates, development has not yet come to an end.

In order to obtain a better understanding of the aging process, two fundamental problems should be addressed: (1) the possible teleological component of aging, that is, the purpose of a life cycle with an endogenous finite component and (2) the mechanisms behind senescent changes during the aging process. In this thesis, the question will be discussed to what extent studies on the induction and processing of DNA damage in the genome of cells may contribute to the solution to these problems.

## 1.2 Why do Organisms Age?

### 1.2.1 Senescence genes

Ideas about a possible teleological component in aging are based on hypotheses which were already formulated at the turn of the century. Some of these ideas are still considered to be valid. The most frequently mentioned "explanation" for the existence of aging in nature is by analogy with the wear and tear of a machine. This popular, but scientifically debatable, comparison was the basis of August Weismann's suggestion, made about a century ago, that senescence and death were adaptations fixed in the genome by natural selection with the objective of discarding old worn-out individuals who had become a burden for the group or species (Weismann, 1891). In this form the argument is essentially circular, since it considers the phenomenon which it seeks to explain (senescence) as the very reason for its own existence.

There is, however, some evidence for the occurrence in nature of programmed life termination by the action of genes that code for senescence and death ("senescence genes"). In the case of Australian marsupial mice (at least six species of the genus Antechinus), synchronous degeneration and death occur in all adult males within a few weeks after mating. In this case it seems indeed likely that environmental constraints (e.g., competition for food) have led to the establishment of a semelparous life cycle (reproduction once in a lifetime), of which programmed senescence and death is an integral part (Diamond, 1982). Semelparity is not uncommon among bacteria, invertebrates and plants. In addition, in higher vertebrates certain embryonic tissues (e.g. pronephros,

mesonephros) undergo programmed cell death as a normal part of their developmental process (Saunders, 1966).

The existence of these cases could easily lead to the assumption that in all species senescence is the result of a genetic program for mortality. However, in most species natural selection acts at the level of the individual. This makes the emergence and fixation of senescence genes that benefit the group or species by the removal of old individuals highly unlikely. Indeed, all other individuals in the group would benefit as much as the direct descendants. In other words, the new genetic trait offers no selective advantage in populations with individuals dying at different times. This will preclude the fixation of senescence genes in most higher animal populations. Moreover, whereas senescence in the case of marsupial mice is an efficient and rapid way of removing a lot of animals simultaneously, this cannot be said of senescence as it occurs in humans or captivated animals. Interestingly, when the above described marsupial mice species are kept isolated in laboratory cages, senescence in these animals is as long and tedious as in other captivated animals or in humans (Diamond, 1982). Apparently, because these marsupial mice are programmed to die at an early age as a part of their reproductive life cycle they have no opportunity to undergo "normal" senescence. In most iteroparous animals in the wild the situation is yet much the same. In nature, senescent animals are rare. Death through predation or other factors usually occurs so frequently that there is no chance to become old. This lack of normal senescence in nature is probably the strongest argument against senescence genes. Indeed, how can natural selection act on a trait that is never expressed? In view of the above considerations it seems unlikely that senescence and death in mammals, including humans, are essential and purposeful components of their genetically programmed life cycle.

### 1.2.2 Aging as differentiation

Somewhat related to Weismann's concept of senescence as a genetically determined program is the idea, originally proposed by Charles Minot at the beginning of the 20$^{th}$ century, that senescence is a part of normal differentiation (Minot, 1907). He assumed that senescence only occurred in multicellular organisms (Metazoa), which were thought to "pay the price" for the benefits of differentiation. This idea is interesting because it distinguishes between two categories of cells with respect to the occurrence of senescence and death, i.e. undifferentiated actively dividing cells and terminally differentiated postmitotic cells.

According to Minot, senescence and death were exclusively associated with terminally differentiated cells which were thought to undergo an irreversible decline of vital functions, ultimately leading to their death. Since even present day gerontological research has not yet answered the question whether or not actively dividing undifferentiated cells undergo senescent changes (Miquel et al., 1979; Knook, 1980), Minot's differentiation hypothesis cannot simply be rejected. In addition, there is no clear cut theoretical argument against it, since senescence could be a by-product of differentiation rather than be programmed as such. This possibility has been adopted by Strehler in his codon restriction theory (Strehler and Barrows, 1970). In this theory it is proposed that the differentiation process in each specific cell type is associated with an irreversible restriction in codon usage by its machinery for protein synthesis. This would lead to the inability of terminally differentiated cells to replace certain long-lived (but nevertheless temporary) proteins, due to the fact that the only decoding capacity left matches the cell's synthetic specializations. Since the fatal events resulting from this defect would only become manifest after the reproductive period (initially, codon restriction would be economically advantageous for the individual), natural selection would have favoured such a developmental plan.

The original "differentiation to death" hypothesis of Minot did not include a mechanism for senescence (or differentiation, which is the same in this theory), but it had a "purposeful component". According to Minot, aging was exclusively associated with multicellular organisms, which was of advantage to them, since it gave them differentiation. However, it is now clear that unicellular organisms also age and undergo senescent changes (Smith-Sonneborn, 1981). Therefore, Minot's theory has lost its purposeful component. Any further discussion of this theory is rather futile as long as both aging and differentiation are vaguely defined processes, the mechanisms of which are incompletely understood.

Although neither Weismann's theory in which aging is encoded in the genes because it is good for the group or species, nor that of Minot in which senescence is an integral part of ongoing differentiation, are at the present time satisfactory hypotheses that explain the existence of aging, they nevertheless contributed greatly to gerontological research. For the first time the aging process was considered as a biological phenomenon which can be discussed in a scientific context, experimentally addressed and eventually be understood. Their work forms the basis of all modern concepts in fundamental research on aging.

### 1.2.3. Aging and the control of lifespan

In an evolutionary perspective it seems likely that senescence, irrespective of its mechanism or origin, cannot be prevented by natural selection. It has been argued that the forces of natural selection strongly diminish after the reproductive period and therefore no mechanism to prevent senescence and death has emerged (Williams, 1957; Kirkwood, 1977; Rose, 1983). In this view, the existence of senescence is intricately interwoven with the existence of a reproductive life cycle. With respect to the origin of both phenomena it can be speculated that the reproductive life cycle emerged as the most successful way to deal with senescence, which may have been present since the origin of life. Even the first self-replicating informational molecules, which are now commonly assumed to be RNAs (Eigen et al., 1981), probably already underwent senescence in the form of physical destruction of their phenotypes (their folded configurations) and their genotypes (their RNA base sequences). Recombination, a rudimentary form of sexual reproduction, could have been the way to overcome the damage (Bernstein et al., 1985).

The apparent incapacity of natural selection to generate immortal species does not exclude the possibility that the lifespan can be modified by evolutionary mechanisms. Lifespan is inherent in an organism's developmental plan and is therefore necessarily subjected to natural selection. Because the senescent phenotype, characteristic for individuals towards the end of the maximum species lifespan, is seldom expressed in nature, the phenotypical characteristics acted upon must merely be those that are expressed before and during the reproductive period.

However, under certain circumstances it is imaginable that natural selection acts on characteristics only expressed at old age. This is illustrated by some cases in which extension of lifespan has become advantageous. Certain higher animal species, such as humans, have adapted to a level at which also aged individuals contribute to the likelihood of survival of the group to which they are kin (Mergler and Goldstein, 1983). An aged cohort in a human population can be advantageous by the transmission of information. There are even examples of species that profit from having senescent individuals in the population. For instance, senescent (slow) saturniid moths are readily caught by birds, thereby teaching them that the species is distasteful. In this way, preying of the predator upon younger (and reproductively potent) individuals is precluded (Blest, 1963).

With respect to the control of longevity it can be inferred from the above that natural selection is not likely to act on hypothetical genes that actively

provoke senescence ("senescence genes") nor on genes that completely prevent it by coding for immortality. The two possibilities that are at the present time seriously considered, viz., the pleiotropic gene theory and the longevity assurance theory, have been considered as diametrically opposed to each other (Sacher, 1978a). According to the pleitropic gene theory, senescence is a negative by-product of ontogeny, due to the activity of so-called pleiotropic genes (Williams, 1957). That is, senescence is thought to be the result of late deleterious effects of genes that have beneficial effects early in life. Such genes would therefore have been favoured by natural selection. In a series of studies with Drosophila melanogaster it has been attempted to test the main prediction of this theory: selection for increased lifespan automatically occurs in populations that reproduce at an advanced age (Lints et al., 1979; Rose and Charlesworth, 1980; Luckinbill et al., 1984). The results of these studies were conflicting. More recently, however, Clare and Luckinbill (1985) elegantly showed that the failure of some attempts to select for greater longevity in Drosophila is due to environmental effects that strongly influence the expression of genes determining lifespan. These authors conclusively demonstrated that in Drosophila (a) lifespan is under genetic control and (b) selection for increased lifespan antagonistically reduces early-life fecundity. Thus, the main prediction of the pleiotropic gene theory appears to be correct.

In the longevity assurance theory (Sacher, 1978b) aging is not considered as a process, evolved during evolution, that the organism passively endures. Instead, it is proposed that the control of longevity by natural selection is mediated by genetic systems that govern positive enzymatic mechanisms for the protection, regulation and repair of the essential information molecules in the organism. It follows that according to this theory, non-functional alterations in information molecules are considered as the main causal factor in senescence. The essence of the longevity assurance theory in an evolutionary perspective, is its commitment to the idea of life extension by natural (evolutionary) means, which makes it most applicable to mammals, the (rapid) evolution of which is associated with a dramatic increase in maximum lifespan (Cutler, 1979).

The longevity assurance theory, which is essentially stochastic, and the pleiotropic gene theory, the characteristics of which are programmatic, represent satisfactory explanations for the existence of senescence in nature. Unfortunately, the pleiotropic gene theory is thought to clash with the longevity assurance theory because accumulation of late-acting senescence genes during evolution suggests that originally, senescence did not exist in nature, and that the living state is intrinsically perfect (Sacher, 1978a). This latter possibility, is, at least at first sight, not wholly unrealistic. Indeed, orga-

nisms equipped with the highly sophisticated mechanisms that are necessary for their development and reproduction must be able to develop maintenance mechanisms that completely prevent deterioration. The question of why this did not occur and why senescent processes are so intricately interwoven with living organisms, are not solved by the longevity assurance theory. Likewise, the pleiotropic gene theory does not provide any rational explanation for the problem of how and why genes would switch from having a beneficial to having a deleterious effect (Sacher, 1978a). The answers to such questions might be derived from deeper insight into the mechanisms of senescence. On the basis of such insight it should be possible to reconcile the longevity assurance theory with the pleiotropic gene theory.

## 1.3 How do Organisms Age?

### 1.3.1 Aging at the physiological level

Aging at the physiological level can be considered as a combination of developmental and deteriorative processes. Clearly, at the beginning of the lifespan the developmental component predominates, whereas at the end the deteriorative aspect becomes more and more prominent. In order to study the basic cause of senescence, fundamental senescent processes must be distinguished from secondary phenomena such as adaptive "positive" changes. This, now, has proven to be extremely difficult.

At the physiological level, one of the most prominent characteristics of aging is its variability. Although aging of humans is associated with a gradual reduction in average functional performances after the age of 40 years, not all functions deteriorate at the same rate or to the same extent (Timiras, 1972; Shock, 1983). In addition, there are large variations among individuals with respect to the rate of occurrence of certain deteriorative changes and the pattern of age-related pathology (Martin, 1979; Shock, 1983). In part, this may be due to genetic differences. Longitudinal studies of aging twins revealed that intrapair differences in lifespan and in the incidence of cancer are significantly smaller for monozygotic than for dizygotic twins (Bank and Jarvik, 1978). Although this is suggestive for heredity as a significant factor in determining human lifespan, the details of the pattern of transmission are not clear (Murphy, 1978). In this context, the possibility should be considered that other intrinsic factors are involved, superimposed on the genetic traits that determine maximum species lifespan. This is illustrated by the observed variation in lifespan and patterns of multiple pathology among rodents of the

same strain and sex kept under identical conditions (Burek, 1978; Zurcher and Hollander, 1982). It is not unlikely that such individual variation among inbred animals is to some extent not present ab initio but emerges from continuous genetic and/or epigenetic alterations during aging in each individual.

Extensive cross-sectional and longitudinal studies on physiological functions in relation to age suggest that senescence can not be ascribed to the failure of any single organ system, tissue or cell type. Instead, it has been suggested that the main deteriorative factor in the aging process at the physiological level is the breakdown of the regulatory processes that provide functional integration between cells and organs (Shock, 1977). The resulting inability to purposefully coordinate several functions in supporting homeostasis might then be responsible for the increased sensitivity of the elderly to internal and external environmental stresses (Shock, 1977). This is in keeping with some general characteristics of the aging process, such as the slowing of response and recovery. For instance, glucose removal from the blood is diminished as a consequence of reduced sensitivity of beta cells in the pancreas, the rates of the various recovery processes after exercise are reduced, tissue regeneration is slower and temperature regulation and control are impaired.

Thus, senescence may be ascribed to alterations in the setting of the control mechanisms that regulate functional activity within and between cells rather than to the diminished capacity of one or more organ systems. This line of reasoning is in keeping with the observation of a gradual linear decline in bodily functions, whereas the chance of dying (as a consequence of pathological changes) increases exponentially (Timiras, 1973). The latter might then be due to progressive impairment of the effectiveness of control mechanisms, the basic cause of which may lie at the cellular level. With regard to this latter possibility, it seems likely that most cell types in an organism are subject to deleterious age-related changes (Frolkis, 1984), possibly with the exception of hematopoietic stem cells and germ cells.

Hematopoietic stem cells may not undergo senescent changes, as was indicated by continuous repopulation of mouse bone marrow upon repeated depletion by exposure to an alkylating agent (Valeriote and Tolen, 1983). Furthermore, stem cell lines from old animals function normally, without any loss of proliferative capacity or other intrinsic defects upon transplantation to young irradiated recipients (Harrison, 1979a). However, three to six serial transplantations lead to exhaustion of proliferative capacity (Harrison, 1979b). Whether this involves senescence or is solely an artefact of the transplantation procedure is as yet not clear (Harrison, 1979b).

A second example of a cell type in a living organism that does not seem to undergo senescence are germ cells. Although there can be no doubt that the germ line has an infinite replicative lifespan, germ cells may nevertheless undergo deteriorative (senescent?) changes. For germ cells this is indicated by the increased risk of the older parent of having a child with a genetic disorder, the best example of which is Down syndrome. Advanced maternal age is related to an increased risk of having a child with Down syndrome (Kram and Schneider, 1978). Assuming that senescent changes in germ cells are involved in the etiology of the parental age-related increase in genetic disorders, the question arises of why such cell populations have an infinite lifespan. The answer to this question may be that germ cells and possibly also hematopoietic stem cells, may be programmed - in accordance with their function - to divide indefinitely. Other somatic cells may either functionally differentiate or undergo terminal differentiation. The latter process, which is not necessarily caused by a deteriorative process, should be sharply distinguished from senescence. It is a major misunderstanding that the cessation of mitotic activity of a cell population represents (or is due to) senescence as it occurs in the intact organism. This problem is further discussed in Chapter 5.

It can be inferred from the above that deteriorative changes can occur in cell populations that do not undergo clonal exhaustion. Apparently, selection processes operate in these cell populations which keep them from extinction. In germ cells, one of the selective mechanisms for viability may be spontaneous abortion (Evans, 1984). The frequency of spontaneous abortions strongly increases with age (Kram and Schneider, 1978). In hematopoietic stem cells the thymus and perhaps also other organs may function in the selection and instruction processes associated with maturation of the immunocytes (Bevan, 1981). In neoplastically transformed cells and in actively dividing cells in general, there is continuous selection for cells with a growth advantage.

A great number of age-related changes in the various somatic cell types have been described. For instance, by Schneider and Mitsui (1976) and by Hayflick (1980), who studied human fibroblasts during passaging in vitro and during aging, and by Knook and collaborators, who worked with rat liver cells. Knook's studies are especially noteworthy since they indicated differential age-related changes in certain enzyme activities in the various cell types of the liver (Knook and Sleyster, 1976; Knook, 1980; Wilson et al., 1982). Thus, age-related changes that had been previously observed in the whole organ could now be traced backwards to phenotypical events in specific cell types, and this has been used to support the idea that organisms or organs

senesce because their cells senesce. Such combined studies at the organ and at the cellular level are essential for the elucidation of the fundamental causes of senescence. Further progress, however, has been severely hampered by the fact that any change in a specific cell type may readily influence proper functioning of other cells or give rise to adaptive changes. Therefore, a search at the phenotypic level for the ultimate cause of cellular senescence is unlikely to succeed. However, since cellular phenotypes ultimately reflect the central dogma of molecular biology (DNA → RNA → protein), senescent changes can theoretically be traced backwards via protein and RNA to DNA, the blueprint of cellular information. It has been suggested by numerous authors that changes in these information macromolecules may underly the basic processes in senescence.

A potential source of "spontaneous" alterations in information macromolecules is easily found in the variety of external agents (e.g., radiation, certain environmental components, products of cellular metabolism) which permanently threaten living organisms. According to the longevity assurance hypothesis (see Section 1.2.3), enzymatic sytems have been developed during evolution which limit the rate at which such alterations take place. Modulation of the efficiency of these enzymatic systems during evolution, in conjunction with and driven by evolutionary modulation of the developmental plan, might have led to the large interspecies variation in lifespan which is observed (Cutler, 1980).

### 1.3.2 Aging at the DNA level

Like physiological phenomena, age-related changes at the DNA level can also be subdivided in deteriorative (senescent) changes and developmental ones. Examples of the latter are the generation of immunoglobulin and T cell receptor diversity by a series of programmed changes in the DNA sequence organization of B and T lymphocytes, respectively. As yet these are the only examples of programmed DNA sequence changes in mammals. Comparable changes, associated with other developmental processes, have thusfar not been found. However, it should be mentioned here that developmental changes in patterns of 5-methylcytosine (the major DNA base modification) have been observed in and around a great number of genes (for a review, see Doerfler, 1983). In view of the proposed relationship between DNA methylation and gene expression, it is not inconceivable that the control of gene activity during development is in part based on this type of DNA modification.

The kind of DNA changes that might be categorized under senescence are likely to include DNA damage, that is, abnormalities in the chemical structure

of DNA that may influence its normal template function. Although in principle all information macromolecules are subjected to damage causing their dysfunction, changes in DNA have often been suggested to be the main factor involved in senescence. Such a view is supported by the argument that changes in DNA, the cell's primary source of information, may lead to a permanently altered pattern of gene expression. In contrast, direct changes in any of the other information macromolecules (RNA and protein) are only temporary, provided an intact DNA template is present. On the basis of this argument, it has been suggested that DNA alterations are involved in the etiology of senescence and the multitude of disorders associated with it (Hirsch, 1978).

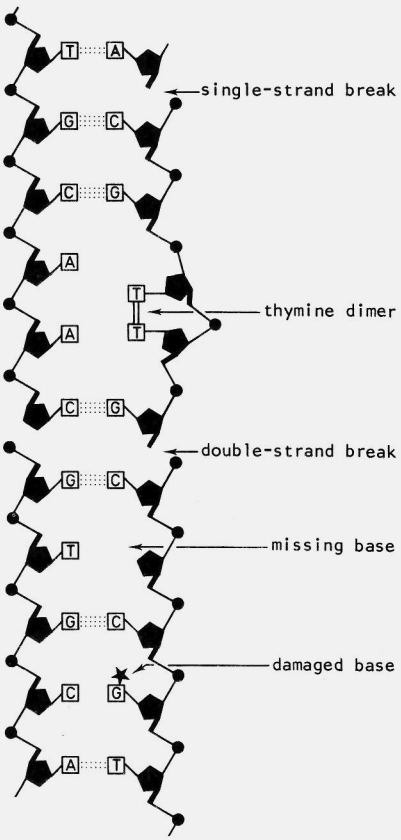


Figure 1.1:
Schematic representation of a part of a DNA molecule containing a number of different lesions.

Most age-related DNA alterations are likely to ultimately result from the continuous activity of damaging agents from the environment or from inside the cell itself. Interaction of these agents with the DNA, either directly or indirectly via cellular processes, may lead to the induction of a spectrum of lesions in the DNA (Fig.1.1). In general, DNA lesions themselves are non-heritable, that is, they are normally not transferred as a genetic trait to daughter cell DNA by semi-conservative DNA synthesis. The fate of induced DNA lesions, in terms of their persistency and potential consequences at the DNA sequence level, is governed by the processing activities of the complex of enzymatic systems collectively termed DNA repair (schematically depicted in Fig.1.2).

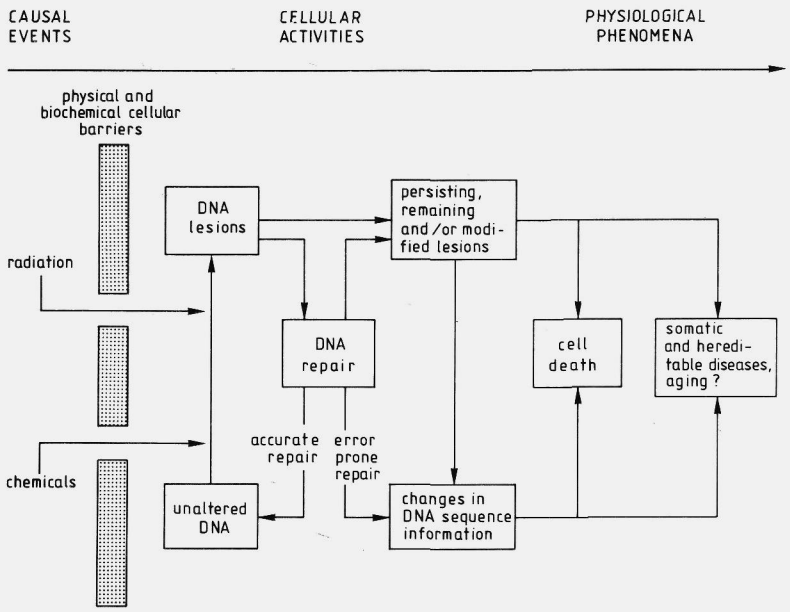


Figure 1.2: Hypothetical pathways via which induced DNA damage may lead to physiological disorders.

### 1.3.3 DNA repair: An overview

In a strict sense the term "DNA repair" should only be applied to those biochemical systems that are involved in the removal of DNA damage with the

aim of restoring the original situation. From a more general point of view, DNA repair encompasses all enzymatic systems involved in the processing of DNA damage. Then it includes those systems that merely act to tolerate the damage and the so-called "SOS response" (see below). Fig.1.3 is a schematic overview of the currently known pathways of DNA repair in a strict sense and their

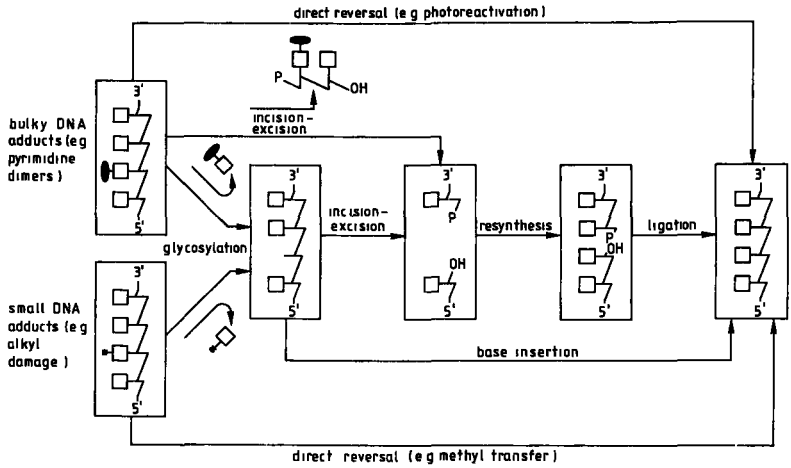


Figure 1.3: Various interconnected pathways thought to be available for the repair of 2 major groups of DNA lesions. A lesion is called "bulky" when it causes distortion of the DNA structure. Unlike most bulky lesions, which are large chemical adducts, pyrimidine dimers involve 2 adjacent bases (only the more general form of a bulky lesion is shown). Such a so-called intra-strand crosslink can greatly distort the DNA structure.

interrelationships. They generally can be divided into two categories, viz. direct reversal and excision repair. Two well-documented examples of direct reversal are photoreactivation of pyrimidine dimers induced by ultraviolet light (UV), and the direct removal of alkyl damage by its transfer to an acceptor protein. Photoreactivation is mediated by a DNA photolyase. This enzyme binds to a pyrimidine dimer-containing site and utilizes visible light energy to split the cyclobutyl ring of the adduct (Eker, 1983). Photoreactivation of pyrimidine dimers has been convincingly shown to occur in many prokaryotes (e.g. Escherichia coli, Streptomyces griseus) and lower eukaryotes (e.g. Saccharomyces cerevisiae). The occurrence of photoreactivation of pyrimidine dimers in living cells from higher eukaryotes is still controversial, although the phenomenon has been reported to occur in embryonic chicken cells in culture

(Wade and Lohman, 1980) and human epidermal cells in vivo (Sutherland, 1980; D'Ambrosio et al., 1981; Vijg et al., 1986a).

The direct removal of alkyl damage, which can for instance be induced by the mutagens N-methyl-N'-nitro-N-nitrosoguanidine, methyl methanesulfonate and N-methyl-N-nitro-ourea, has been best described for E.coli. Methyl groups at $O^6$-guanine, resulting from treatment with these mutagens, are removed (without removal of the base itself) by their direct transfer to a cysteine residue in an acceptor protein, DNA methyl transferase, to form S-methyl-cysteine. In E.coli, this DNA repair activity has been shown to be inducible and adaptive (Samson and Cairns, 1977; Karran et al., 1979; Cairns, 1980). That is, DNA damage itself induces the expression of the gene product (DNA methyl transferase), and the level of expression is higher in cells that have received multiple treatment with non-lethal doses of the alkylating agent than in cells treated for the first time.

In contrast with photoreactivation, alkyl transfer has been demonstrated also to occur in various cell types from humans and rodents (Lewis and Swenberg, 1980; Sklar and Strauss, 1981). Interestingly, differences were observed in the capacity of alkyl repair between different cell types. It was for instance shown by Lewis and Swenberg that non-parenchymal cells of the rat liver are deficient in their ability to remove $O^6$-methylguanine as compared to hepatocytes (Lewis and Swenberg, 1980). At the present time it is not known whether in mammalian cells this response is inducible and adaptive, like in E.coli.

Besides photoreactivation and alkyl repair two other less documented forms of direct reversal exist. One is the repair of breaks in the phosphodiester backbone, which is, in both pro- and eukaryotes, mediated by a ligase and is actually part of replicational, recombinational and excision repair processes. The existence of the other, the direct repair of depurination by means of a purine insertase, is still controversial. Some evidence exists for the presence of purine insertase activity in extracts of E.coli and cultured human fibroblasts (Deutsch and Linn, 1979).

DNA excision repair. A second category of DNA repair responses is known as excision repair. Excision repair is a collective term for a number of interrelated pathways via which a large variety of frequently induced lesions in cellular DNA of both pro- and eukaryotes can be repaired. As shown in Fig. 1.3, repair of DNA damage via excision repair can be divided into 5 steps: (1) recognition of the initial lesion in the DNA; (2) initiation, either by direct incision or via glycosylation followed by strand-nicking; (3) removal of a small piece of the DNA containing the damaged site (or part of it) by the

action of an exonuclease; (4) filling of the gap with new nucleotides by the action of a DNA polymerase under guidance of the sequence in the intact opposite strand; (5) subsequent sealing of the gap by the action of a ligase.

DNA excision repair is often subdivided into two categories on the basis of the initiation step. If excision repair is initiated by glycosylase action, one speaks of base excision repair since the lesion is removed as a damaged free base. If, on the other hand, the damage is removed as nucleotides (for instance in the form of an oligonucleotide) the process is termed nucleotide excision repair. However, it should be noted that also in the case of base excision repair the apurinic or apyrimidinic sites are eventually removed via incision, and excision of a number of nucleotides (see Fig.1.3).

At the present time there is information that in bacteria a number of specific enzymes can operate (e.g., endonucleases and DNA glycosylases) that are capable of initiating excision repair by recognizing and acting upon various types of DNA damage (Lindahl, 1982). Insight into the characteristics of such enzymes provided the basis for the identification of excision repair pathways with either a narrow or a broad specificity towards the induced lesions. An example of a specific action is the incision 5' to a pyrimidine dimer by the UV-specific endonuclease derived from bacteriophage T4 or from Micrococcus luteus bacteria. These enzymes have both an N-glycosylase activity that cleaves the N-glycosyl bond between the 5' pyrimidine of a dimer and the corresponding sugar, and an apyrimidinic/apurinic (AP) endonuclease activity that cleaves the phosphodiester bond 3' to the newly created AP site (Haseltine et al., 1980). As yet, no information is available about the subsequent enzymatic steps required for complete repair (e.g. excision, resynthesis, ligation).

It is not known to what extent the above described incision activities participate in the removal by excision repair of UV damage or other lesions from the DNA of other pro- and eukaryotes. However, there is evidence against the occurrence of initiation of excision repair by damage-specific glycosylases in E.coli and cultured human fibroblasts (La Belle and Linn, 1982). In E.coli, excision repair of pyrimidine dimers is encoded by the uvrA, uvrB and uvrC genes. As was revealed from a series of experiments by Sancar and Rupp (1983), the uvrABC endonuclease complex generates a strand break on either side of a dimer, thereby releasing a fragment with a length of 12 to 13 nucleotides that contains the damage. Presently, the various enzymatic properties of the different uvr gene products are being unraveled (Yeung et al., 1983), as are the subsequent steps in this repair process. In contrast to the M.luteus and the T4 endonucleases, which specifically recognize pyrimidine

dimers, the uvrABC endonuclease acts upon a broad spectrum of damages, probably by recognition of a distortion of the DNA structure commonly associated with bulky adducts. Thusfar there is no direct evidence that a system comparable to the uvrABC system also acts in mammalian cells, although this has been suggested on the basis of some common features (Haseltine, 1983).

The above described mechanisms illustrate the advances that have been made in identifying the various excision repair pathways operating in bacteria. For mammalian cells the situation is much less clear. On the basis of the number of substituted nucleotides in the "repair patch", two forms of excision repair have been identified: long-patch and short-patch repair. For mammalian cells in culture it has been shown that UV as well as a variety of "UV-like" chemicals (such as polycyclic aromatic hydrocarbons), characteristically induce long-patch repair whereas X-rays evoke short-patch repair (Regan and Setlow, 1974). Although this distinction is considered to reflect the difference between nucleotide versus base excision repair, it is not based on a clear-cut difference in mechanism since there is at present no detailed insight into the mechanisms of the complicated DNA excision repair pathways in mammalian cells. The progress that has been made is to a large extent the result of the discovery of patients with heritable diseases associated with DNA repair defects (Setlow, 1978).

The best known example of such natural mutants is xeroderma pigmentosum (XP), an autosomal recessive disorder characterized by progressive degeneration of the skin, eyes and nervous system and an increased risk of malignancy (Cleaver, 1968; Cleaver and Bootsma, 1975). It has been demonstrated that fibroblasts (or other cells) from such patients are unable to remove pyrimidine dimers via the excision pathway (Cleaver, 1968; Zelle and Lohman, 1979). In a series of elegant studies it was established that XP patients can be categorized into so-called complementation groups (Kraemer et al., 1975; Cleaver and Bootsma, 1975). That is, when cells from two different patients are fused, in many cases the fusion product regains the capacity to perform excision repair. If so, the patients are assumed to belong to two different complementation groups (Bootsma, 1978). Thusfar, there is convincing evidence for nine complementation groups, which illustrates the genetic complexity of excision repair. This complexity becomes especially striking when it is realized that much, if not all, of this complexity concerns the initial step(s) of the process. Analogous to bacterial DNA-repair mutants, XP cells of various complementation groups as well as comparable mutants isolated from established rodent cell lines (Thompson et al., 1981) are valuable tools in the genetic and biochemical analysis of DNA repair pathways in mammalian cells.

Another important development is formed by attempts to clone genes involved in DNA repair processes in mammalian cells (Westerveld et al., 1984; Rubin et al., 1985). As in E.coli, where the availability of the purified uvrA, uvrB and uvrC genes and gene products provided important new insight into how such cells repair damage by means of excision repair, comparable efforts can be expected to shed some light on the situation in mammalian cells. Gene cloning is also being applied in the study of the above decribed direct DNA repair modes, such as alkyl repair (Ding et al., 1985).

Tolerance of DNA damage during replication. In confluent cultures of human fibroblasts, pyrimidine dimers are completely removed by excision repair in 10 to 20 days, depending on the UV dose (Kantor and Setlow, 1981). However, in actively dividing cell populations induced damage has to be removed more quickly, otherwise it would block DNA replication. In order to circumvent DNA damage present during replication, cells may utilize so-called tolerant "DNA repair" pathways. In UV-irradiated E.coli, for instance, DNA synthesis is arrested at the site of the damage, but is reinitiated at some point beyond it. The resulting gaps formed in newly synthesized daughter strand DNA are corrected by means of a recombinational insertion of the corresponding segment of parental DNA. This parental DNA is then replaced by a repair type synthesis (Rupp and Howard-Flanders, 1968; Rupp et al., 1971). The process has been termed postreplication repair or daughter strand gap repair. Next to this recombinational type of mechanism, bacteria possess an inducible mechanism, categorized among the SOS repair mechanisms (see below), that permits replication of DNA that contains damage (trans-lesion DNA synthesis). Because this system involves insertion of nucleotides opposite a noninstructive site on the template, it is likely to cause mutations (misincorporations). Thus, the cell tolerates what otherwise might be a lethal lesion at the cost of the introduction of mutations ("error-prone" DNA repair).

At the present time it is not known whether mammalian cells tolerate damage by discontinuous or by continuous (trans-lesion) DNA synthesis. The replication process in mammalian cells is organized in many independent units, termed replicons. Each time, in two adjacent replicons semiconservative DNA synthesis is started from a joint initiation point to proceed outward (bidirectional synthesis). It has been shown that initiation of replication is sensitive to DNA damage; specific gene products appear to be present which inhibit initiation after treatments with DNA-damaging agents (Kaufmann et al., 1980). This process is altered in cells from ataxia telangiectasia (AT) patients. They have a genetic disease which is characterized by abnormalities in the nervous system, the skin and the immune system as well as by X-ray hypersensitivity

(Paterson and Smith, 1979). Such cells show much less inhibition of replicon initiation upon treatment with DNA-damaging agents and this may reflect the DNA-repair defect thought to underly the disease (Jaspers et al., 1982; Painter, 1985).

Besides induced inhibition of replicon initiation, mammalian cells treated with genotoxic agents also undergo inhibition of DNA synthesis due to blocks to chain elongation. After low doses, transient inhibition of DNA synthesis occurs, as a consequence of single lesions between replicons. In this situation, two approaching replication forks are prevented from normal termination when one fork is blocked at the lesion. The other fork then may pass its normal termination site and replicon termination is completed at the site of the lesion, which functions as an abnormal terminus (Park and Cleaver, 1979). The cell may then be programmed to insert a nucleotide opposite the damaged base(s) with an error frequency that is determined by the characteristics of the polymerases and nucleases present at the point of the lesion (Strauss et al., 1983).

After high doses of a genotoxic agent, the probability of two lesions between adjacent replicons increases. It has been suggested that this situation may result in a long-lived unreplicated region or LLUR (Park and Cleaver, 1979). A mechanism that describes the eventual replication of LLUR's by activation of otherwise unused origins within the LLUR's has recently been proposed (Painter, 1985 and personal communication).

The SOS response in E.coli. In E.coli, situations of physiological stress, caused by the inhibition of replication by DNA damage or by nucleoside starvation, induce a complex response that may include enhanced excision and postreplication repair, enhanced mutagenesis, prophage induction, cell-division delay and the shut-off of cellular respiration. This reaction of the cell to immediate danger has been termed "SOS response" and the associated repair phenomena are designated as "SOS repair" (Radman, 1975). The SOS response is regulated by the recA and lexA genes. The recA gene encodes a protease which, upon activation by a situation of distress, inactivates the lexA protein. The lexA protein represses a set of genes, the activity of which represents the actual SOS response. Among the activated genes are the uvrA and B genes of the excision repair system and various genes that code for recombinational activities (Little and Mount, 1982).

The consequences of SOS repair are enhanced survival of the damaged cell in combination with enhanced mutagenesis. These two characteristics of the SOS response can be observed in split-dose experiments. In such experiments, an initial low dose of a genotoxicant is used to evoke the SOS response, so

that after a second, higher, dose the survival is much higher than it would be without the inducing effect of the first dose. As mentioned above, enhanced survival is accompanied by enhanced mutagenesis, suggesting that the induced recA/lexA regulated system overrules the normal constraints of the various (error free) repair responses, thereby turning them into error prone systems. This error proneness of the SOS system is explained by assuming that SOS repair protects the survival of the genetic substance rather than its integrity. DNA sequence changes are of no importance as long as the survival of (some of) the genetic entity is warranted. This may not appear a rational strategy, but it could be a sine qua non for the generation of DNA sequence diversity which is essential for evolution to proceed.

Extensive study of the SOS response and its regulation has been made possible by the availability of SOS deficient mutants and the successful cloning of genes encoding SOS functions. For instance, a dominant lexA mutation blocks the expression of almost all the SOS phenotypes and therefore precludes the above described phenomena of enhanced survival and mutagenesis. Such cells are extremely sensitive to DNA-damaging treatments (Mount et al., 1972).

In mammals, the induction of DNA sequence variations is, as in E.coli, thought to be mainly due to error-prone processing of induced DNA lesions. In mammalian cells, a number of reactions to the application of stress (e.g. treatment with genotoxic agents, heat shock) has been observed. Such reactions include enhanced postreplication repair, sister chromatid exchange, abnormal initiation of DNA replication and chromosomal rearrangements (Sarasin, 1985). With respect to the driving force that underlies such events, the concept of "genome shock" has been introduced for those situations in which stress instigates genome modifications by mobilizing available cell mechanisms that can restructure genomes (McClintock, 1984). However, an inducible recovery system, comparable to the SOS response in E.coli, has not yet been found in mammalian cells. It has been argued that stress-induced phenomena in mammals cannot be simply reduced to an inducible error-prone reaction, analogous to that in E.coli, which leads to mutagenesis of the genome (Rossman and Klein, 1985). In the next sections, error-prone DNA repair in mammals is defined as alterations in the genetic code as a consequence of the processing of DNA damage by the cell's repair, replication and recombination systems.

### 1.3.4 DNA repair as a determinant of cellular aging

Spontaneous DNA damage. With respect to the spectrum of lesions induced in the DNA of the aging genome, a considerable amount of information has recently become available. Lesions that are suspected to be most relevant for the aging process are those that are induced by active oxygen species, generated in vivo as a consequence of normal metabolism and detoxification processes (Ames, 1983; Cerutti, 1985; Cutler, 1985). Thymine residues in DNA are very susceptible to damage by oxidizing agents and ionizing radiation, and a major detectable product is thymine glycol, although other base damages are also formed (Hariharan and Cerutti, 1972). Interestingly, both human and rat urine have recently been shown to contain a considerable amount of thymine glycol and thymidine glycol (Cathcart et al., 1984). Evidence was presented that these compounds, that are formed in DNA in vitro as a consequence of chemical oxidation or ionizing radiation are derived from repair of oxidized DNA.

A second physiologically relevant DNA-damaging agent is heat. For instance, body heat in mammals is responsible for a considerable number of depurinations. With respect to the biological consequences of such damage, it has been shown that in E.coli unrepaired AP sites can be lethal and cause base-substitution errors under conditions in which the SOS response has been induced (Schaaper and Loeb, 1981; Kunkel, 1984).

Evidence has been obtained for the occurrence of a third type of "spontaneous" DNA damage. Sargentini and Smith (1981) found that in E.coli a deficiency in excision repair, which enhances the spontaneous mutation rate of these cells, renders them more mutable by UV irradiation but has little effect on X-radiation mutagenesis. These results led them to suggest that in E.coli "spontaneous" DNA damage resembles the damage induced by UV (Smith and Sargentini, 1985 and personal communication). With respect to the mechanism by which this "UV-like" damage is produced in DNA, it has been suggested that enzymatic reactions are involved that require oxygen and produce excited-state molecules that can damage DNA (Cilento, 1980). The possibility that part of the spontaneous DNA damage in mammalian cells is also "UV-like" was suggested by a comparative study on spontaneous and ionising radiation-induced mutations in the HPRT locus of V79-4 Chinese hamster cells. In this study it was demonstrated that spontaneous DNA damage in the hamster cells is not X-ray-like (Brown and Thacker, 1984).

Primary effects of DNA damage. Assuming that the above described lesions (and perhaps other as yet unidentified forms of spontaneous DNA damage) are

continuously induced over the lifespan, they either become a substrate of the cell's DNA repair systems or persist in the genome. In the case of abortive repair they may persist in a modified form. Consequently, the capacity to efficiently remove lesions from the DNA would be an ideal candidate for a longevity assurance system (Sacher, 1982). Accordingly, the suggestion that the organism with the most efficient repair systems throughout its lifespan has the highest longevity (Hart and Setlow, 1974) became an important concept in experimental aging research. Gensler and Bernstein (1981) have recently discussed the possibility that in non-dividing cells the (accumulated) DNA lesions themselves are solely responsible for senescence, via cell death or interference with transcriptional processes. These authors argue that spontaneous DNA damage accumulates in those cell types which have a rate of repair that is lower than the incidence of the damage. Postmitotic cells were considered good candidates to undergo such a process of DNA damage accumulation in view of their relatively low level of DNA repair synthesis (Hahn et al., 1971; Kidson, 1978). However, a low response in an experimental system measuring a specific step in a DNA repair pathway does not necessarily indicate a general DNA repair defect in the cell type under study. This is illustrated by the observation that, in terms of colony forming capacity, cultured rodent fibroblasts are as resistent to cell killing by UV irradiation as cultured human fibroblasts, in spite of their far lower DNA repair response (Ganesan et al., 1983; Chapter 3).

With respect to DNA damage accumulation, there is some experimental evidence for an increase in alkali-labile sites in the DNA of various tissues with age (Price et al., 1971; Ono et al., 1976; Su et al., 1984). However, reports in this field are often contradictory (Dean and Cutler, 1978), which may be due to the lack of techniques to adequately assess the occurrence of low levels of DNA damage. Recently we have found that liver parenchymal cells of old rats contain about 800 DNA alkali-labile sites more on a per cell basis, than those isolated from young rats (unpublished results). Such an age-related difference was not found in non-parenchymal (still actively dividing) liver cells. The age-related increase in alkali-labile sites in parenchymal cells found by us, is about 50 times smaller than that reported by the above mentioned authors, which illustrates the difficulties in drawing any general conclusions in this field. Only recently, reliable techniques became available to adequately address the problem of whether damage accumulates in the DNA of various organs and tissues with age (Chapter 2).

Secondary effects of DNA damage. Genome functioning may be impaired by induced DNA lesions in a direct way, but also in an indirect way via their

conversion by the error-prone component of DNA repair into stable changes in the genetic code (see Fig.1.2). The idea that somatic mutations could be causal to senescence arose in a time when nothing was known about the role of DNA repair systems in the removal of damage and the generation of mutations. The somatic mutation theory, first introduced at the end of the fifties by Failla (1958) and Szilard (1959) and later in a more elaborate form by Burnet (1974), states that senescence is due to the inactivation or dysfunctioning of genes by accumulated changes in DNA sequence organization. Such a process was considered to result in cell death or in the production of altered proteins. The increased number of chromosomal aberrations in various cell types with age (Brooks et al., 1973; Hedner et al., 1982; Evans, 1986) and an observed age-related increase in the frequency of 6-thioguanine resistant cells (Morley et al., 1982) support this idea. However, conflicting evidence exists in the latter case (Strauss and Albertini, 1979; Horn et al., 1984).

If the error-free component of DNA repair is considered as a longevity-assurance system (and therefore as beneficial), its error-prone component (its genome-restructuring activities) can be considered as the deleterious component of a pleiotropic gene system. In discussing SOS repair in Section 1.3.3, it was already mentioned that in E.coli or in the germ cells of mammals the interaction between induced DNA lesions and error-prone DNA repair could be favourable for the generation of a sufficient level of genetic diversity. Theoretically, evolutionary modulation of the DNA repair systems, active in somatic cells, towards ever higher levels of perfection is possible. However, as discussed in Section 1.2.3, the possibility for natural selection to act beyond the reproductive period is likely to be limited, which could explain the apparent absence of an evolutionary strive after immortality on the basis of near-perfect DNA repair systems. Immortality in terms of a static non-replicating cellular system may never have existed in nature in view of the essential evolutionary function of "imperfect" DNA repair. In this perspective, both senescence and the arise of new forms of life are a consequence of DNA sequence changes. Such a "DNA repair theory of aging" also explains how and why pleiotropic genes would 'switch' from having a beneficial effect towards having a deleterious effect. Indeed, the action of DNA repair is predominantly beneficial early in life because it removes damage from DNA, while the number of DNA sequence changes at that time is too low to have any physiological effect. However, in old age the effects may become more and more harmful when the accumulation of DNA sequence changes lead to altered patterns of gene expression, reflecting the senescence phenotype. In this concept the ratio between error-prone and error-free DNA repair would determine lifespan.

If this balance would change during the aging process towards higher error-prone activities (for instance as a consequence of accumulated DNA lesions) a progressive age-related increase in DNA sequence changes would be the result. DNA repair would thus assure longevity by efficiently removing DNA damage and simultaneously lead to senescence by processing damage to DNA sequence changes.

Specificity in the induction and repair of DNA damage. A major problem in considering DNA sequence changes as the basis of senescence is that of the likely functional sequelae. For instance, it is difficult to see how random induction of DNA damage or mutations may lead to impairment of specific cellular functions such as the reduced sensitivity of beta cells in the pancreas to a glucose load. In order to impair this function in a considerable fraction of the cell population, a tremendous number of lesions or mutations would be required, which would have killed all the cells long before this specific age-defect could have become manifest. In other words, what is lacking in somatic mutation theories is the aspect of consistency (the programmatic component), which is as characteristic for senescence as the stochastic aspect. If it is assumed that the physiology of senescence finds its ultimate cause at the DNA level, one has to postulate a gradual process of site-specific DNA alterations which specifically affect regulatory functions leading to the impaired effectiveness of control mechanisms that is observed during aging. DNA repair could control such a putative process.

Whereas the actual occurrence of DNA alterations with age is not being questioned, their frequency and site-specificity, which determine whether or not such events may lead to the physiological decrements and cluster of diseases associated with senescence, are as yet unknown. On the basis of the available data on mutation frequencies in pro- and eukaryotes, it seems highly unlikely that a somatic-mutation theory in the classical sense, that is, random inactivation of genes by point mutations, chromosomal aberrations etc., is responsible for the senescence phenotype. Instead, DNA changes can only explain senescence if they are to some extent consistent, that is, if they occur at the same sites in all or most of the cells of a tissue or organ. Moreover, such sites should in some way govern the level of expression of one or more genes so that alterations in its sequence will not completely block a specific cellular function (this would kill the cell), but merely affect its regulation.

With respect to the consistency of DNA changes, it is clear by now that neither the induction of DNA lesions nor their conversion into mutations is random. Within bacterial genes, hotspots for specific DNA lesions and for mutations have been reported (Miller, 1983). With respect to the intragenomic

distribution of DNA damage, it has been shown for various carcinogens that binding occurs preferentially to template active chromatin (Yu, 1984), nucleosomal linker DNA (Kaneko and Cerutti, 1980), matrix-bound DNA (Mironov et al., 1983) and repetitive DNA sequences (Gupta, 1984). While the structure of DNA and chromatin can affect the initial distribution of DNA damage, its processing by DNA repair systems can generate even more specificity. This could be a consequence of differential accessibility of various genomic sites to repair enzymes, or through the sequence specific action of DNA repair systems. As will be illustrated below by some examples, DNA excision repair appears to distinguish between coding and non-coding DNA sequences in the mammalian genome with respect to its efficiency in removing induced lesions. In this respect, there is evidence that excision repair of chemical adducts in African green monkey cells is less active on highly repetitive α DNA sequences as compared with the overall genome (Zolan et al., 1982). Comparable results were obtained by Gupta (1984), who showed that repair of carcinogen-DNA adducts from certain repetitive sequences was deficient as compared to the overall repair.

More recently, it has been shown that UV-induced pyrimidine dimers are more efficiently removed from the coding region of the dihydrofolate reductase gene in Chinese hamster ovary cells than from its 5' flanking sequences or from the genome overall (Bohr et al., 1985). In addition, recent data revealed a non-random persistence of the adduct N-(deoxyguanosin-8-yl)-2-aminofluorene in liver DNA of rats injected with the hepatocarcinogen 2-acetylaminofluorene. Removal of this adduct from DNA loops was markedly inefficient in the vicinity of their association with the matrix (containing predominantly regulatory regions of structural genes), in comparison with regions within the loops (which may be associated with the matrix), where the transcribed portions of the genes are thought to be present (Gupta et al., 1985).

The data mentioned above provide direct evidence for the site-specificity of DNA damage induction and repair. In addition, they suggest that protein-coding sequences are generally a better substrate for excision repair processes than flanking DNA sequences or certain repetitive DNA sequence families. In this regard one may speculate that the processing of lesions in non-protein-coding DNA sequences often lead to errors. This possibility is supported by a number of recent observations. For instance, mutations in the $\beta_2$-microglobulin gene, generated independently by two different chemicals and cobalt irradiation, were all within a short region in the first intron (Parnes et al., 1986). The authors suggested that the mutations at this site, which involved major gene rearrangements, were the result of recombinational events taking place

during the repair process. The site could be a hotspot for recombinations, possibly because of the presence of B1 repeat sequences near the breakpoints of the rearrangement events. Although there is no direct evidence for this latter possibility, it should be noted that repetitive sequences are often associated with mutational events (Jagadeeswaran et al., 1982).

Interestingly, sequences containing inverted repetitions are often unstable or completely inviable upon cloning in phage lambda (or plasmids) in wild type E.coli hosts, possibly because they are prone to rearrangements (Lilley, 1981; Leach and Stahl, 1983). However, when such sequences are cloned in E.coli hosts with mutations in their recB and/or recC and sbcB genes they can be efficiently propagated (Leach and Stahl, 1983; Wyman et al., 1985; for a review, see Wyman and Wertman, 1987). Sequences containing direct repetitions may also be lost when the clones are grown on a wild type host. A well known example of a mammalian DNA sequence that is unstable upon cloning in an E.coli host is the 5' upstream region of the human insulin gene, a highly polymorphic sequence (Bell et al., 1981). In this case the instability could be assigned to a region containing short direct repeats. The polymorphism of this region is due to variations in the copynumber of the repeat. Instability of this sequence in E.coli is prevented by subcloning and growing on a $recA^-$ host (Wyman and Wertman, 1987).

Thus, bacterial SOS functions seem to recognize and act upon certain mammalian DNA sequences. Using a $recB^-C^-sbcB^-$ host for the construction of human genomic libraries, an overrepresentation of polymorphic repetitive sequences was found in the recBCsbcB-sensitive clones of about a factor of three (A.R. Wyman, personal communication). This suggests that also in the mammalian genome repetitive sequences are more prone to recombinational events than others, for instance by the specific action of error-prone DNA repair systems. Interestingly, certain species of extrachromosomal DNA, which have been shown to increase in number during aging of rat lymphocytes and fibroblasts (Kunisada et al., 1985) contain specific repetitive sequences and are highly unstable upon cloning in normal E.coli hosts (Riabowol et al., 1985).

It has been suggested that repetitive sequences, often present near the flanking sites or in the introns of various coding sequences (Jelinek and Schmid, 1982), may play a role in the coordinated regulation of genes (Britten and Davidson, 1969; Davidson and Britten, 1979; Davidson and Posakony, 1982; Davidson et al., 1983). Such a function would coincide with the relatively rapid evolutionary changes in sequence organization undergone by these elements. The rapid evolution of mammals is thought to be almost exclusively

associated with changes in regulatory sequences. The relatively few differences in coding sequences among different mammalian species do not explain the often huge differences in their complex developmental pathways (Wilson et al., 1974; Bush et al., 1977). In this regard, speciation has been considered as reprogramming of the genome by changes in the positions and sequences of regulatory elements, thereby influencing developmental pathways in complex and coordinated fashions (Davidson and Britten, 1979; Rose and Doolittle, 1983).

Assuming that regulatory sequences are the predominant substrates for genome-restructuring activities during evolution, it follows that in relation to the aging process attention should be focussed on the same kind of changes in aging somatic cells. This is in keeping with the conclusion of Cairns (1981) that cancer, the incidence of which increases progressively with age, is generally caused by DNA rearrangements leading to a disturbed pattern of gene control. Although there is as yet no indication for an age-related increase in tumor-initiation events, a direct relationship between aging and carcinogenesis is well conceivable.

Thusfar studies on mutation frequencies with age mainly have involved selectable genes (HGPRT). In view of the above, it is not unlikely that major changes occur in the network of repetitive sequences which may coordinately regulate the expression of coding sequences. Furthermore, if in non-coding sequences certain types of DNA lesions accumulate due to deficient error-free repair, genome stress may be the result. Consequently, error-prone (genome-restructuring) activities may progressively increase, resulting in the collapse of the gene regulatory network. A situation may have been created in which the balance of activities of the various cell types in an organism is greatly disturbed, due to small changes in the expression of certain enzyme or receptor systems. Under these circumstances, the organism may no longer be able to cope with even small burdens from the environment. Such a situation is characteristic for an aged individual.

In the hypothesis presented above it is proposed that an important aspect of senescence is the disturbance of the regulatory network of repetitive DNA sequences that may coordinate gene expression. The driving force of this process could be the continuous induction of ("spontaneous") DNA lesions in conjunction with their differential processing by the enzyme systems for repair, replication and recombination. The specificity of these systems warrants the rapid evolutionary creation of novel coordinately regulated structural gene networks, in which the coding regions themselves hardly undergo changes. Unfortunately, they may simultaneously be primary responsible for senescent changes in the somatic cells.

## 1.4 Objective and Design of the Experimental Work

One aspect in the search for the molecular basis of aging, and in part the subject of the experimental part of this thesis, is the analysis of DNA repair activities in species or individuals with different lifespans in order to test whether (error-free) DNA repair may function as a longevity-assurance system. The major problem in such an attempt to determine "the DNA repair capacity" of an individual is the great complexity of DNA repair and the limited insight in the variation in DNA repair responses between different cell types, differentiation states and DNA sequence substrates. Therefore, criteria for "the DNA repair capacity" of an individual organism do not exist. Moreover, during aging changes in DNA repair activities may occur, varying from increased dysfunctioning of repair enzymes to switches in the utilization of repair pathways.

The purpose of our study was to analyse some experimental endpoints of UV-induced DNA excision repair in fibroblasts derived from aging individuals of the same inbred strain of rats. Fibroblasts offer the advantage that they represent a relatively homogeneous cell population that can be established in culture from the same individual over a long time period by taking skin biopsies. Moreover, the use of this cell type allowed us to address the additional problem of potential changes in DNA excision repair characteristics during successive subculturing. The limited in vitro lifespan of somatic cells is considered by many authors to reflect the in vivo aging process (Hayflick, 1980). Thus, in this way DNA excision repair could be studied as a function of (1) the chronological age of the cell donor and (2) in vitro passaging of the established cell samples (Fig.1.4).

In view of the reported differences in DNA excision repair between rodents and humans we first analyzed DNA excision repair activities in fibroblasts from these two species. Differences in DNA excision repair have been suggested to reflect the differences in lifespan between these organisms, which would be in keeping with the essence of the longevity assurance theory (Hart and Setlow, 1974).

The results obtained in this study on DNA excision repair in fibroblasts in vitro are provided in Chapters 3, 4 and 5. They indicate the absence of a consistent relationship between DNA repair synthesis and pyrimidine dimer removal in rat cells relative to the situation in human cells. That is, rat cells were well able to perform DNA repair synthesis, but almost completely lacked the capacity to remove pyrimidine dimers. In human cells such a discrepancy was much less apparent. These results were interpreted in terms of possible

interspecies differences in the utilization of DNA excision repair pathways.

Substantial changes in DNA repair synthesis were found during passaging of rat fibroblasts in vitro, whereas during in vivo aging virtually no changes in DNA repair synthesis in this cell type were observed. It was concluded that in rat fibroblasts quantitative changes in DNA repair synthesis accompanied

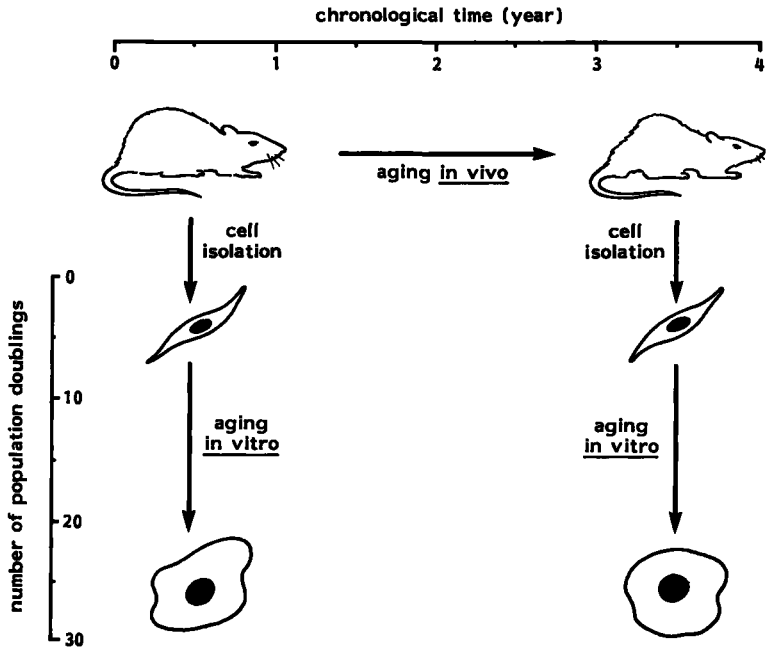


Figure 1.4: Schematic representation of rat skin fibroblasts during aging and passaging in vitro. The use of these cells allowed us to longitudinally study UV-induced DNA excision repair as a function of both the age of the donor and the population doubling level of the established fibroblast culture. The latter is considered by many authors as an in vitro parallel of the chronological age level of the individual.

terminal differentiation in vitro, whereas no such changes occurred during aging in vivo or immortalization (in vitro or in vivo).

In view of the results obtained with fibroblasts in vitro, an attempt was made to establish how far the repair characteristics identified in cultured cells were representative for cells in their natural environment. To address this question, reliable techniques, which are unbiased by differences in cell

morphology or animal physiology, were required for comparing DNA repair activities in different cells in vivo and in vitro. To this end, the alkaline sucrose gradient procedure used to determine DNA breaks had to be adapted for application to cells in vivo which were not previously radioactively labelled. The resulting immunochemical method was applied in combination with the UV-endonuclease enzymatic method to monitor the induction and removal of UV-induced pyrimidine dimers in rat skin in vivo. Interestingly, it was found that whereas rat epidermal cells in vitro were, like fibroblasts, extremely slow in removing pyrimidine dimers, such lesions in the same cell type in the intact skin in vivo were removed rapidly. No significant differences in dimer removal from epidermal cells were observed between old and young rats.

# CHAPTER 2

# METHODS FOR STUDYING DNA EXCISION REPAIR IN MAMMALIAN CELLS*

## 2.1 Introduction

Two developments are of importance for the future unraveling of the complicated excision repair pathways in mammalian cells: the acquirement of mutant cell lines, deficient in the ability to carry out one or more steps in excision repair, and the genetic cloning of DNA sequences that, upon transfection into the mutant cell lines, neutralize the enhanced sensitivity of these cell lines for certain genotoxic agents. In addition, sensitive and specific techniques have to be developed to analyse the various steps in excision repair processes. The techniques should be sensitive enough to allow DNA repair determinations after exposures to low doses of DNA-damaging agents. The use of low doses is essential to avoid possible inhibition of the repair processes by the damage itself. Specificity of the methods is required because the endpoints should not be differentially influenced by secondary (cellular or physiological) factors when, like in this thesis, different cell types or organisms are compared. Currently, however, sensitive and specific methods to determine DNA excision repair activities are still scarce or unreliable.

The most frequently used methods for the measurement of DNA excision repair in mammalian cells are listed in Table 2.1. They will be discussed as to their application in accurately quantifying excision repair activities in comparative studies involving either cultured cells or the intact animal.

## 2.2 Determination of Excision Repair in Cultured Cells

The most frequently used methods to analyse and quantify part of the excision repair process in mammalian cells are assays of so-called unscheduled DNA synthesis (UDS), the resynthesis step in excision repair. UDS can be visualized in individual cells grown on glass or plastic coverslips by autoradiography following the incorporation of tritiated thymidine into the DNA.

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* Parts of this work have been published previously (Vijg et al., 1984a).

TABLE 2.1 METHODS FOR MEASURING DNA EXCISION REPAIR IN MAMMALIAN CELLS

| Stage in the excision repair process | Method | Endpoint measured |
|---|---|---|
| Incision | Ara-C-inhibition assay | SSB in the presence of ara-C |
| Repair synthesis | UDS | Amount of incorporated $^{3}H$ thymidine in non-S-phase cells |
| | Repair replication | Amount of incorporated $^{3}H$ thymidine in low density DNA |
| | BrdU-photolysis | Number of SSB as a function of the dose of 313-nm light |
| Damage removal | Chromatographical | Modified bases |
| | Immunological | Modified bases |
| | Enzymatic | SSB at enzyme susceptible sites |

UDS has been studied most intensively in cultured mammalian cells exposed to ultraviolet light (UV). Under these circumstances UDS reflects the process of long-patch excision repair (see Section 1.3.3). Experimentally, UDS is measured by counting the number of grains in the photographic emulsion above nuclei of cells that were not in the DNA synthesis phase (S-phase) of the normal cell division cycle. The latter cells are easily distinguished from repairing ones because they are much more heavily labeled (Fig.2.1). The counting can also be done automatically by using a grain counter (Kraemer et al., 1980). With this apparatus the grain surface area is determined, per nucleus or as a percentage of the nuclear area. An example of a set of data for irradiated cells and unirradiated control cells is shown in Fig.2.2.

Alternatively, UDS can also be determined by liquid scintillation counting as the incorporation of radioactive thymidine. However, in that case no discrimination is possible between S-phase and repairing cells. To circumvent this problem UDS is allowed to proceed in the presence of hydroxyurea (HU), a compound that is thought to inhibit semiconservative DNA synthesis without affecting DNA repair synthesis. Unfortunately, there is some evidence that HU may have an influence on the DNA repair endpoint, as was shown by Clarkson (1978), Francis et al. (1979) and ourselves (see below). Moreover,

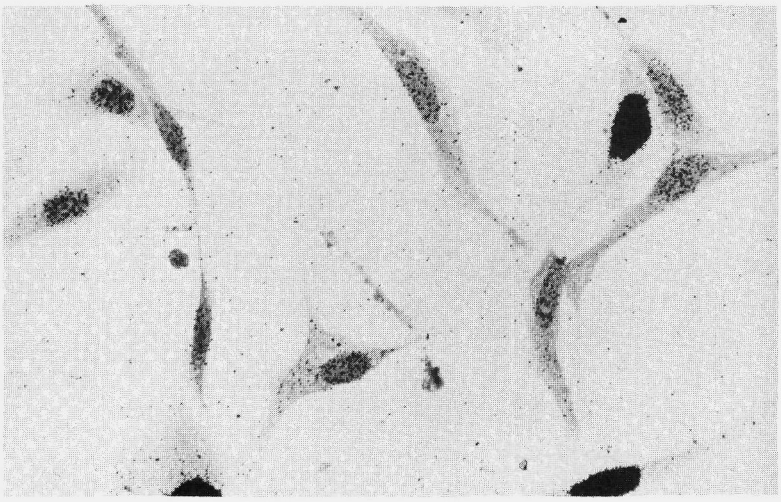

Figure 2.1: Scheduled (replicative) and unscheduled (repair) DNA synthesis in rat skin fibroblasts. Early passage cells were grown attached to glass coverslips, irradiated (after discarding the medium) with 10.3 J/m$^2$ 254-nm UV and subsequently incubated in fresh medium containing $^3$H-thymidine. After a 5 h repair period, the cells on the glass coverslips were fixed, mounted on glass slides and dipped into a photographic emulsion. After 4 days of exposure DNA synthesis is visible as grains above the cell nuclei. Repairing cells (lightly labelled) can be easily distinguished from S-phase cells (completely black).

with the liquid scintillation method a certain amount of semiconservative DNA synthesis is inevitably included in the measurements.

UDS is often used synonymously with DNA repair replication. Although both UDS and DNA repair replication most likely reflect the same step in the excision repair process (Painter and Cleaver, 1969), the determinations are based on different methodologies. While UDS is defined as the amount of radioactivity incorporated by non-S-phase cells determined directly in the cells, either autoradiographically or by liquid scintillation counting, DNA repair replication involves the separation of replicated DNA from nonreplicated repaired DNA, for instance by equilibrium density centrifugation (Hanawalt and Cooper, 1971; Lohman et al., 1973). Both UDS and DNA repair replication are often referred to as DNA repair synthesis.

The methods described above cannot always be considered as reliable quantitative indicators for excision repair activities. The amounts of resynthesis observed, for instance, can be influenced by the thymidine-pool size, the

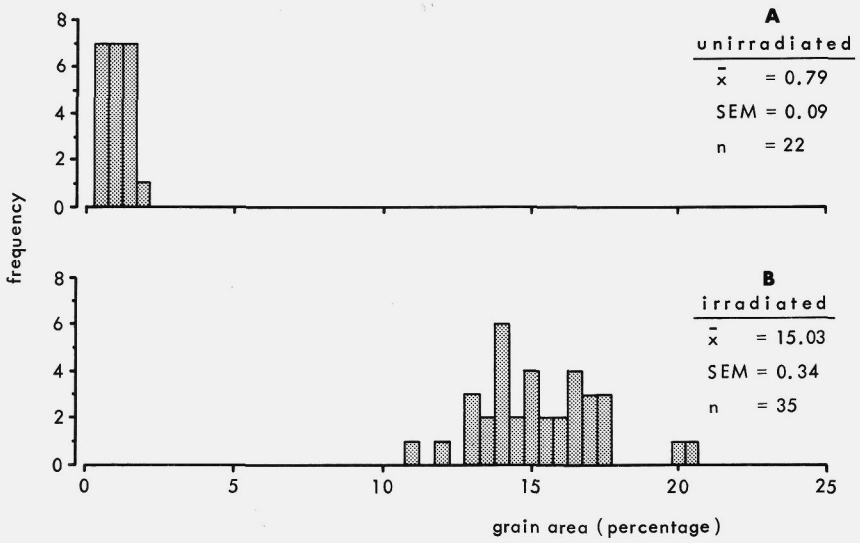


Figure 2.2: Frequency distributions of the grain surface areas (in percentages of the areas of the nuclei) of UDS performing cells (B) and unirradiated control cells (A).

thymidine-kinase activity, the repair-patch size and cell geometry and morphology (in case of autoradiographically determined UDS). It should also be realized that the observation of repair synthesis reveals only a part of the total excision repair process. Therefore, the repair process should always be studied by a variety of methods to allow the identification of the underlying mechanism.

A sensitive technique that has been propagated for the determination of one of the initial steps in excision repair, the endonucleolytic incision, is based on the use of the inhibitor 1-β-D-arabinofuranosylcytosine (ara-C), the presence of which, together with HU, during the repair process prevents complete resynthesis (Collins et al., 1977). Thus, in this technique the repair sites are held open, which allows quantification of the number of patches created over a given time period (incision rate) by determining the number of single-strand breaks (SSB) in the cellular DNA. SSB in DNA can be measured by sedimentation through an alkaline sucrose gradient after lysis of the cells on top of the gradient (or after application of the isolated DNA), or by alkaline elution through membrane filters, after lysis of the cells on the filter (van der Schans et al., 1982). With the latter technique, one lesion in $10^8$ basepairs can be detected.

Thus, in the ara-C inhibition assay the determination of SSB provides direct information about the number of repair patches created during the initial repair period. The major problem with this technique is the uncertainty as to the precise effects of the inhibitors HU and ara-C on cellular DNA metabolism. There is evidence that ara-C brings about premature chain termination by fixing the repair enzyme at the site of repair (Snyder et al., 1981). Such repair enzymes are prevented from participating in other repair events. This inhibition of the repair process itself leads to an underestimation of the number of damaged sites that can undergo repair. Consequently, incision break determinations are only accurately reflecting excision repair rates over the shortest possible time intervals after addition of the inhibitors (for an extensive discussion, see Johnson et al., 1984). However, the characteristics of repair saturation may vary as a function of cell type, donor species etc. This could for instance be due to differences in pool sizes of DNA precursors. Expanded pools of DNA precursors might reduce the efficiency of inhibition (Johnson et al., 1984).

The situation becomes even more complicated when HU is added to enhance the repair inhibition by ara-C. The nature of this enhancement is, like the mechanisms of action of inhibitors in general, as yet far from clear (for a review, see Collins and Johnson, 1984). Therefore, repair inhibition is more useful in illuminating the molecular mechanism of the incision stage in excision repair in a given cell type than in accurately monitoring relative excision repair activities in different cell types.

Thus, neither thymidine incorporation nor incision break accumulation in the presence of inhibitors are fully reliable as methods for quantifying excision repair activities in mammalian cells. A more complete technique to examine excision repair in mammalian cells is the bromodeoxyuridine (BrdU) photolysis assay (Fig.2.3). This assay involves incubation of radioactively labeled cells in medium containing BrdU after the treatment with the genotoxic agent. BrdU is a thymidine analogue that is incorporated in the newly synthesized DNA stretches. After incubation, the cells are irradiated with 313-nm UV, which makes the BrdU-containing repair regions alkali labile. Thus, in alkali, breaks appear in the DNA at the sites of BrdU insertion. The number of strand breaks as a function of 313-nm UV irradiation is measured by alkaline sucrose gradient centrifugation (Regan and Setlow, 1974) or alkaline elution (Rosenstein et al., 1985). Multiple breaks within one repair patch are scored as one SSB. From the so-called photolysis curve obtained in this way, the following information can be derived (Regan and Setlow, 1974). The number of breaks, B, approaches a plateau at the value N (the number of

repaired regions). From the formula $B=N(1-e^{n\sigma F})$, in which n is the number of BrdU residues per repair patch, σ the cross-section for strand breaks by 313-nm radiation and F the fluence, both n and N can be derived.

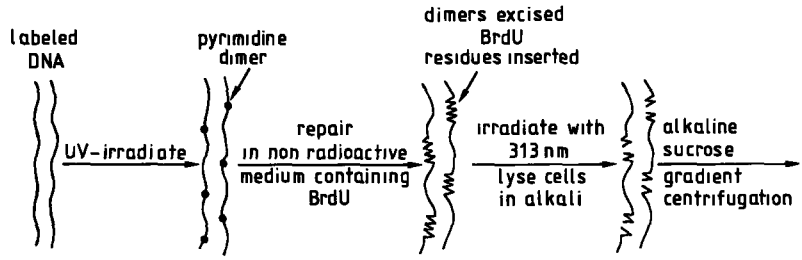


Figure 2.3: Principle of the BrdU photolysis assay.

The advantage of this technique for the assessment of DNA repair synthesis in comparison to the techniques mentioned above is twofold. The BrdU photolysis assay requires no inhibitors and provides information on both the number of repair patches and their average size. Its usefulness in comparison to other less specific methods for assaying the repair synthesis phase of the excision repair process is demonstrated in an experiment in which UV-induced DNA repair synthesis in rat and human fibroblasts is analysed in the presence and in the absence of 2 mM HU. Autoradiographically determined UDS values are not influenced by the presence of HU in this concentration (unpublished results). However, with rat cells the BrdU method showed that HU reduces the number of repair patches and simultaneously enlarges the repair patch size without affecting the total repair synthesis, i.e., the product of the two (see Fig.2.4 and Table 2.2).

The same tendency was seen with human fibroblasts, but the effect was not significant (Table 2.2). Francis et al. (1979) have shown earlier that for higher HU concentrations the effect on human fibroblasts is comparable with

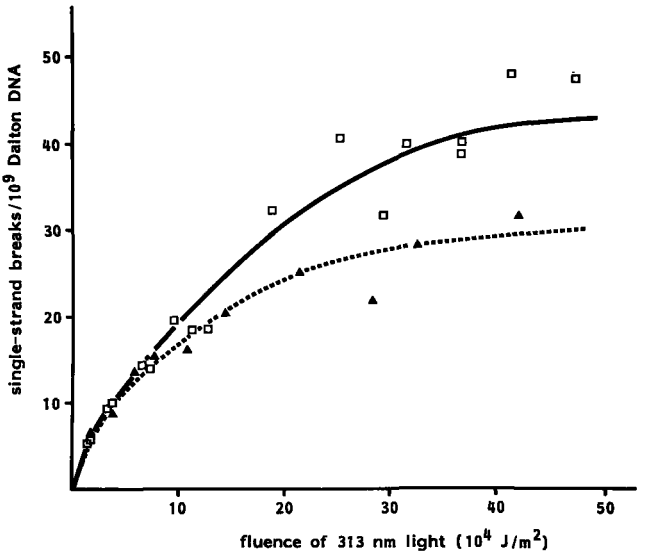


Figure 2.4: BrdU photolysis curves for rat fibroblasts at 24 h after irradiation with 4.6 J/m$^2$ of UV, in the presence (▲) and in the absence (□) of 2 mM HU. HU was added 30 min prior to irradiation.

that on the rat cells presented here. In addition, these authors showed that the reduced number of repaired patches is reflected by a reduction in the number of pyrimidine dimers removed.

The comparative experiment described above illustrates the value of the BrdU photolysis method in that it dissects the overall repair synthesis step in two discrete parameters: the number of repair patches and the average number of nucleotides they contain. Whatever the precise effect of HU on excision repair may be (for an extensive discussion of this topic, see Collins and Johnson, 1984), its differential effect on rat and human cells exemplifies the danger of using inhibitors in comparative studies.

The removal of UV-induced damage (and other types of DNA damage) can directly be measured by various chromatographical procedures (Carrier and Setlow, 1971; Cook and Friedberg, 1976). However, in general such detection methods are rather insensitive, possibly with the exception of recently developed HPLC methods (Niggli and Cerutti, 1983). Indirectly, dimers can be measured by using a dimer-specific endonuclease, the M.luteus endonuclease or the T4 endonuclease. The protocol for this assay (Fig.2.5) includes the extraction of DNA from radioactively labelled UV-irradiated cells, the

TABLE 2.2 INFLUENCE OF HYDROXYUREA ON UV-INDUCED EXCISION REPAIR IN RAT AND HUMAN FIBROBLASTS

| Donor species | HU (2 mM) | Number of repaired sites per $10^9$ mw DNA | Number of nucleotides per repair region |
|---|---|---|---|
| Rat | - | 52 ± 10 | 59 ± 10 |
| | + | 31 ± 10 | 86 ± 10 |
| Human | - | 90 ± 10 | 63 ± 10 |
| | + | 89 ± 10 | 79 ± 10 |

The UV dose and the repair period were 4.6 J/m² and 24 h, respectively. The errors were estimated from the photolysis curves of 2 experiments.

subsequent treatment of the isolated DNA with the pyrimidine dimer-specific endonuclease preparations and quantification of the enzyme-induced breaks by alkaline sucrose gradient centrifugation (Paterson et al., 1973) or alkaline elution (Fornace, 1982). The number of strand breaks is an indirect measure of the number of pyrimidine dimers (Paterson et al., 1973). Interestingly, by the application of the recently isolated uvrABC excision nuclease (Sancar and Rupp, 1983), Van Houten et al. (1986) were able to demonstrate the repair of either benzo(a)pyrene DNA adducts or UV-induced pyrimidine dimers in human cells, analogous to the UV-endonuclease assay. The wide specificity of the uvrABC excision nuclease with respect to bulky adducts could make this variant of the UV-endo method applicable for studying a number of different carcinogen-induced DNA lesions.

By the combined application of BrdU photolysis and enzymatic endonuclease assays in principle all but the initial (recognition) steps of excision repair are covered.

## 2.3 Determination of Excision Repair In Vivo

Excision repair activities in organisms of different ages or from different species can be compared by isolating cells from a specific organ or tissue, which are then treated with a test agent in vitro whereafter determinations as described above can be performed. This offers the advantage that under standardized conditions various steps in the excision repair process can be studied separately. Such a procedure, however, does not have to be representative for

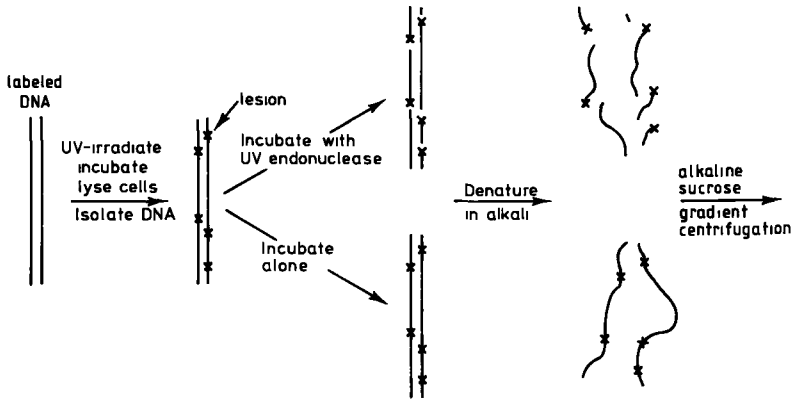


Figure 2.5: Scheme for the assay for pyrimidine dimer sites in the DNA of UV-damaged cells.

the actual excision repair activities in the donor tissue or organ. Various characteristics of the in vivo situation such as hormonal activities, specific cell to cell contacts, metabolic conditions, the level of oxygenation and the status of differentiation may influence excision repair activities of the cells under study. Therefore, attempts have been made to measure excision repair in vivo. Such attempts were hampered by the fact that the in vitro techniques described above, such as those based on nucleotide analogue incorporation or on break accumulation by inhibition, cannot easily be applied on intact animals.

For in vivo measurement of excision repair, the available methods are often inadequate because radioactive labelling of DNA is required. Alternative methods, however, have been developed. For instance, monitoring of the removal of UV damage induced in the skin has been made possible by the development of UV-endonuclease based methods, which do not require the use of radioactivity. In these methods the size of the DNA fragments resulting from treatment with the UV-endonuclease is measured by electrophoresis in alkaline agarose gels (Sutherland and Shih, 1983) or by alkaline sucrose gradient centrifugation (D'Ambrosio et al., 1981). The DNA is detected by fluorescent dyes. These published methods, however, are either not very accurate (alkaline gel electrophoresis) or rather laborious (DNA detection in alkaline sucrose gradient fractions). In addition, their sensitivity in detecting DNA is rather low. As will be described in Chapter 6, an alternative method has been developed recently, based on alkaline sucrose gradient centrifugation and immunochemical detection of DNA, which allows accurate and sensitive

measurement of the induction and repair of dimers after in vivo treatment with UV.

After adaptation to the in vivo situation, detection methods involving the action of a lesion-recognizing endonuclease in principle are applicable on all DNA lesions that can be converted into single-strand breaks. Their sensitivity can be great, their specificity in distinguishing various types of lesions, however, is limited. An alternative possibility of assessing the induction and removal of DNA damage in vivo (also applicable in vitro) is by monitoring the presence of the damages in a more direct way, via a detection based on the binding of lesion-specific antibodies. On the basis of antisera raised against UV-irradiated DNA, radioimmunoassays and enzyme-linked immuno sorbent assays have been developed for monitoring the induction and removal of UV damage (Seaman et al., 1972; Cornelis et al. 1977; Mitchell and Clarkson, 1981; Wani et al., 1984). Like the adapted enzymatic detection methods, these assays do not require in vivo radiolabelling of DNA. In addition, the use of specific antibodies offers the possibility to sharply distinguish between the various types of DNA damage since in principle each type can be detected separately through binding of an antibody directed only against that specific lesion. With respect to UV damage, antisera that specifically recognize TT dimers have been prepared (Klocker et al., 1982). Moreover, variations in the relative binding capacity of polyclonal antisera raised against UV-irradiated DNA, allow to distinguish between various photoproducts (Ley, 1983; Mitchell and Clarkson, 1984). The importance of being able to distinguish between different lesions becomes clear when it is realized that the mutagenic (and probably carcinogenic) effect of an agent can be brought about by only one of the various lesions induced by the agent. A good example is the recent finding that in E.coli the (6-4)photoproduct and not the cyclobutane pyrimidine dimer is predominantly responsible for mutation induction (Haseltine, 1983). Interestingly, Mitchell et al. (1985) have obtained evidence that (6-4)photo-products are removed from the DNA of human and rodent cells more efficiently than are cyclobutane pyrimidine dimers.

Immunochemical detection can also be applied on other carcinogen-DNA adducts. Recently it has been shown that for the experimental liver carcinogen 2-acetylaminofluorene (2-AAF), adducts can be detected in isolated rat liver chromosomal DNA after dosages as low as 1 mg per kg body weight (Baan et al., 1985). The detection limit reached in that study was 1 adduct per $10^8$ nucleotides. This makes it sensitive enough for biomonitoring humans exposed to genotoxic agents, which is an important potential application of immuno-chemical detection methods (Lohman et al., 1985).

An alternative method for the detection of DNA lesions in vivo is the post-labeling method, developed by Gupta et al. (1982). This method, the application of which is restricted to aromatic carcinogen-DNA adducts, is based on a two-dimensional thin-layer chromatography system involving multiple developments. Its high sensitivity is obtained through enzymatic digestion of DNA to deoxynucleoside-3'-monophosphates, which are then radioactively labelled by T4 polynucleotide kinase-catalyzed $^{32}$P-phosphate transfer from adenosine-($\alpha$-$^{32}$P)triphosphate. Separation of the adduct-containing nucleotides from the normal ones is accomplished by 2 chromatography steps. A third and a fourth step provide a two-dimensional adduct fingerprint. By the application of this procedure one adduct in $10^7$-$10^8$ nucleotides can be detected. The sensitivity can even be further increased by the isolation of the adduct-containing nucleotides with 1-butanol in the presence of a phase transfer agent prior to the radioactive labeling. With this modification, first described by Gupta (1985), a sensitivity of one adduct per $10^{10}$ nucleotides can be reached when 10 µg of DNA is used. This is the highest sensitivity ever reported for assay systems for DNA lesions. Unfortunately, application of the postlabeling method is restricted to aromatic carcinogen-DNA adducts. Furthermore, the high amounts of radioactivity used in this method requires special precautions (Gupta, 1985).

## 2.4 Summary and Discussion

In summary, various methods exist for quantifying DNA excision repair activities in mammalian cells. Isolated cells, cultured in vitro under standardized conditions, are good model systems for comparative studies of excision repair responses in relation to the actual removal of the damage induced in the DNA. Their main disadvantage is the uncertainty as to whether they accurately resemble the in vivo situation, which is of major importance in studies on the possible relationship between aging and DNA repair. For the analysis of DNA excision repair in vivo, one depends on sensitive detection methods for DNA lesions that are removed via the excision pathways, which do not require prelabeling of the DNA. At the present time immunochemical detection methods seem to be the most versatile; specific antibodies can be made against all possible DNA lesions. Furthermore, immunochemical assays for DNA damage are sensitive, rapid, accurate and do not require the use of radioactivity. Removal of the damage itself is seen by many as the most reliable endpoint in assessing a repair pathway. However, also this approach has its limitations. Modifications of the induced lesions can often not be

distinguished from repair (Baan et al., 1985). Therefore, even when antibodies of high specificity and high affinity are available it is still necessary to study the induction and repair of the lesion(s) of interest with more than one method.

# CHAPTER 3

## KINETICS OF UV-INDUCED DNA EXCISION REPAIR IN RAT AND HUMAN FIBROBLASTS*

### 3.1 Introduction

The level of excision repair occurring in mammalian cells after exposure to ultraviolet light (UV) depends on the donor species. In general, human derived cells are more competent in performing the DNA synthesis step of the excision repair process than are rodent derived ones (Painter and Cleaver, 1969; Hart and Setlow, 1974; Kato et al., 1980; Francis et al., 1981). Also, the actual removal of pyrimidine dimers, the main UV photolesion, is much slower in rodent cells than in human cells (Ganesan et al., 1983; Takebe et al., 1983). These findings have been interpreted as evidence for "DNA repair" as a longevity-assurance system (Chapter 1). However, the difference in excision repair capacity between UV-irradiated human and rodent cells is not reflected in a corresponding difference in survival, e.g., the colony forming capacity of mouse cells is similar to that of human cells (Ganesan et al., 1983). Moreover, it has been reported that primary mouse embryonic fibroblasts, in contrast to late passage cells, excise dimers rather well and also perform high levels of repair synthesis (Peleg et al., 1977). Therefore, the question arises of whether rodent cells are truly defective in excision repair or are simply able to remove DNA damage in another way than do human cells, by a different mechanism of excision repair or via alternate repair pathways. For a further understanding of this problem, it is necessary to analyse the differences in excision repair between rodent and human cells in more detail and preferably by several methods. To this end, we have studied the excision of UV-induced photoproducts from the DNA of rat and human skin fibroblasts by (i) autoradiographical analysis of unscheduled DNA synthesis (UDS), (ii) analysis of DNA repair synthesis by means of the bromodeoxyuridine (BrdU) photolysis assay, (iii) monitoring the disappearance of Micrococcus luteus UV-endonuclease sensitive sites (ESS) and (iv) determining the number of induced and removed thymine dimers by high pressure liquid chromatography

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* Parts of this work have been published previously (Vijg et al., 1984b).

(HPLC). The results obtained are discussed in relation to the capacity of these cells to survive the toxic effects of the applied UV doses.

## 3.2. Materials and Methods

### 3.2.1 Cell isolation and culture

Rat primary fibroblasts were obtained from 10-mm surgical ellipse biopsies from the skin of the anterior part of the back of inbred Wistar-derived rats (WAG/Rij ♀). The rats were maintained under "clean conventional" conditions. The biopsies were cut into fragments that were placed in 3.5-cm diameter petri dishes (Greiner) and supplied with a droplet of growth medium. This consisted of Dulbecco's modification of Eagle's medium (DMEM, Gibco), supplemented with 20% fetal calf serum (FCS) and antibiotics. Cell growth was usually observed within 4 days. The primary explant cultures were supplied with fresh medium every two days and when they had reached confluency removed by trypsinization and transferred to 25-$cm^2$ culture bottles (Greiner). Although some groups of epithelial cells were initially observed near the explant, these were completely lost after the first trypsinization cycle. This was determined by electron microscopy. At this point, the medium was changed to DMEM with 10% FCS. Human fibroblast strains were obtained in the same way from the skin of the upper arm of normal donors. Rat and human cells were studied between 3 and 5, and 15 and 25 cumulative population doublings, respectively. Preliminary experiments were performed with several different rat and human cell strains. All the final comparisons, however, were made between rat and human strains R33 and Mb, respectively.

### 3.2.2 Survival assay

Cells were seeded in 6-cm diameter petri dishes (Greiner) at a density of 200 cells per dish and cultured for 24 h. After this period the medium was removed and the cells were rinsed with phosphate buffered saline (PBS), drained and exposed from above to 254-nm radiation supplied by a low pressure mercury vapour lamp (Philips TUV, 15W) at a fluence rate of 0.38 $W.m^{-2}$ for various time intervals. After irradiation the cells were cultured for 2 to 3 weeks, fixed with 70% ethanol and stained with 0.05% Coomassy brilliant blue G250 (Serva) in 20% methanol, 7.5% acetic acid. The colonies were counted and the relative survival was determined.

### 3.2.3 Determination of UDS

Cells ($1x10^5$) were seeded onto 11-mm diameter glass cover slips in 3.5-cm diameter petri dishes (Greiner) and cultured for 48 h. Then, they were rinsed with PBS and irradiated with varying doses of UV as described for the survival assay. Exposed and unirradiated control cells were both incubated with ($^3$H) thymidine (10 µCi/ml, 25 Ci/ mmole, Amersham) in medium with 10% dialyzed FCS for varying periods of time. At the end of the incorporation period, the cells were rinsed 3 times with medium without serum and fixed in acetic acid-ethanol (1:3). The cover slips were then rinsed with 70% ethanol, air dried and mounted onto glass slides. After dipping in NTB2 liquid emulsion (Kodak), the slides were stored in light-proof boxes at 4°C for 4 days. They were then developed and stained with toluidine blue. The amount of UDS was determined with an Artek Model 880 automatic grain counter. For each slide, the total nuclear area, the area above the nuclei occupied by silver grains and the percentage of the nuclear area occupied by silver grains were measured in at least 30 lightly labelled cells (which excludes cells performing semi-conservative DNA synthesis). The results were plotted as a histogram. As a rule, 2 or 3 slides were counted for the same dose and labelling time. UDS values were corrected for background labelling found in unirradiated controls.

To inhibit the synthesis of TTP, UDS was also measured in the presence of aminopterin. This compound was added to a final concentration of 0.2 µM 24 h before UV irradiation. For UDS determinations during confluency, cells were cultured for 10 days after seeding on cover slips.

### 3.2.4 Determination of ESS

Cells were seeded in 6-cm diameter petri dishes (Greiner) at a density of about $2x10^5$ cells per dish, allowed to grow for 24 h and subsequently labelled for 3 days with 1 µCi ($^3$H) thymidine per 3 ml of medium (25 Ci/mmole, Amersham). The cultures were then rinsed with PBS and irradiated as described for the survival assay. Afterwards, the cells were incubated with fresh unlabeled medium for periods of from 0 to 24 h, then rinsed with PBS, drained, immediately frozen on dry ice and stored at -70°C for not more than 3 days. The procedure used to determine the number of ESS was described earlier (Paterson et al., 1973; Wade and Lohman, 1980). Enzymatic photoreactivation of extracted DNAs was performed by treatment with an extract from Streptomyces griseus plus white fluorescent light (Zelle et al., 1980), which preceded the treatment with the Micrococcus luteus UV-endonuclease.

### 3.2.5 HPLC analysis

Cells were seeded in 9-cm tissue culture dishes (Greiner) at a density of about $7.5 \times 10^5$ cells per dish. The cells were allowed to grow for 24 h, after which period they were labelled for 3 days with ($^3$H) thymidine (1 µCi/ml of medium, 25 Ci/mmole, Amersham). The cultures were then rinsed with PBS and irradiated as described for the survival assay. Subsequently they were incubated with fresh unlabeled medium for 0 or 24 h, then rinsed with PBS and harvested by trypsinization. The cell suspension was then layered onto 2 µm polycarbonate filters (not more than $1.5 \times 10^6$ cells per filter) for DNA isolation according to the procedure of Leadon and Cerutti (1982). DNA was released from the filter by using DNase I. The DNA samples obtained were evaporated to dryness in a speed vac concentrator (Savant). They were hydrolysed with 98% formic acid at 170°C for 90 min and again evaporated. The residues were dissolved in 5 mM Na-formiate, pH 6.0, and analysed on a Beckman liquid chromatograph, using a 250x5 mm Lichosorb RP18 reverse phase column with 1% methanol (in 5 mM Na-formiate, pH 6.0) at a flow rate of 1 ml/min. A small precolumn, packed with $C_{18}$ Corasil (37-50 µm diameter), was used to protect the analytical column from resinous material. Fractions of 0.5 ml were sampled by a modified Redirac fraction collector (LKB). The six fractions eluting directly before thymine (containing the TT dimers) were pooled, evaporated to dryness, and rechromatographed under the conditions described above. The radioactivity-content was determined by liquid scintillation counting.

### 3.2.6 BrdU photolysis

Cells were seeded in two 9-cm tissue culture dishes (Greiner) at a density of about $7.5 \times 10^5$ cells each. They were allowed to grow overnight, after which the cells in one dish were labelled with ($^3$H) thymidine (1 µCi/ml of medium, 25 Ci/mmole, Amersham) and in the other with ($^{14}$C) thymidine (1 µCi/ml of medium, specific activity 56.5 mCi/mmole, Amersham). After a 3-day incubation period, the radioactive medium was replaced by normal medium and the incubation was continued for one day. Then, the normal medium was replaced by medium containing 3% FCS and 0.1 mM BrdU (the $^3$H labelled cells) or 0.1 mM thymidine (the $^{14}$C labelled cells). Both BrdU and thymidine were obtained from Sigma. After 30 min of incubation, the cells were irradiated as described for the survival assay and subsequently incubated in the same medium for 24 h. After this period, they were washed with PBS, trypsinized and suspended

in 5 ml PBS-containing glucose (1 mg/ml). Cells from the two dishes were now mixed and placed into a quartz cuvette. The cells in the cuvette (placed in a holder cooled with tap water) were irradiated with 313-nm light with a XeHg lamp (Hanovia) operated at 1000 W. The power supply, lamp housing, collimator, filter and monochromator were from Oriel. The entrance and exit slits had a width of 1.8 mm, resulting in a half-maximum bandwidth of 12 nm. Between the monochromator and the cuvette, a 5-mm thick glass filter WG 305 (Schott) was placed to absorb the radiation below 290-nm. The fluence rate (about 60 $W.m^{-2}$) was determined in each experiment by means of a radiant flux meter (Hewlett Packard). During irradiation the cell suspension was continuously stirred. At different times, a 50-µl sample was taken and sandwiched between two layers of 75 µl of lysis solution (1 M NaOH, 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). The first sample was collected at the beginning of the irradiation, the last at 2 h thereafter. After a lysis and denaturation period of 1 h, tubes with the gradients were centrifuged at between 25,000 and 35,000 rpm (depending on the number of breaks expected) in a Beckman SW60Ti rotor for 180 min at 20°C. These rather high rotor speeds are justified by the range of molecular weights. The number of breaks was derived from the size distribution of the DNA in the same way as in the UV-endonuclease experiments (Wade and Lohman, 1980). The maximum number of breaks resulting from the photolysis indicates the number of repaired sites. The kinetics of the photolysis reaction are used to calculate the average number of nucleotides inserted into the repaired regions. For this purpose, a computer programme was developed to determine the best fit for the data obtained in the equation describing the kinetics of the photolysis reaction (Regan and Setlow, 1974; Chapter 2). The value for sigma, representing the efficiency of 313-nm light in inducing alkali-labile sites in BrdU-containing regions, was determined (for rat as well as for human cells) on fully substituted ($^3$H)-labelled DNA irradiated with three different doses of 313-nm light. The values obtained for rat and human fibroblasts were $0.32\pm0.01 \times 10^{-6}$ and $0.36\pm0.02 \times 10^{-6}$ $m^2/J$, respectively. The assumption was made that the percentage of thymidine nucleotides in the DNA of both rat and human cells is 30%.

### 3.2.7 Thymidine kinase assay

Thymidine kinase (TK) activities were measured as described by Olashaw et al. (1983) with some modifications. Briefly, cells were harvested by trypsinization and resuspended in 10 mM Tris, pH 8 at a density of $3x10^5$/100 µl.

Subsequently, Triton X100 was added to a final concentration of 0.02%. Then 100 µl of this suspension was mixed with 100 µl TK-buffer (50 mM Tris, pH 8.5, 5 mM $MgCl_2$, 10 mM ATP, 10 mM β-mercaptoethanol). The reaction was started by adding 1 µCi $^3$H-thymidine (25 Ci/mmole, Amersham) and 4 nmole cold thymidine. The mixture was incubated for 0 to 30 min at 37$^0$ C. The reaction was terminated by boiling for 3 min, after which period 3 ml DEAE-cellulose suspension (1 g/60 ml of 1 mM ammonium formiate, pH 7) was added. The mixture was vortexed for 10 min and centrifuged at 2000 rpm for 5 min. The pellet was washed 6 times with 1 mM ammonium formiate, pH 7, resuspended in 1 ml 1 N HCl and centrifuged. The amount of radioactivity in the supernatant was determined by liquid scintillation counting.

## 3.3 Results

### 3.3.1 Survival

The cytotoxic effect of UV-irradiation of early passage rat fibroblasts was compared with that of normal human fibroblasts. Figure 3.1 shows that the capability of the two cell lines to form colonies upon UV-irradiation falls in the same range.

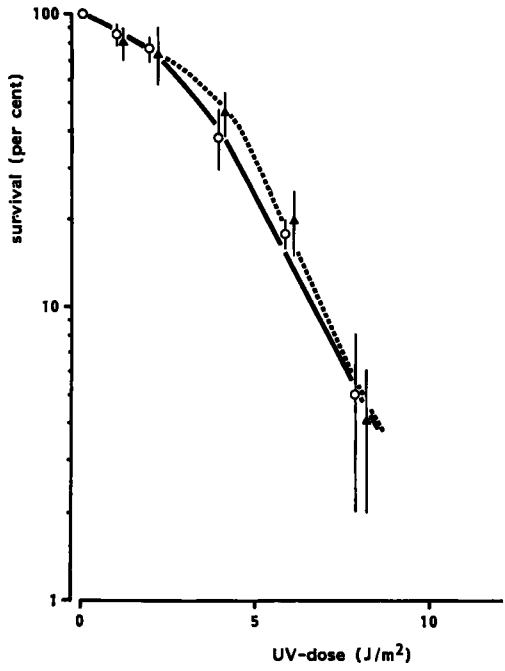


Figure 3.1:
Colony forming ability of rat (▲) and human (o) fibroblasts after various doses of UV.

### 3.3.2 Unscheduled DNA synthesis

A time course study of UDS in UV-irradiated fibroblasts is shown in Fig.3.2. It illustrates that human cells are more capable of repair synthesis than are rat cells, especially in the initial phase of the process. Initially, the rate of UDS in both rat and human cells is high, but gradually the slope of the curve becomes less steep. Since these kinetics could be due to a decrease in repair activity, for instance by a loss in cell viability, a split dose experiment was performed. Seven hours after a first UV dose of 4.6 J/m², rat fibroblasts were irradiated a second time with the same dose (Fig.3.3). The

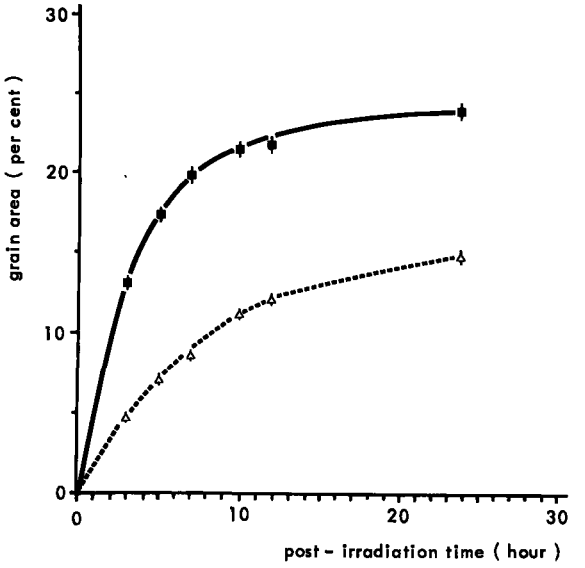


Figure 3.2:
UDS occurring in rat (Δ) and human (■) fibroblasts as a function of repair time after exposure to 4.6 J/m² of 254-nm UV. Points in each curve were obtained by determining the amount of UDS occurring during the preceding time interval and adding each value to the preceding ones. Corrections for the background were made by subtracting the value obtained for unirradiated control cells. Each point is the mean determined from 60 cells belonging to two separate cell cultures. Bars indicate the standard error of the means.

reaction to this second dose (indicated by the broken line) is the start of a new rapid process, with a 24-h level significantly lower than that obtained when both doses are given at the same time (upper solid line in Fig.3.3). However, since it can be expected that dose fractionation leads to a certain delay in reaching the 24-h level, complete additivity of UDS is assumed. It is obvious that rat cells are still fully able to perform repair synthesis at 7 h after irradiation. Comparable experiments with two doses of 10.3 J/m² showed that in both human and rat fibroblasts UDS after the second dose was reduced by about 50% as compared to the first (results not shown).

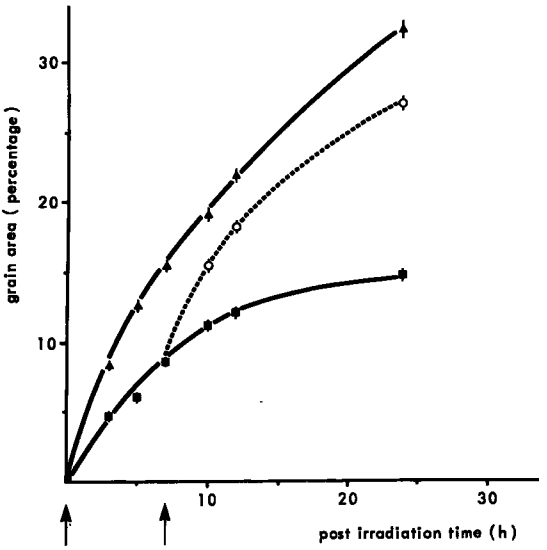


Figure 3.3: UDS in rat fibroblasts as a function of repair time after exposure to 9.2 J/m² (▲), 4.6 J/m² (■) and 2 times 4.6 J/$m_2$ with a 7-h interval (o). The times of irradiation are indicated by the arrows. Data points were obtained as in Fig.3.1.

### 3.3.3 Removal of ESS

With the UV-endonuclease method, the number of UV-induced ESS in rat and human fibroblasts after irradiation with different UV doses can be determined. A dose-response curve for the induction of these sites in the DNA of both species is presented in Fig.3.4. All three doses induced slightly more ESS in human than in rat cells (50 ± 4 and 47 ± 2 induced ESS per J/m² per $10^9$ molecular weight of DNA, respectively). This might be a reflection of differences in nucleotide content between the two species.

From the results on UDS, one can conclude that human cells are more active with regard to UV-induced DNA repair synthesis than are rat cells. The difference varies from a factor of 2.7 at 3 h after irradiation to one of 1.6 at 24 h after irradiation (Table 3.1). As it is generally assumed that UV-induced DNA repair synthesis reflects the removal of pyrimidine dimers, one would expect the difference in this latter process to be similar. To determine whether this indeed was the case, we followed the removal of ESS (pyrimidine dimers) in both cell types. The results (Fig.3.5) show a distinct difference between

TABLE 3.1 COMPARISON OF UDS, BrdU PHOTOLYSIS, UV-ENDO AND HPLC OVER 3 AND 24 h PERIODS AFTER IRRADIATION WITH 4.6 J/$m^2$ OF 254 nm UV

| Cell type | Postirr. time (h) | UDS | BrdU photolysis | | UV-endo | HPLC |
|---|---|---|---|---|---|---|
| | | grain area (%) | number of repaired sites per $10^9$ mw DNA | number of nucleotides per repair region | number of removed dimers per $10^9$ mw DNA | removed T<>T dimers (% T<>T/T) |
| Human fibroblasts | 3 | 13.1 ± 0.4 | 83 ± 10 | 73 ± 10 | 50 ± 10 | ND |
| | 24 | 17.5 ± 0.7 | 90 ± 10 | 63 ± 10 | 121 ± 7 | 0.010 ± 0.001 |
| Rat fibroblasts | 3 | 4.8 ± 0.1 | 24 ± 10 | 66 ± 10 | 0 ± 7 | ND |
| | 24 | 12.5 ± 0.5 | 52 ± 10 | 59 ± 10 | 26 ± 7 | 0.002 ± 0.002 |
| Ratio rat/human | 3 | 0.37 ± 0.01 | 0.3 ± 0.1 | 0.9 ± 0.2 | - | ND |
| | 24 | 0.71 ± 0.04 | 0.6 ± 0.1 | 0.9 ± 0.2 | 0.21 ± 0.06 | 0.2 ± 0.2 |

The errors in UDS, UV-endo and HPLC were estimated from 4 experiments.
The errors in BrdU photolysis were estimated from the photolysis curves of 2 experiments.

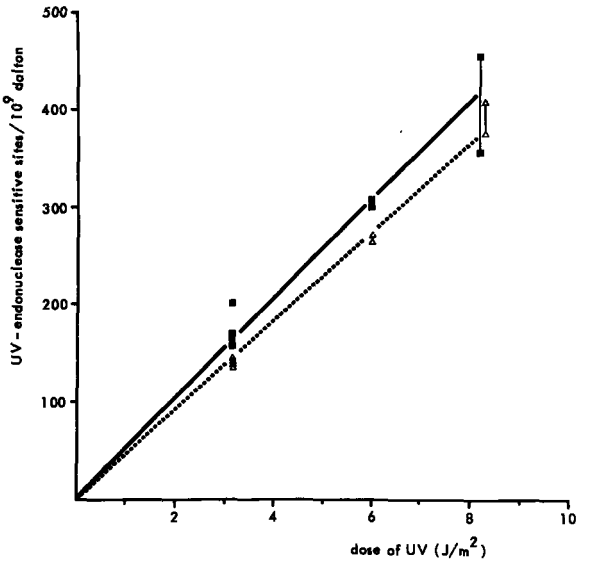


Figure 3.4:
Dose response for ESS induction by 254-nm UV in rat (Δ) and human (■) fibroblasts. For each dose, two determinations were made.

the two species with respect to the removal of ESS which, however, is much greater than was expected. While the human cells removed more than 70% of the total number of induced lesions within 24 h, the rat cells during the same period and under identical conditions removed less than 20% of the dimers originally present. After 5 days, rat cells still contain 71% of the dimers originally induced (results not shown). This indicates that, although rat cells are quite capable of performing DNA repair synthesis, they are much less able to remove pyrimidine dimers (Table 3.1). To check whether the ESS still present in rat cells at 24 hours after irradiation were genuine pyrimidine dimers, their photoreactivability was determined. As indicated in Fig.3.5, virtually all sites determined as ESS were photoreactivable both immediately after irradiation and 24 h later. To investigate whether there was a correspondence between the number of dimers present in rat cells at certain times after irradiation and the rate of DNA repair synthesis, we determined the amount of UDS (over a period of 4 h) as possibly evoked by: (1) the dimers still present at 24 h after irradiation; (2) the same number of dimers as in (1) consisting half of old (still present at 24 h after irradiation) and half of newly introduced dimers; and (3) the same number of dimers as in (1) and (2), all of them newly introduced. The results presented in Table 3.2 show that hardly any UDS is induced by 24 h old dimers. However, with the same number of new

dimers present, rapid UDS is obtained, about twice as high as the amount of UDS observed when the same number of dimers is present, half of which being introduced 24 h earlier. While these results could already be predicted from the time course curve (Fig.3.2) and the split dose curve (Fig.3.3), they unequivocally demonstrate that newly induced dimers appear to be able at any time to induce rapid UDS, whereas old dimers are unable to do so.

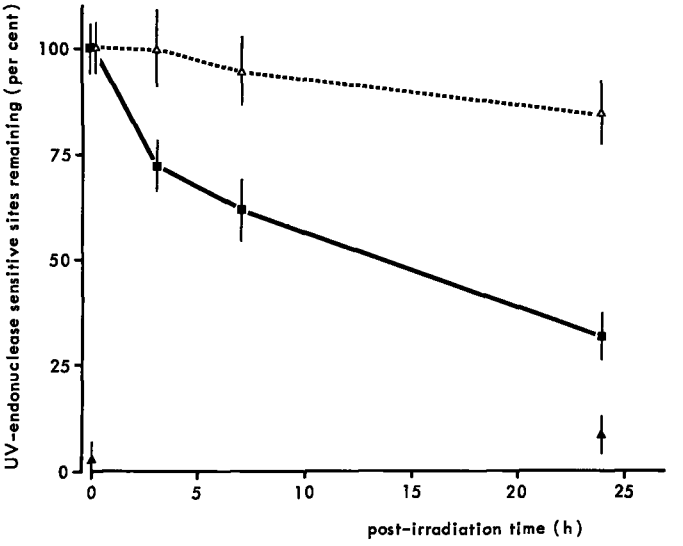


Figure 3.5: Relative time course of disappearance of ESS from the DNA of rat (Δ) and human (■) fibroblasts exposed to 4.6 J/m² of 254-nm UV. The numbers of ESS determined after treatment of the extracted DNAs with photoreactivating enzyme (PRE) at 0 and 24 h after irradiation are also indicated (▲). Bars indicate the standard deviations as determined from four determinations.

In view of the absence of a relationship between DNA repair synthesis and dimer removal, in rat cells, we compared the kinetics of UDS with ESS removal in both rat and human cells. For this purpose, their ratios of UDS vs ESS removal were calculated at 3, 7 and 24 h after irradiation (the data were the same as presented in Fig.3.2 and Fig.3.5). The results presented in Table 3.3 show that neither in rat nor in human cells do the kinetics of UDS and ESS removal differ significantly. However, it is obvious that in both cell types the kinetics of ESS removal tends to be slower than the kinetics of UDS. This is indicated by the fact that for both cell types the ratio of UDS to ESS

removal is the smallest at 24 h after irradiation. Furthermore, from Table 3.3 it can be derived that the ratio of UDS to ESS removal for human cells is significantly smaller than that for rat cells.

TABLE 3.2 INDUCTION OF UDS BY "OLD" AND "NEW" DIMERS

| t=0 Number of dimers | t=24 Number of dimers | | | t=24-28 UDS |
|---|---|---|---|---|
| induced[a] | remaining[b] | induced[a] | total | (grain area)[c] |
| 414 ± 15 | 352 ± 18 | 0 | 352 ± 18 | 0.4 ± 0.1 |
| 207 ± 8 | 176 ± 9 | 175 ± 6 | 351 ± 11 | 7.2 ± 0.2 |
| 0 | 0 | 352 ± 13 | 352 ± 13 | 13.7 ± 0.4 |

a The numbers of induced dimers were calculated from the dose-response curve in Fig.3.4. The errors were derived from linear regression analysis.
b The numbers of removed dimers were derived from the percentage of dimer removal in Fig.3.5. The errors are composed of the estimated errors in the number of removed dimers (4 Expts.) and the errors in the number of induced dimers.
c UDS was determined over 4 h as described under Materials and Methods. The errors are estimated from 4 experiments.

### 3.3.4 Monitoring of dimer removal by HPLC

Instead of a reflection of the incapacity of rat cells to remove pyrimidine dimers by excision repair, the observed persistency of ESS in these cells could be an artefact of the dimer detection method used. This is not wholly inconceivable, since with the enzymatic assay used dimers are detected in an indirect way, as substrates of a crude bacterial extract. As long as the dimers themselves have not been shown to persist in the DNA of rat cells the possibility should be taken into account that the persistent ESS (in spite of their photoreactivability) represent some other kind of UV damage. In order to address this problem we applied a chromatographical method to measure the induction and removal of UV damage. Generally, chromatographical methods are not very sensitive and their application therefore requires high doses of UV (10 J/m$^2$ or higher). Since we have shown that at such high doses UDS is being inhibited, we considered it necessary to work with the same low doses as in the UV-endo assay. For that purpose we made use of a recently developed HPLC assay,

TABLE 3.3 RATIO OF UDS TO ESS REMOVAL IN RAT AND HUMAN FIBROBLASTS

| | Human (Mb) | | | Rat (R33) | | |
|---|---|---|---|---|---|---|
| Postirr. time | UDS | ESS removed | UDS/ ESS removed | UDS | ESS removed | UDS/ ESS removed |
| 3 h | 13.1 ± 0.4 | 50 ± 7 | 0.26 ± 0.04 | 4.8 ± 0.1 | 0 ± 7 | - |
| 7 h | 20.0 ± 0.4 | 67 ± 8 | 0.30 ± 0.04 | 8.6 ± 0.2 | 9 ± 7 | 1.0 ± 0.8 |
| 24 h | 24.1 ± 0.5 | 121 ± 7 | 0.20 ± 0.01 | 14.9 ± 0.3 | 26 ± 7 | 0.4 ± 0.1 |

All data are derived from Figs. 3.2 and 3.5. The errors are estimated from four experiments.

which allows the quantification of UV damage after doses below 5 J/m² (Niggli and Cerutti, 1983).

A typical HPLC elution profile of hydrolysed radioactivity-labelled DNA from human fibroblasts, irradiated with 4.6 J/m², is shown in Fig.3.6. The position

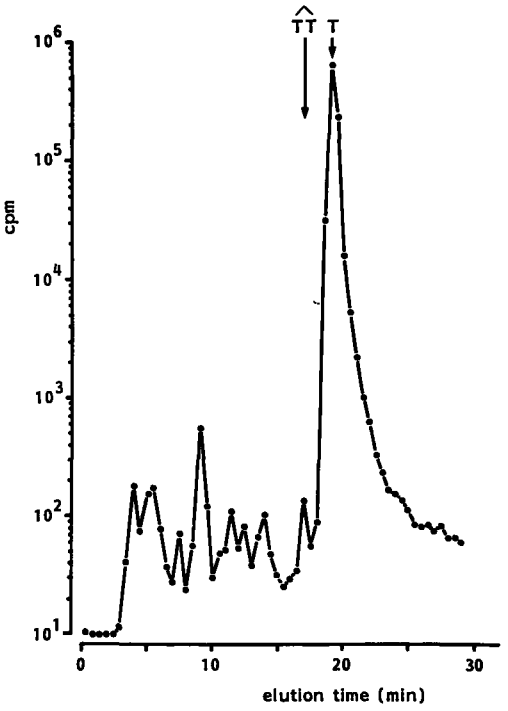


Figure 3.6: HPLC elution profile of hydrolyzed radioactivity-labelled DNA from human fibroblasts, irradiated with 4.6 J/m² of UV.

of the co-chromatographed thymine and TT dimer markers (the latter was obtained by irradiating a frozen thymine solution with 20 kJ/m² of 254-nm of UV) were detected by spectrophotometry (220-nm UV absorption). In the radioactivity profiles, the small peak directly before thymine appeared to be the TT dimer. This peak was not present in the DNA of unirradiated control cells. The 0 h elution profile of the rat cells was identical to that of the human cells shown in Fig.3.6. Quantification of the radioactivity contents of the fractions containing the TT dimers (described under Section 3.2.5) in comparison to those containing the radioactive thymine (which served as an

internal standard) provided information on the number of TT dimers induced and repaired. The results were essentially the same as those obtained with the UV-endo enzymatic method. Human cells appeared to remove about 70% of the dimers originally induced, whereas rat cells remove only about 20% over the same time period (Table 3.1). The number of dimers induced was found to be less as compared to those detected with the UV-endo method. After correction for the contribution of CC, CT and TC dimers, which also form a substrate for the M.luteus extract, about 29 dimers per $10^9$ molecular weight of DNA per $J/m^2$ were found. However, in view of the systematic errors in the two methods (the error in the calibration of the UV-endo method using T4 DNA may already amount to 50%; Van der Schans, unpublished results) this difference is not statistically significant.

### 3.3.5 The number and average size of repair patches

One factor that may influence UDS but not dimer removal is the size of the repair patches. To find out whether a difference in repair patch size between rat and human cells modulates the difference in UDS, the BrdU photolysis assay was applied. This assay offers the possibility of determining both the number and the size of repair patches in one experiment (Regan and Setlow, 1974). Figure 3.7 shows the photolysis curves for rat and human fibroblasts at 24 h after a 254-nm UV dose of 4.6 $J/m^2$. From these curves, obtained by fitting the numbers of breaks after various doses of 313-nm light into the equation describing the kinetics of the photolysis reaction (Regan and Setlow, 1974), the number as well as the mean size of the repair patches was derived. Such data were also obtained for the period between 0 and 3 h after irradiation. The results are shown in Table 3.1, in comparison to the values for UDS and dimer removal over the same time periods. Since all three methods are based on different principles comparison among them may be biased by systematical errors. However, determinations using one technique on rat and human cells were always done simultaneously. Therefore conclusions can be drawn from the ratios rat to human, which are also provided in Table 3.1. The results provided in Table 3.1 indicate that a) the average repair patch size remains fairly constant over the whole repair period; b) this size is not different for the two species; c) the ratio rat to human in dimer removal is significantly smaller than that in DNA repair synthesis, both at 3 and 24 h after irradiation (confirming the discrepancy observed between dimer removal and UDS); d) in both species, DNA repair synthesis seems to proceed considerably faster than dimer removal.

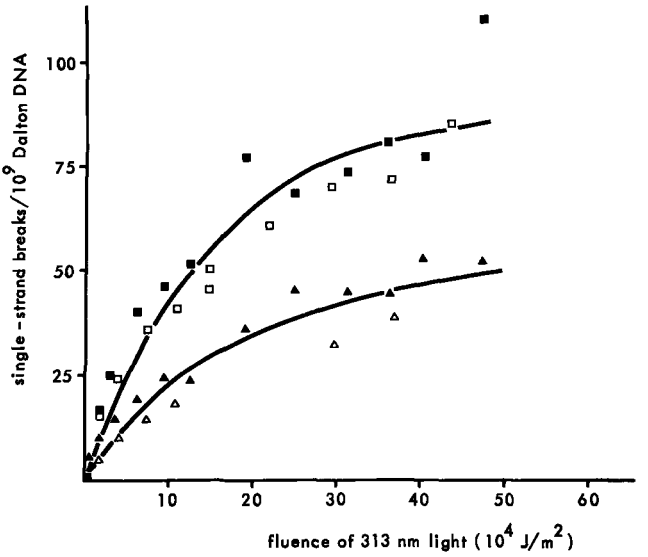


Figure 3.7: BrdU photolysis curves for rat (Δ▲) and human (□ ■) fibroblasts irradiated with 4.6 J/m² of 254-nm UV and allowed a 24-h repair period in medium with BrdU. Each point indicates the number of single strand breaks above the number of background breaks (breaks in the non-BrdU containing control cells). The results of two independent experiments are presented (open and closed symbols); the points were fitted into the equation describing the photolysis kinetics.

### 3.3.6 Effect of thymidine pool size

A different thymidine pool size could be involved in the observed discrepancy between UDS and dimer removal. Whether it may also influence the number of repair patches, which is one of the endpoints measured in the BrdU photolysis assay, is unlikely. Instead, it may influence the number of BrdU substitutions per repair patch, which will manifest itself in this assay as differences in patch size. Such differences were not observed in this study. However, in view of the rather large experimental error in patch size determinations using the BrdU photolysis assay (Table 3.1), the possibility of pool size differences could not be entirely ruled out. To check whether differences in thymidine pool size between rat and human cells could have been modulated the observed difference in UDS level, UDS was determined at different concentrations of ($^3$H) thymidine. Figure 3.8 shows that, at the normally used concentration of 10 μCi/ml, saturation of the thymidine pools of the two cell

types was almost reached but not complete. This indicates that this factor can not be neglected in comparative studies on UDS. However, the fact that the dependency of UDS in rat and human cells on the concentration of radioactive thymidine does not appear to be different, indicates that no substantial pool size difference exists. This conclusion was confirmed by the finding that the amount of ($^3$H) thymidine incorporated during semiconservative DNA synthesis is the same for the two cell types (results not shown).

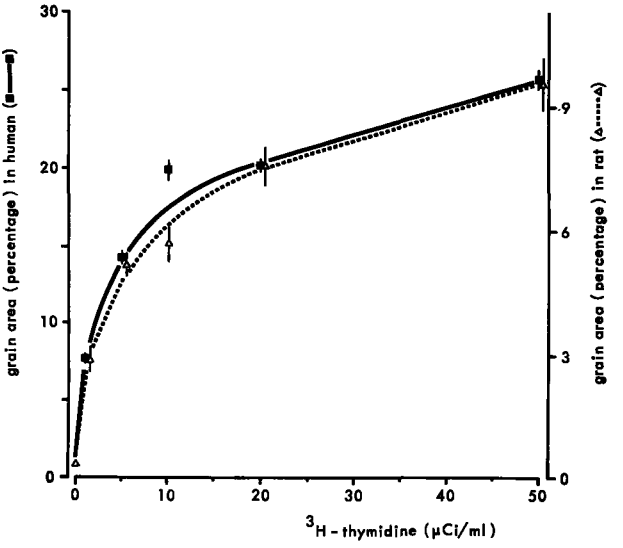


Figure 3.8: UDS in rat (Δ) and human (■) fibroblasts as a function of $^3$H-thymidine concentrations. Apart from the added $^3$H-thymidine no thymidine was present in the medium. UV dose, 4.6 J/m$^2$; postirradiation time, 3 h.

In order to completely rule out the possibility that pool size differences are responsible for the observed discrepancy between dimer removal and repair synthesis, we determined the amount of UDS in rat and human cells in the presence of aminopterin and during confluency. Aminopterin completely blocks the utilization of endogenous thymine by inhibiting the synthesis of TTP from UTP. Consequently, the radioactive thymidine added is no longer diluted by endogenous thymidine. In confluent cells, thymidine pool sizes are considerably reduced in comparison to actively proliferating cells (Olashaw et al., 1983). If such relatively large changes in pool size would not affect the level of UDS, it was assumed that the concentration of radioactive thymidine in our study was

high enough to preclude any pool effects.

The effects of aminopterin and confluency on UDS in rat and human fibroblasts are shown in Table 3.4. In the presence of aminopterin, the levels of UDS in the two cell types were significantly increased as compared to the situation in the absence of aminopterin. The increase in UDS in human was slightly larger than that in rat cells, indicating a smaller pool size in the latter. However, since even in the presence of aminopterin the ratio rat to human in UDS is still significantly larger than that in dimer removal (compare Table 3.4 with Table 3.1), the small difference in pool size indicated by these results does not explain the observed discrepancy between these two DNA repair endpoints. In addition, in the course of this study it was found that at higher concentrations of aminopterin UDS in rat cells is inhibited, whereas that in human cells is not influenced. In view of this difference, the possibility should be taken into account that the relatively small effect of aminopterin on UDS in rat cells reflects the greater sensitivity of this cell type to the toxic effects of aminopterin rather than a difference in pool size. Moreover, the UDS level of confluent cells, which have a very low pool size, is hardly different from that in exponentially dividing cells (Table 3.4). This suggests that under the experimental conditions normally applied (the absence of aminopterin) there is no effect of possible pool size differences. In this respect, UDS might be a far better measure for excision repair than is often assumed.

TABLE 3.4 UDS IN POLIFERATING VS CONFLUENT HUMAN AND RAT FIBROBLAST POPULATIONS IN THE PRESENCE AND IN THE ABSENCE OF AMINOPTERIN

| Species | Repair time (h) | Proliferating -AP[1] | Proliferating +AP[1] | Confluent -AP[1] |
|---|---|---|---|---|
| Human | 3 | 14.0 ± 0.6 | 27.1 ± 1.0 | 18.5 ± 0.7 |
| | 24 | 17.5 ± 0.7 | 31.1 ± 1.2 | - |
| Rat | 3 | 7.9 ± 0.3 | 12.2 ± 0.5 | 8.3 ± 0.3 |
| | 24 | 12.5 ± 0.5 | 18.3 ± 0.7 | - |
| Ratio Rat/Human | 3 | 0.56 ± 0.03 | 0.45 ± 0.03 | 0.45 ± 0.03 |
| | 24 | 0.71 ± 0.04 | 0.59 ± 0.03 | - |

[1] AP = Aminopterin

### 3.3.7 Thymidine kinase activity

Another factor that has often been suggested to be a modulator of the experimental endpoint of DNA repair synthesis assays is the thymidine kinase activity. The possibility that our results were influenced by differences between human and rat cells in TK activity was taken into account. For that purpose we measured TK activities of the two cell types. If the small difference between rat and human cells in UDS as compared to the much larger difference in dimer removal would be due to a difference in TK activity, a relatively high level of activity should be found in rat cells. Surprisingly, the human cells had far larger TK activities than the rat cells (Fig.3.9). Therefore we conclude that the level of TK activity in this specific case does

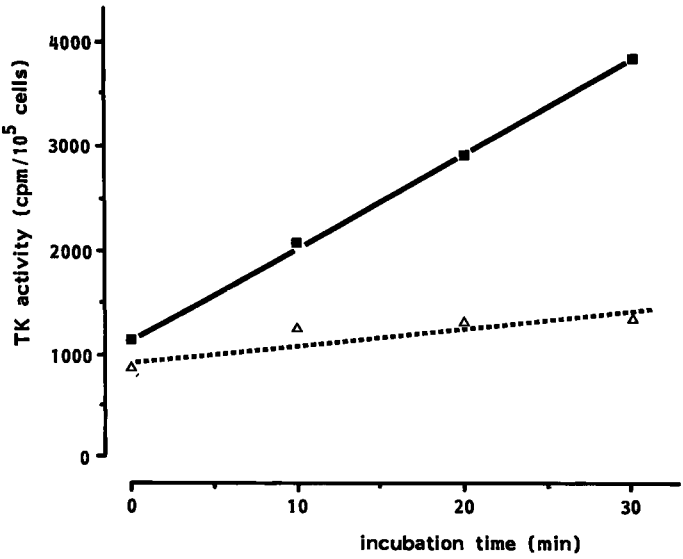


Figure 3.9: Thymidine kinase activity in rat (Δ) and human (■) fibroblasts.

not influence UDS at all. In Chapter 5 data are presented which indicate that even confluent cells, without any detectable TK activity (which does not mean that there is no such activity), have the same level of UDS as exponentially dividing cells. In view of these data it seems that also the putative effect of TK activity on UDS has often been overestimated.

## 3.4 Discussion

A comparison of the UV-induced DNA excision repair process in cells of different mammalian species is important for an eventual understanding of the relationship between induced DNA damage, its repair and physiological phenomena such as aging and carcinogenesis. Whereas much attention has been recently paid to inter- and intraspecies differences in DNA repair responses, there is as yet no consensus with respect to the mechanism of excision repair of UV-induced DNA damage in mammalian cells. To gain a further understanding of this problem, we have compared the kinetics of UDS and the disappearance of pyrimidine dimers in UV-irradiated fibroblasts from rats and humans, being representatives of the two groups that are most dissimilar with respect to excision repair level. The results obtained on the kinetics of UDS in cells from these species indicate that the rate of the repair process in rat cells is much slower than in human cells. However, during the course of the process, the difference becomes less. This suggests that, although human cells are more rapid in recognizing and removing UV-induced DNA damage, there may not be a difference in the end level of the repair process between the two cell types.

When the excision repair capacity of both cell types was assessed by following the removal of UV-endonuclease susceptible sites (pyrimidine dimers) instead of determining UDS, an apparent discrepancy between the two techniques became obvious. While the difference in UDS between the two cell types is approximately a factor of 2, the difference in their capacity to remove pyrimidine dimers is much more striking. Human cells remove 4-5 times as many dimers during the same time interval than do rat cells. Essentially the same results were obtained when pyrimidine dimer removal was analysed by HPLC instead of using the UV-endo enzymatic assay. This means that, although rat cells are quite capable of performing DNA repair synthesis, they are much less able to perform the process thought to underly this, i.e., the removal of pyrimidine dimers. Our results made it likely that the discrepancy between UDS and dimer removal is not due to a species specific difference in thymidine pool size, thymidine kinase activity or repair patch size. This last finding is in agreement with data from Francis et al. (1981), who did not find interspecies differences in repair patch size in a study with cells from 21 animal species, including humans and rodents. Furthermore, the difference between human and rat cells in the number of repaired sites as determined by the BrdU photolysis assay closely corresponds with the difference in UDS. These findings indicate the absence of a consistent relationship between UV-induced DNA repair synthesis and pyrimidine dimer removal in rat cells. The

absence of such a relationship was suggested earlier by Lohman et al. (1976), who demonstrated that hamster cell lines show a markedly reduced capacity in the removal of pyrimidine dimer sites as compared with normal human cells, whereas their levels of DNA repair synthesis were not significantly different.

While our study shows that a similar discrepancy is found in normal rat cells, it also provides additional information with respect to the nature of this phenomenon, which is best demonstrated by the results presented in Table 3.2. It is tempting to conclude from this experiment that newly induced dimers are able to evoke rapid repair synthesis while the same amount of old dimers (dimers that have been present in the cell for a period of 24 h after irradiation) is clearly not. It should be noted that all of these dimers seem to be authentic, that is, they are all photoreactivable and elute to the same position after HPLC. This makes an explanation of this phenomenon in terms of a modification of induced dimers during postirradiation incubation, as has recently been described for xeroderma pigmentosum group D cells (Paterson, 1982a), unlikely. This and the alternative possibility that unremoved dimers in rat cells are simply inaccessible to the repair enzymes should also be considered unlikely in another respect. Indeed, if this were to be the case, the small number of dimers removed in rat cells should give rise to a relatively high level of UDS. This would imply that pyrimidine dimers induce more UDS in rat cells than in human cells (indicated by the difference in the ratio of UDS vs ESS removal between the two cell types), for instance, as a consequence of large repair patches or a small thymidine pool size. This is not supported by our results with respect to these parameters.

Another possible explanation for our present findings is that the observed UDS in rat cells reflects the removal from the DNA of other, as yet unknown, UV lesions instead of being due to the removal of dimers. Results of experiments with embryonic chick cells, which have an efficient photoreactivation repair system, suggest that excision repair of other lesions may occur. When these cells were incubated in the dark after irradiation with UV, removal of pyrimidine dimer sites, associated with DNA repair synthesis occurred. Surprisingly, the same level of DNA repair synthesis was found after the removal of pyrimidine dimers by exposing the cells to photoreactivating light (Paterson et al., 1974). However, contrary to these results, it has been shown recently that photoreactivation in embryonic chick cells is accompanied by a decrease in the end level of UDS (Roza et al., 1985). Moreover, there is no present evidence that pyrimidine dimers do not represent the vast majority of UV-induced damage.

In relation to the above considerations, our findings are best explained

by assuming that in the rat (1) DNA repair synthesis is a rapid process that is almost ended at 24 h after irradiation and (2) pyrimidine dimer removal is a slow process and occurs after DNA repair synthesis. Furthermore, there is no reason to assume that the situation in human cells is much different from that in rat cells. Indeed, a comparison of the kinetics of UDS and dimer removal in human cells (Table 3.3) also suggests that most of the UDS in human cells occurs within the first few hours, while dimer loss occurs later. Essentially the same conclusion can be drawn when the creation of repair patches is compared to dimer removal; whereas the number of patches at 3 h after irradiation is significantly larger than the number of dimers removed, this is just the opposite between 3 and 24 h (Table 3.1). In this respect, our data do not differ from those of Ehman et al. (1978), who compared UDS with the chromatographically determined removal of acid precipitated dimers in UV-irradiated human cells. These authors suggest a "patch and cut" type of mechanism to explain the rapid kinetics of repair synthesis as compared with the slower loss of thymidine dimers in these cells. However, others (Williams and Cleaver, 1978) found the loss of T4 endonuclease V-sensitive sites in African green monkey cells to be at least as rapid as the occurrence of repair synthesis. Nevertheless, a "patch and cut" type of mechanism for excision repair in which repair synthesis occurs before the dimers are actually excised should be taken into consideration. As has been demonstrated with bacteria, the initial step in such a process might be the action of a dimer specific glycosylase which disrupts one of the two bonds between the dimer and the DNA phosphoribosyl backbone (Haseltine and Gordon, 1982). If mammalian cells would primarily (but not necessarily solely) initiate excision repair in this way, one might assume that in subsequent steps the resulting apyrimidinic site is removed, followed by resynthesis and ligation. According to this speculative model, schematically depicted in Fig.3.10, the dimers are not removed but remain semidetached in the DNA. In view of its small size, such a structure may still be able to basepair and therefore not form a block during replication. In addition, the semidetached dimer may then still be recognized by M.luteus UV-endonuclease and by photoreactivating enzyme. This would explain why in rat cells most of the initially present dimers are still found after most of the repair synthesis has already been accomplished. The model may also explain the discrepancy between immunochemical and enzymatic monitoring of dimer removal. It has been shown that the kinetics of dimer removal in Chinese hamster ovary cells as determined with antibodies are even more rapid than DNA repair synthesis in these rodent cells (Mitchell et al., 1982; Clarkson, 1983). This may be explained by assuming that, in contrast to the M.luteus

extract, the antibodies are able to select between genuine dimers and semidetached ones. Removal of antibody binding sites would then rather be a measure for glycosylase action than for dimer removal.

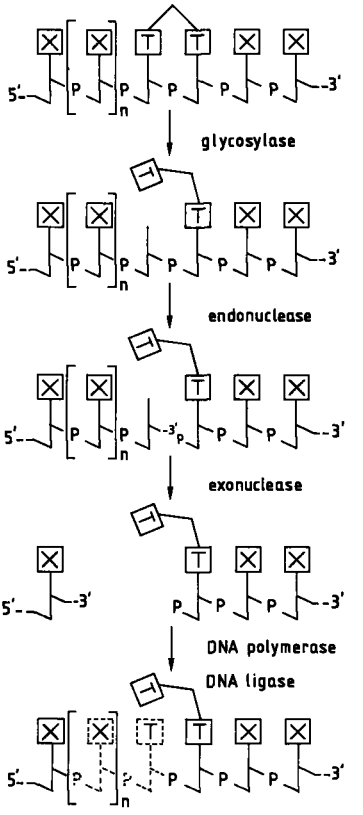


Figure 3.10: Possible sequence of events in an hypothetical pathway of UV-induced excision repair in rat and human fibroblasts.

As already mentioned above, we do not consider the speculative pathway shown in Fig.3.10 to be the only one utilized by mammalian cells. Instead, it is not inconceivable that mammalian cells utilize a number of different excision repair pathways. The utilization of these pathways could be species (or even cell type) specific. Rat cells may for instance rely more heavily on the pathway proposed above than human cells. Species specific utilization of DNA

repair pathways may also explain why no difference in survival is observed between human and rodent cells. One might assume that the latter cell type is more dependent on error prone DNA repair pathways. In this context, it is of interest to mention that in human cells the extent of excision repair before semiconservative DNA synthesis determines the mutagenic but not the lethal effect of UV (Konze-Thomas et al., 1982). In keeping with this are the observations of Menck and Meneghini (1982) that the recovery in survival of UV irradiated mouse 3T3 cells cannot be accounted for by excision of pyrimidine dimers. As to the nature of the recovery process, they considered the activity of systems resembling bacterial SOS repair. According to this line of reasoning, the ratio of error free to error prone repair activities may differ between human and rodent cells. While this evidently does not lead to a difference in survival capacity between the two cell types, it may lead to an increased frequency of genetic changes in rodent cells. The increased rate of evolution of rodents in comparison to primates supports this idea (Britten, 1986).

# CHAPTER 4

## UV-INDUCED DNA EXCISION REPAIR IN FIBROBLASTS OF AGING INBRED RATS*

### 4.1 Introduction

It can be hypothesized that DNA damage is the primary cause of aging. Aging may then be envisaged as the consequence of two processes the occurrence of which is not mutually exclusive: (1) a direct process in which DNA lesions accumulate in the genome of the constituent cells of aging organisms and, interfering with transcription and replication, lead to loss of cell function and death (Gensler and Bernstein, 1981); and (2) an indirect process in which induced lesions lead to genetic alterations such as base pair mutations and DNA rearrangements, changing the normal pattern of gene expression (Chapter 1).

Much attention in aging research has been focused on certain steps in the complex of DNA repair pathways thought to determine to a large extent the rate at which lesions accumulate in the aging genome (Williams and Dearfield, 1981). Mainly on the basis of the correlation found by Hart and Setlow (1974) between the lifespan of a species and the activity of the excision repair pathway in its UV-irradiated fibroblasts, it has been suggested that "DNA repair" may be a determinant of lifespan. This hypothesis is supported by the fact that both cancer and immunological defects, the incidences of which increase greatly with age, have been shown to be associated with deficiencies in certain DNA repair pathways (Setlow, 1978; Paterson, 1982b; Harris, 1983). However, a connection between biological aging and age-related changes predisposing to fatal disease is not clear at present. In this context, direct evidence for the implication of any DNA repair pathway as a determinant of individual longevity is still lacking.

A possible relationship between DNA repair activities and lifespan may be twofold. First, cellular repair systems might remain constant throughout the lifespan of an organism, but might vary from individual to individual. In this case, relatively proficient DNA repair systems in early age would be predictive

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* Parts of this work have been published previously (Vijg et al., 1985b).

for a long lifespan. Alternatively, cellular DNA repair systems might deteriorate with age. The rate of this deterioration could vary from individual to individual, in which case a rapid rate of deterioration would be predictive for a short lifespan and vice versa. Complicating factors are that the two possibilities are not mutually exclusive and that switches in the utilization of the many different DNA repair pathways may occur during the lifespan, that is, the relative importance of the different DNA repair pathways may vary with age. To address these questions it would be necessary to test in a lifespan study each of the various DNA repair pathways individually and in relation to each other.

Recently, a number of investigators have attempted to correlate the amount of DNA repair synthesis occurring in human cells after treatment with radiations or chemicals with the age of the donor (Pero et al., 1978; Lambert et al., 1979; Nette et al., 1984; Kovacs et al., 1984). According to Setlow (1983 and personal communication), the most striking results of these studies were not so much the age-related changes in this DNA synthesis step of the excision repair pathway that were observed in most of these cases but the large variations among apparently normal individuals. Hence, age-related changes in DNA repair synthesis can only be established on the basis of determinations on many individuals. Even then, it is not allowed to draw any definite conclusion from cross-sectional analyses in which groups of young subjects are compared with groups of aged subjects. It should be realized that in this type of approach each group has its own unique characteristics based on both individual and generational differences. Therefore, the best way to study a DNA repair pathway in relation to donor age is through longitudinal studies in which the same population is followed over many years. In humans, such an approach is complicated by the length of the human lifespan. A good model system that may be influenced to a much lesser extent by the above-mentioned factors is offered by relatively short-lived mammals such as rats or mice with a maximum lifespan of about 4 years. Diversity due to genetic and environmental variables can be minimized by the use of inbred rodents kept under well defined conditions (Hollander, 1976). In addition, the short maximum lifespan of these animals makes it possible to perform a longitudinal study.

In this paper, the strategy for a lifespan study of DNA repair pathways will be outlined. The results of the first two time points (separated by a time interval of 9 months) in a time sequential cross-sectional (longitudinal) study of UV-induced DNA repair synthesis in skin fibroblasts isolated from rats of the same inbred strain will be presented.

## 4.2 Materials and Methods

### 4.2.1 Animals

The animals used in this study were female Wistar-derived WAG/Rij rats, one of the inbred strains kept especially for aging research at the TNO Institute for Experimental Gerontology. The survival characteristics of the strain are shown in Fig.4.1. The animals were well defined in terms of age-related pathology and health status (Burek, 1978). They were maintained under "clean conventional" conditions as described by Hollander (1976).

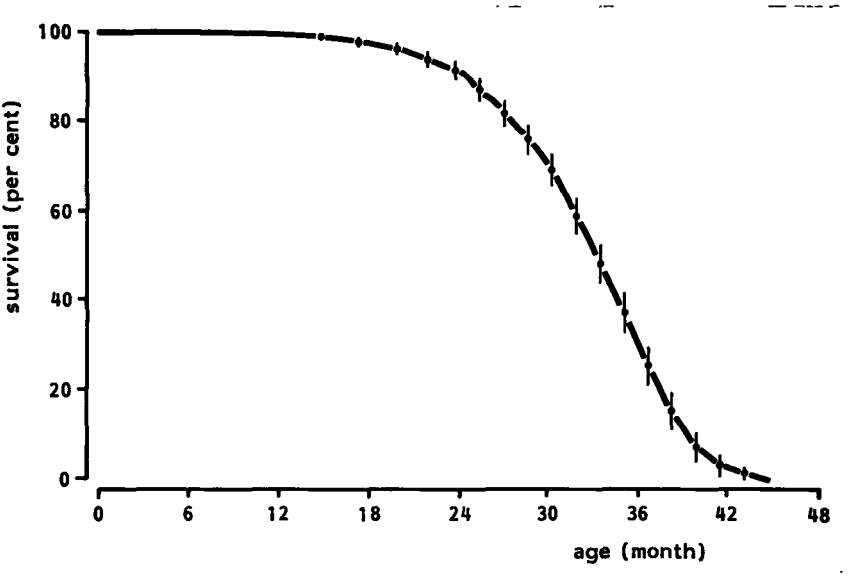


Figure 4.1: Survival characteristics of Wistar-derived WAG/Rij female rats.

### 4.2.2 Cell isolation and culture

Primary rat fibroblasts were isolated and cultured as decribed in Chapter 3. Primary cells were trypsinized and used for the determinations of unscheduled DNA synthesis. The normal human (HAN) and xeroderma pigmentosum (XP2CA) cells were early passage skin fibroblasts and kindly provided by Dr. A. Westerveld (Erasmus University, Rotterdam).

### 4.2.3 Determination of UDS

Unscheduled DNA synthesis (UDS) was determined as described in Chapter 3. In each cross-sectional study, all handlings on the different cell samples such as cell isolations, cell culture, UV-irradiation, fixation and dipping were performed together. The sequence of the individual samples undergoing each operation was random. Grain areas were determined with good agreement by two observers who were unaware of the identity of the cells.

## 4.3 Results

To investigate possible variations in DNA repair synthesis among aging inbred rats of the same strain and sex, we initiated a lifespan study of UV-induced UDS in isolated skin fibroblasts. UDS was determined autoradiographically; this assay is generally accepted as an appropriate, relatively simple and rapid technique for the detection of variations in the excision repair process (Cleaver and Thomas, 1981; Mitchell et al., 1983). However, its potential application in longitudinal studies is limited because no quantitative comparisons can be made between determinations not performed together at the same time (Cleaver and Thomas, 1981). Therefore, in our opinion, the only suitable data base for a lifespan study on UDS is a time sequential sampling plan. This involves the replication of cross-sectional studies at successive determination points and permits the segregation of interindividual differences from those attributable to the age level of the sample (see Fig 4.2 and Schaie and Parr, 1983).

To get an impression of the occurrence of age-related differences and to select the optimal parameters for a longitudinal study, a pilot experiment was performed with fibroblasts from a young adult rat (3 months of age) and from an old one (40 months of age) using different doses of UV and postirradiation times. Normal human skin fibroblasts from an adult donor were used as positive control cells (Fig.4.3C) and xeroderma pigmentosum skin fibroblasts as negative controls (Fig.4.3D). The results shown in Fig.4.3 suggest the absence of large differences in UDS values between cells of the young (Figure 4.3A) and the old (Figure 4.3B) rat.

Subsequently, experiments with groups of animals were performed in which two UDS values were studied: UDS over the first 3 and the first 24 h after UV irradiation. The first can be considered a measure for the initial rate of UDS. The latter value, in our opinion, can be considered a good measure of the total UDS performed in response to the irradiation. This can be concluded

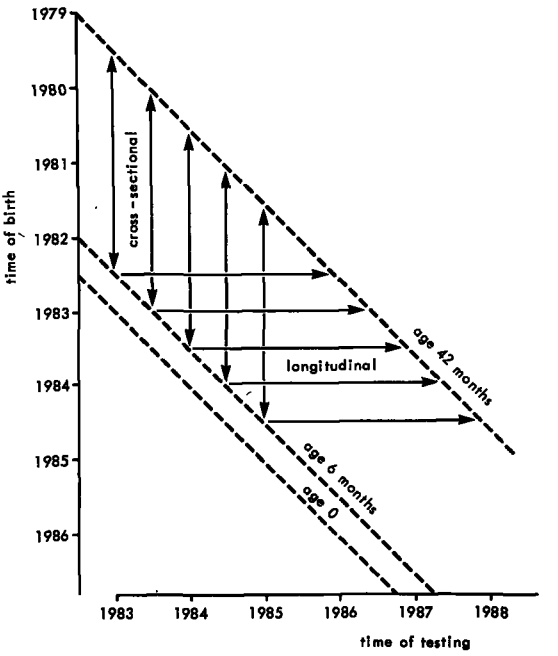


Figure 4.2: Time-sequential data base. Such data involve the replication of cross-sectional studies of the same animals at successive determination points (indicated on the abscissa). After Schaie and Parr (1983).

from the observation that at 24 h after irradiation hardly any UDS is taking place while the excision repair system is still intact at that time, that is, the cells are able to react to a new UV dose with the same kinetics as to the first one (Vijg et al., 1984; Chapter 3).

Figure 4.4 shows the dose response curves for the two selected UDS values. On the basis of these results, the dose to be used in the aging study was chosen. In our opinion, the most suitable dose for such a study should be the one that burdens the cell as much as possible without having an adverse effect on its excision repair system. In this way the capacity of an aging cell to perform UDS is fully tested, which is not the case when lower, more "physiological" doses, are used. Thus, a dose of about 10 $J/m^2$, just saturating the repair system, seemed to be optimal for this cell type. We have previously shown that such a dose leads to the induction of about 450 pyrimidine dimers per $10^9$ molecular weight of chromosomal DNA (Vijg et al., 1984; Chapter 3).

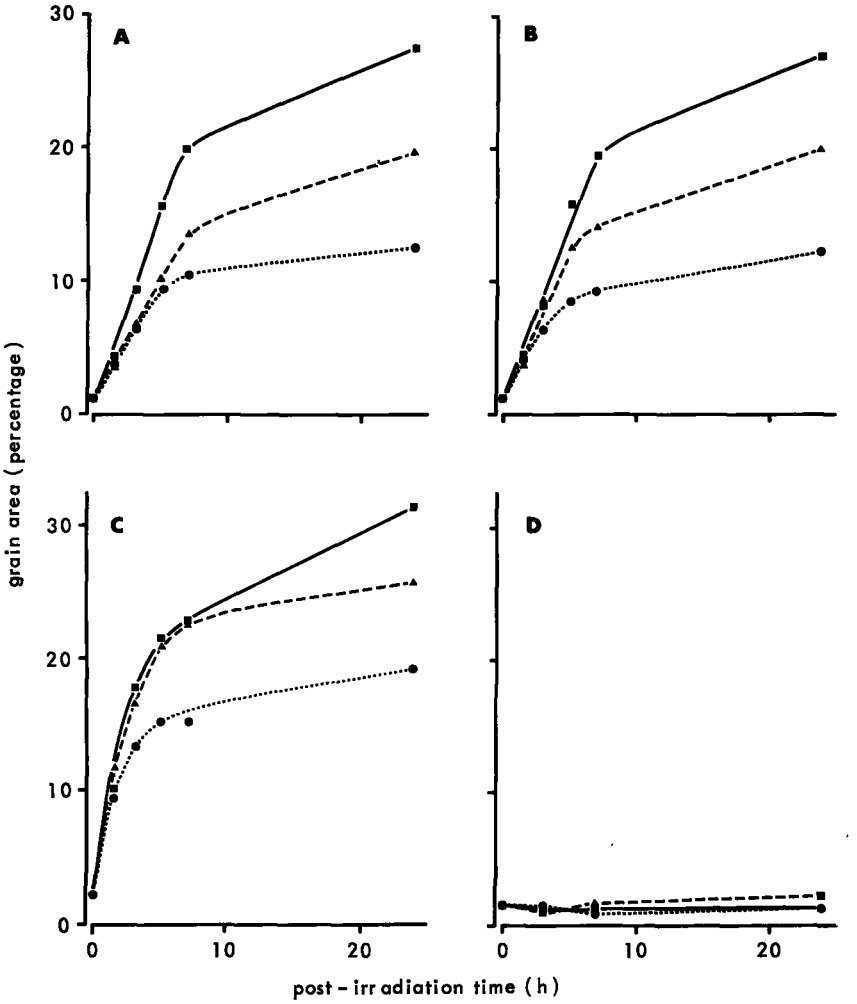


Figure 4.3: Dose response and time course study of UV-induced unscheduled DNA synthesis in normal skin fibroblasts from a 3-month- old rat (A), a 40 month-old-rat (B), a normal human (C) and a xeroderma pigmentosum patient (D). Each point is the mean of two determinations. The doses of UV were 4.6 J/m² (....), 10.3 J/m² (-----) and 22.7 J/m² (———).

In view of the above considerations, we have determined the UDS level in rat fibroblasts at 3 and 24 h after a UV dose of 10.3 J/m$^2$ in two cross-sectional studies separated by a time interval of 9 months, in which for a part the same animals participated. The results of both cross-sectional studies are

shown in Fig.4.5. Each point is the mean of 3 determinations (3 slides); as described under Materials and Methods, the cell samples involved in each study underwent each handling together in a random sequence to avoid systematic errors. No age-related changes in the cell to cell variation in UDS (reflected by the standard deviation or the width of the histograms) were observed (Table 4.1).

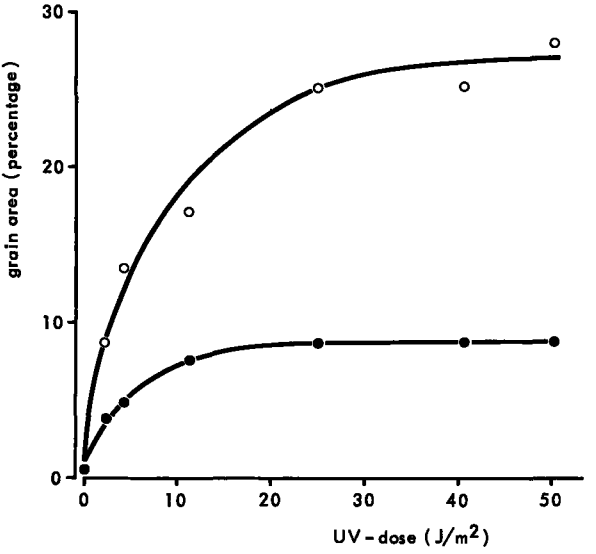


Figure 4.4: Dose response curve for UV-induced UDS in rat fibroblasts over 3h (●) and 24h (o).

Figure 4.5 and Table 4.2 show that the second cross-sectional study yielded values for the repair levels which generally were lower than those of the first study. The interindividual variation too, differed; it was greater in the second study than in the first (Table 4.2). This illustrates the fact that no direct comparisons can be made between UDS determinations performed at different times (for a discussion of the factors that may be involved in this fluctuation with time, see Cleaver and Thomas, 1981).

An indication of the intraindividual variation in this type of comparative study on UDS was obtained from determinations (in triplicate) on 7 cell samples isolated from different adjacent skin biopsies of the same animal. These determinations were performed together with the second cross-sectional study. The standard deviation in UDS among the 7 cell samples of one animal (which can be considered as the methodological error in determi-

nations on different animals) was compared with that among the cell samples of the different animals participating in the two cross-sectional studies (Table 4.2). The results suggest that the interindividual differences are relatively

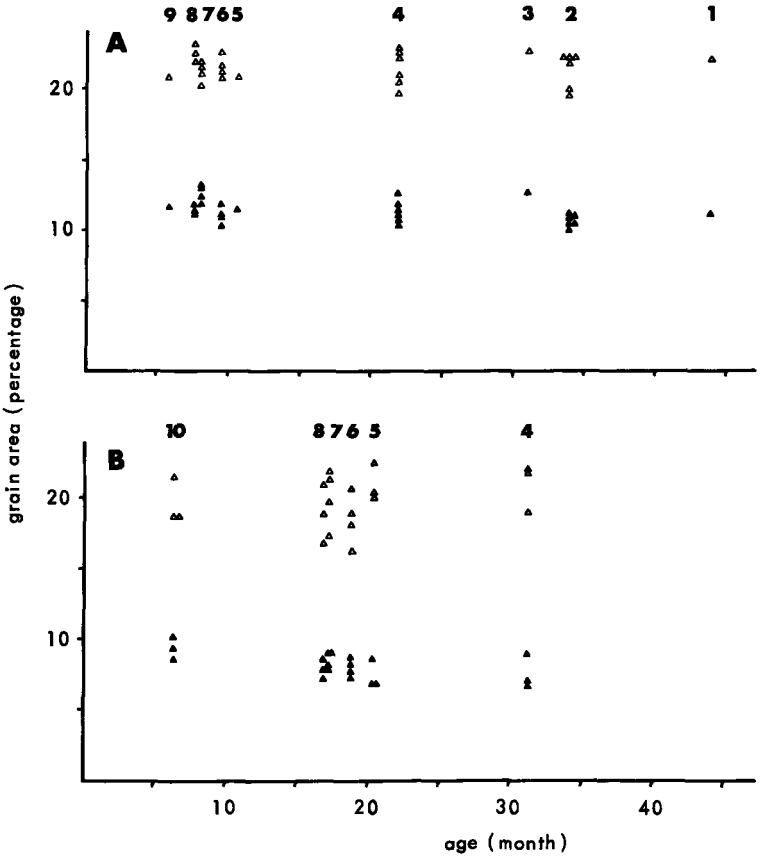


Figure 4.5: Two cross-sectional studies of UV-induced UDS in fibroblasts from rats varying in age from 6 to 44 months. The second study (B) was performed at 9 months after the first (A). Each point represents the mean value of a triplicate determination in one rat. Each group of points on a vertical line represents a different cohort, indicated by a number. A cohort is defined as the rats of a particular strain born during the same week. Animals belonging to cohorts 4 to 8 were included in both studies. Cohort 10 was newly introduced into the second study. The animals belonging to cohorts 1 to 3 died during the interval between the first and the second study. Closed symbols, UDS over the first 3 h after irradiation; open symbols, UDS over 24 h after irradiation.

TABLE 4.1 CELL-TO-CELL VARIATIONS IN UV-INDUCED UDS IN FIBROBLASTS FROM RATS OF VARIOUS AGES

| Cohort(s) | Age (mo) | Post-irradiation time (h) | Standard deviation among 30 cells (%)[a] |
|---|---|---|---|
| A First cross-sectional study | | | |
| 5-9 | 6-10 | 3 | 24 |
| | | 24 | 15 |
| 4 | 22 | 3 | 24 |
| | | 24 | 15 |
| 1-3 | 32-44 | 3 | 26 |
| | | 24 | 15 |
| B Second cross-sectional study | | | |
| 10 | 6 | 3 | 29 |
| | | 24 | 20 |
| 5-8 | 16-20 | 3 | 28 |
| | | 24 | 19 |
| 4 | 32 | 3 | 30 |
| | | 24 | 17 |

a The mean of all standard deviations in the age group.

TABLE 4.2 THE VARIATION IN UV-INDUCED UDS IN FIBROBLASTS WITHIN AND AMONG RATS

| Number of rats | Post irradiation time (h) | UV-induced UDS ± S.D.[a] |
|---|---|---|
| 27[b] | 3 | 11.4 ± 0.9 (7.9) |
| | 24 | 21.6 ± 1.0 (4.7) |
| 20[c] | 3 | 8.1 ± 0.9 (11.1) |
| | 24 | 19.8 ± 1.8 (9.1) |
| 1[d] | 3 | 6.8 ± 0.5 (7.4) |
| | 24 | 19.1 ± 1.3 (6.8) |

a The average value of the means of triplicate determinations. Numbers in parentheses indicate the SD in percentage of the mean.
b First cross-sectional study (Fig.4.5A).
c Second cross-sectional study (Fig.4.5B).
d Seven adjacent skin biopsies of one rat.

small. The impression of a somewhat larger interindividual variation in UDS at 3 h than at 24 h after irradiation can be attributed to the age effect described in the next paragraph.

When the results shown in Fig.4.5 were examined for a possible influence of aging on the level of UDS a small, though statistically significant age-related decrease was found in the 3-h values, of about 12% in the first and 20% in the second cross-sectional study. Application of the distribution-free test for ordered alternatives of Jonckheere and Terpstra (Daniel, 1978) showed that the decrease in the 3-h value is statistically significant ($p < 0.01$ in both studies), while no statistically significant change occurred in the 24-h level. Essentially the same significance was obtained after linear regression analysis. Thus, a statistically significant age-related decrease in UDS was found in both cross-sectional studies, but only in the initial rate of this process.

## 4.4 Discussion

A considerable amount of data is presently available with respect to the activity of a few DNA repair steps in some of the constituent cell types of aging animals (for a review, see Williams and Dearfield, 1981). However, many of the results obtained are contradictory and no clear picture has yet emerged. This may be explained in part by the use of different cell types. Very limited information is recorded on the occurrence of age-related changes in DNA repair steps in different cell types from one individual. To a large extent, however, contradictory results can be attributed to shortcomings and/or limitations in the design of performed studies on the relationship between aging and DNA repair pathways. For instance, in many studies with experimental animals, the so-called old animals were not truly old (for a discussion of this subject, see Knook and Hollander, 1978). A second prerequisite that has not always been fulfilled, is to define the DNA repair pathway to be analysed to such an extent that a reasonable impression is obtained with respect to the characteristics of the endpoint that is measured by the applied technique. In this respect, it should be mentioned that in most of the aging studies on DNA repair synthesis another endpoint has been measured, namely, UDS in the presence of hydroxyurea. In those studies, repair synthesis is usually determined by liquid scintillation counting, which inevitably introduces a certain amount of semiconservative DNA synthesis in the endpoint. In addition, hydroxyurea may have an influence on the DNA repair endpoint, as shown by Clarkson (1978), Francis et al. (1979) and ourselves (Chapter 2). Therefore, hydroxyurea may confuse comparative

studies of this kind and should be omitted. The argument that in autoradiographic analysis of UDS the lack of an agent to arrest cell division will result in the inclusion in UDS determinations of cells which are undergoing the initial stages of replication is not valid. Normally, S-phase cells can be easily distinguished from repairing cells and the number of those that are not recognized appears to be negligible, as can be concluded from many aspects of this and former studies such as the negative response of unirradiated control cells and xeroderma pigmentosum cells.

We feel confident to have determined in the present study the initial rate and the end level of UV-induced DNA repair synthesis in fibroblasts isolated from young, adult and aged inbred rats which were well defined in terms of survival, health condition and patterns of age-related pathology. Previously, we have studied the kinetics of this DNA repair step in a comparison of rat cells with human cells by applying both autoradiographically determined UDS and the BrdU photolysis assay and we related the results to the induction and removal of pyrimidine dimers, the main UV photolesion (Vijg et al., 1984; Chapter 3). From that study, it became clear that the autoradiographical determination of UDS is a good method for studying the DNA synthesis step in the excision repair process in rat cells. In the present study, we found the variation in both the initial rate and the end level of UDS among different rats to be small. Since individual variation in lifespan among inbred rodents of the same strain and sex is large (see Fig.4.1) and of the same relative extent as that in normal human populations (Burek, 1978), we conclude that UDS appears not to be correlated with individual lifespan. The large variation in UDS among humans may then reflect genetic differences that may predispose to certain diseases but does not tell anything about aging rate per se.

An important observation in the present study is the statistically significant age-related decrease in the initial rate of UDS. This finding is in reasonable agreement with recent results obtained by Kempf et al. (1984). These authors, studying aging mice, found an age-related decrease (between 7 and 132 weeks) of approximately 30% in UV-induced UDS determined at 5 h after irradiation (the 24-h values were not determined in that study). The fact that in our study no corresponding decrease is observed in the 24-h level is interesting, since it suggests that, although "old" cells are slower than "young" ones in repairing UV damage, they are nevertheless able to repair the same number of lesions. In this context, it is interesting to mention that the difference in UDS between rat and human cells is also much more pronounced in the initial rate than at the 24-h level (Vijg et al., 1984; Chapter 3). In keeping with this is our recent observation of a decline of about 50% in the initial rate of UDS in

rat fibroblasts "aged" in vitro, accompanied by a decline of only about 30% in the end level (Vijg et al., 1986b; Chapter 5). This strongly suggests that observed differences in DNA repair synthesis (between species, "old" and "young" cells, etc.) are not absolute, that is, differences occur only in the rate at which the repair process is being carried out.

By now it seems fairly well established that the rate of UV-induced UDS is significantly lower in cells from old animals than in those from young ones. We do not think it is justified, however, to conclude that this is indicative of a significant decrease in the capacity of fibroblasts to maintain the integrity of their DNA: firstly, because we have measured only one step in a DNA repair pathway of which the mechanism and function are not at all clear at present and, secondly, because in our opinion the decrease is too small to be of any biological significance, that is, it might be a consequence but not a cause of aging. However, the possibility cannot be ignored that other cell types may undergo a much more pronounced decrease in UDS. Indeed, decreases in UV-induced UDS of up to 80% were recently reported to occur in rat liver parenchymal cells during aging (Plesko and Richardson, 1984). However, the dosimetry in that study was obviously wrong (UV doses of up to 2100 $J/m^2$ were used). Furthermore, UDS was determined in the presence of hydroxyurea. Still, it should be taken into consideration that long-lived non-dividing cells may exhibit more pronounced symptoms of cellular senescence than actively dividing cells (Knook, 1980).

Finally, in view of the results obtained a continuation of the initiated lifespan study on this parameter, with this specific cell type, does not appear very promising. Instead, it is our intention to apply the developed (and partially applied) strategy on alternative molecular genetic parameters that may be more subjected to age-related and/or inter-individual variability.

# CHAPTER 5

# UV-INDUCED DNA EXCISION REPAIR IN RAT FIBROBLASTS DURING IMMORTALIZATION AND TERMINAL DIFFERENTIATION IN VITRO*

## 5.1 Introduction

It has been hypothesized that the loss of proliferative capacity (growth crisis) of fibroblasts in vitro, widely accepted as a model of cellular aging, is related to DNA alterations that result from a decline in DNA repair activities during culturing (Little, 1976). In human fibroblasts Hart and Setlow (1976) and Bowman et al. (1975) found a gradual decrease of UV-induced DNA repair synthesis during passaging in vitro. On the other hand, Goldstein (1971) and Painter et al. (1973) could only detect a moderate decline at very late passages.

Rodent fibroblasts also have been reported to go through a growth crisis. However, in contrast to human cells, rodent cell populations form immortal cell lines during culturing in vitro (Todaro and Green, 1963; Meek et al., 1980). Declining DNA repair activities have been suggested to be involved in this immortalization process, which is considered as a preneoplastic state (Elliott and Johnson, 1985). Unfortunately, the available data on DNA repair activities in mouse fibroblasts, most extensively studied in this respect, are conflicting. In embryonic mouse cells an almost complete loss of UV-induced DNA excision repair activity was found after 7-8 passages by Peleg et al. (1977) and by La Belle and Linn (1984), who determined dimer removal and unscheduled DNA synthesis (UDS), and by Meek et al. (1980), who only determined UDS. La Belle and Linn found some restoration in the rate of dimer removal and a complete restoration of UDS after spontaneous immortalization. The latter was observed also by Meek et al. (1980). In contrast with these results Yagi (1982) found for embryonic fibroblasts from three mouse strains an increase in UDS during the first passages followed by a decrease upon immortalization. Comparable results were obtained by Elliott and Johnson (1985) with respect to

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* Parts of this work have been published previously (Vijg et al., 1986b) or are in press (Vijg et al., 1987b).

the level of incision breaks after UV irradiation. Thus, no comprehensive picture exists of the DNA excision repair levels during passaging of mouse fibroblasts in vitro.

For rat fibroblasts, no information is presently available on UV-induced DNA excision repair during in vitro culturing. In one study with fibroblasts from newborn rats, a loss of the capacity to perform DNA repair synthesis upon treatment with 4-nitroquinoline-1-oxide was reported (Chan and Walker, 1977). Recently, we have demonstrated a slight decrease in the initial rate of UV-induced DNA repair synthesis in rat skin fibroblasts during in vivo aging (Vijg et al., 1985; Chapter 4). Therefore, we considered it of interest to monitor UV-induced DNA excision repair activities in the same cell type during passaging in vitro.

Here we report on UV-induced DNA excision repair activities in skin fibroblasts of adult rats in relation to their culture characteristics during passaging. Data are presented on (i) unscheduled DNA synthesis (UDS); (ii) the number and sizes of repair patches; and (iii) the excision of pyrimidine dimers.

## 5.2 Materials and Methods

### 5.2.1 Cell isolation and culture

Primary fibroblasts were derived from skin biopsies taken from the anterior part of the back of 6-month old inbred Wistar-derived WAG/Rij female rats (see Chapter 3). Cell cultures were initiated by transferring 1000 or 100,000 primary cells to a 25-$cm^2$ culture bottle (Greiner). Thus, the initial cell densities were 40 and 4000 per $cm^2$, respectively. Arbitrarily, cells from the first confluent 25-$cm^2$ bottle were designated as passage (p) 1 cells. Cells were transferred only when they fully had reached confluency. When observation of the cells under the microscope suggested confluency, they were supplied with fresh medium and cultured for another 48 h, during which period cell numbers still increased; thereafter no significant increases occurred.

Cells were subcultured with a split ratio of 1:2 with the exception of cells in growth crisis, which were subcultured 1:1, and some established (immortal) cell lines for which split ratios of 1:4 were found adequate. The specific procedures followed for the determinations of UDS, the number and average size of repair patches, the number of pyrimidine dimers and thymidine kinase activity have been described extensively in Chapter 3. Confluent and quiescent

(low serum arrested) cells were obtained from densely inoculated early passage fibroblasts (p5), which were seeded on cover slips as described in Chapter 3 and cultured for 10 days in normal medium (with 10% FCS) and medium containing 0.5% FCS, respectively, before UV irradiation.

### 5.2.2 Morphological analysis

For morphological examination fibroblasts, grown on culture bottles, were fixed in acetic acid/ethanol (1:3). They were stained for 5 sec in 0.05% Coumassie brilliant blue G250 (Serva) in 20% methanol, 7.5% acetic acid, rinsed with water and stained for 30 min in a 4% Giemsa solution in phosphate buffered saline (PBS).

### 5.2.3 Determination of $^3$H-thymidine labeling index

The percentage of S-phase cells, the $^3$H-thymidine labeling index, was determined by incubating cells, grown on cover slips for 24 h, with $^3$H-thymidine (0.1 µCi/ml; 25 Ci/mmole, Amersham) for 24 h (Cristofalo and Sharf, 1973). At the end of the incorporation period the cells were rinsed 3 times with medium without serum and fixed in acetic acid/ethanol (1:3). After rinsing with 70% ethanol the cover slips were processed for autoradiography as described in Chapter 3 for the determination of UDS. The percentage of labelled nuclei (containing 5 or more silver grains) was determined by scoring at least 200 cells in random fields throughout the cover slip.

## 5.3 Results

### 5.3.1 Culture characteristics

During studies on the effects of successive subculturing on rat fibroblasts, it was observed that the density at which the cells were inoculated determined whether or not a growth crisis would occur. Data are provided for 2 representative cell lines, RF61, established from an initial inoculum of about 100000 primary cells (4000/cm$^2$) and RF55, established from 1000 primary cells (40/cm$^2$). The difference in growth capability is illustrated by the percentage of cells in S-phase (Fig.5.1), which rapidly declined in cell line RF55 after initiation of the culture, whereas no significant decline was observed in RF61. In RF55 (the thinly inoculated culture) immortalization was clearly indicated by small areas of rapidly growing cells in a population of large non-dividing

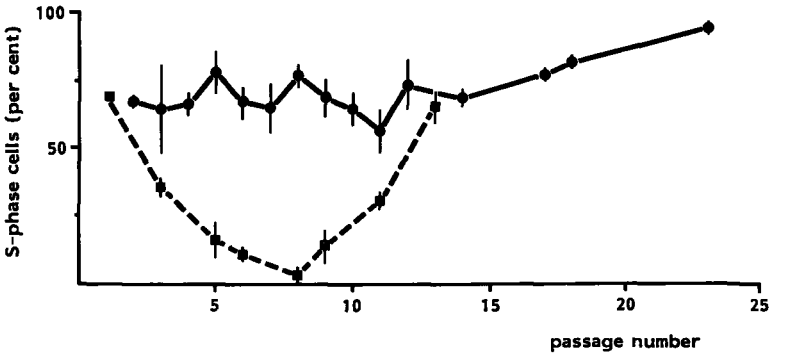


Figure 5.1: $^{3}$H-thymidine labeling index (percentage of cells in S-phase) as a function of passage number of rat fibroblast strains RF55 (-----) and RF61 (———). Each point is the mean of a triplicate determination. Bars indicate the standard deviation.

pleiomorphic cells. This resulted in a restoration of the percentage of cells in S-phase (Fig.5.1). In the densely inoculated cultures such a phenomenon did not occur and the time when immortal cells took over the population was only indicated by the morphological changes observed. Like thinly inoculated cultures also dense ones underwent a shift from small spindle shaped cells to large pleiomorphic ones (Fig.5.2). However, densely inoculated cultures kept containing a considerable number of small spindle shaped cells, while most of the large cells could still go into S-phase. It was assumed that in such cell populations immortal cells had begun to take over the population after p 12, when the average cell surface area started to decrease again, resulting in an immortal cell population with about the same morphology as primary cells (Fig.5.2).

### 5.3.2 Unscheduled DNA synthesis

UV-induced UDS was studied during the successive subculturing of rat fibroblasts inoculated at a high and at a low density. Again, data are provided for the 2 representative cell lines RF55 (thin inoculum) and RF61 (dense inoculum). Fig.5.3 shows that in both RF55 and RF61 the average amount of UDS per nucleus, measured over the first 3 h as well as over 24 h after irradiation with 10.3 J/m$^{2}$, increased about twofold over the first 5 passages. The UDS autoradiographs left no doubt about the fact that the increase in UDS is associated with the shift in the population of small spindle shaped cells to

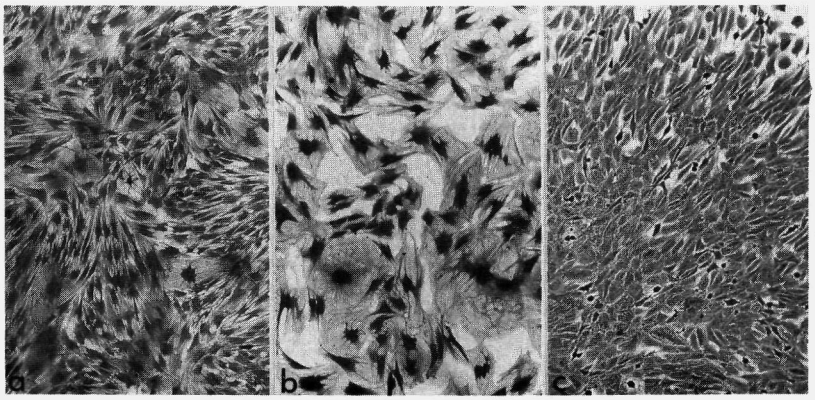


Figure 5.2: (a) Primary rat fibroblasts from a skin biopt 10 days after placing it in a petridish; (b) secondary rat fibroblasts at passage 8 (RF61); and (c) immortalized rat fibroblasts at passage 23. X43.

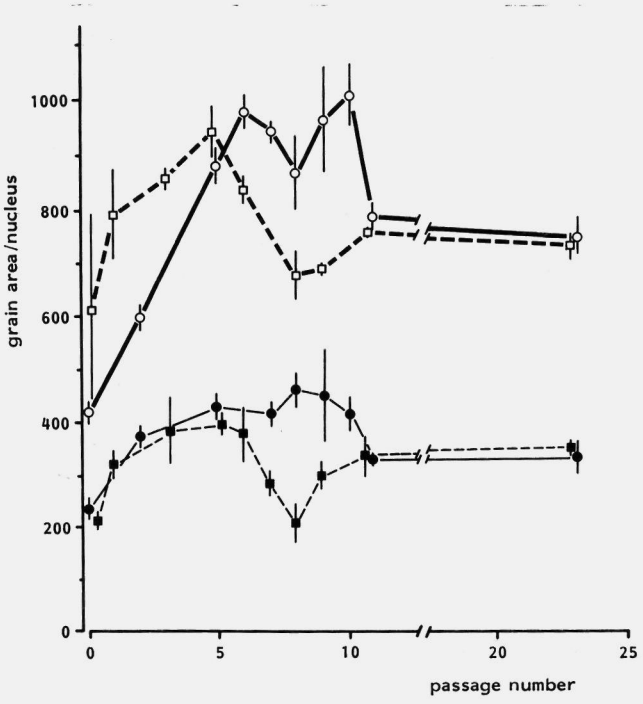


Figure 5.3: UV-induced UDS as a function of passage number in rat fibroblast strains RF55 (■ □) and RF61 (●○) after 10.3 J/m² of UV and 3-h (closed symbols) and 24-h (open symbols) of repair. Each point is the mean of at least 3 determinations. Bars indicate the estimated errors (SD).

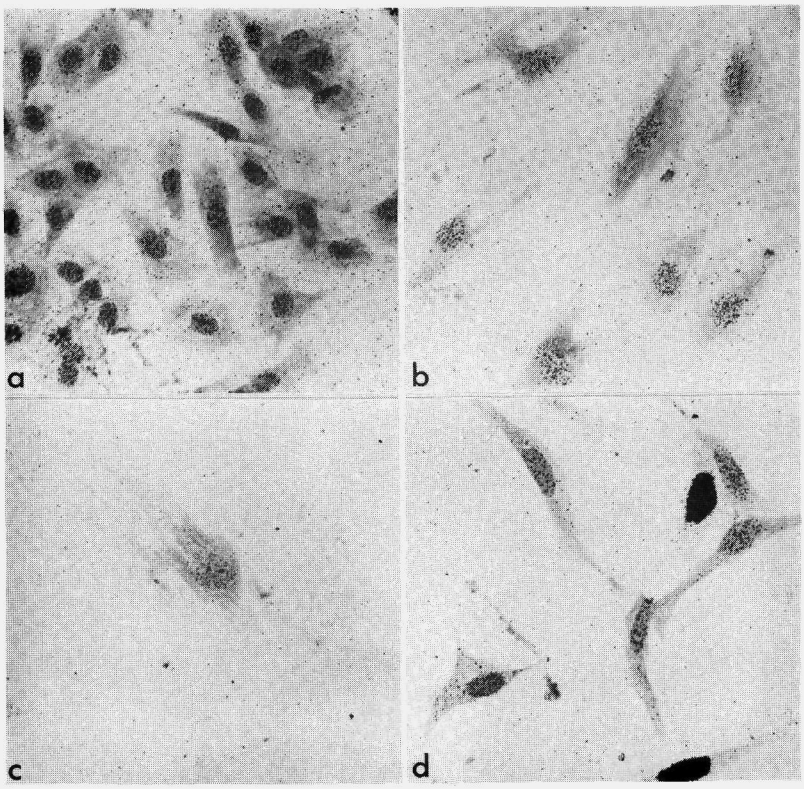


Figure 5.4: Autoradiographs of UDS over 24 h after irradiation with 10.3 $J/m^2$ of UV in primary (a), secondary (b), terminally differentiated (c) and immortalized (d) rat fibroblasts (RF55). In d, 2 cells in S-phase are present. X270.

large pleiomorphic ones (Fig.5.4a,b). When these 2 cell types were discriminated within one slide, UDS was about twice as high in the latter cell type (results not shown).

The morphological change especially relevant to autoradiographical UDS determinations is the increase in nuclear size (Fig.5.5). This increase corresponds with the increase in UDS (Fig.5.3). Comparison of the UDS autoradiographs of primary cells (Fig.5.4a) with those of secondary cells (Fig.5.4b) shows that, although in the latter the total area occupied by grains is much larger than in the former, the grain densities are about the same. UDS, expressed as the average grain area per nuclear area (grain density), is constant over this culture period (results not shown). This raised the question whether the UDS levels were genuinely higher in secondary cells as compared

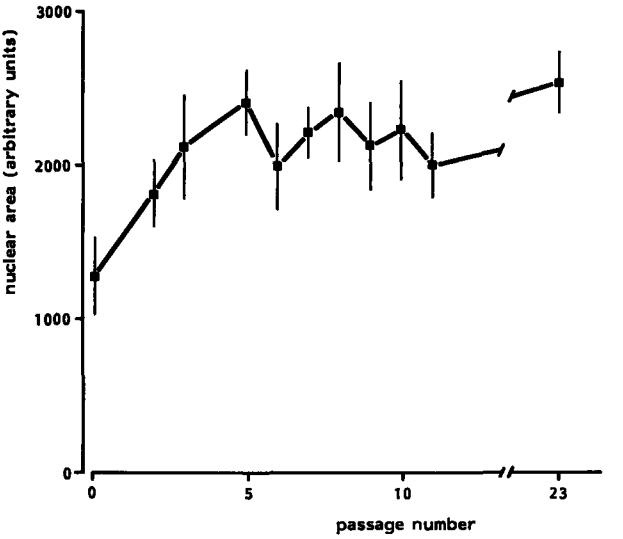


Figure 5.5: Nuclear area as a function of passage number in rat fibroblast strain RF61. Each point is the mean of at least 3 determinations. Bars indicate the estimated errors (SD).

with primary cells or that we were dealing with decreased self-absorption of $^{3}H$ β particles, due to a decrease in nuclear thickness. Electronmicroscopical determination of nuclear thickness (after sectioning at right angles) revealed that nuclei from primary cells were not significantly thicker than those of secondary cells (results not shown). In both cell populations nuclear thickness was about 1 μm. Therefore we conclude that differences in self-absorption, the most important single factor in considering autoradiographical efficiency (Rogers, 1969), are not likely to be involved in the observed increase in UDS over the first 5 passages.

A drastic decline in both the 3-h and the 24-h level of UDS was observed in strain RF55 upon entering the growth crisis period. At p8 the initial rate (3 h) and the end level (24 h) of UDS was only about 50% and 70%, respectively, of the average level at p 5 (Fig.5.3). Cell populations in growth crisis consist predominantly of terminally differentiated cells. In these populations both total and relative grain areas were lower as compared to actively dividing secondary populations (compare Fig.5.4b with 5.4c). After spontaneous immortalization of strain RF55, a restoration of the UDS levels was observed of up to 80% of the level at p 5 (Fig.5.3). In strain RF61 no growth crisis occurred and changes in the UDS level were only observed upon immortalization. The immortal esta-

blished cell line (p 23) appeared to have undergone a slight (20-30%) reduction in both the initial rate and the end level of UDS as compared to passages 5-8 (Fig.5.3). This reduction is not accompanied by a decrease in nuclear area, probably because of the larger DNA content of the immortal cells (see next section). Over the whole culture period, also during growth crisis, all cells not engaged in normal DNA replication were able to perform UDS.

### 5.3.3 The number and average size of repair patches

The BrdU photolysis assay offers the possibility to determine both the number and the average size of the repair patches per amount of isolated DNA. This method is therefore not subjected to artefacts due to differences in morphology or average repair patch size. Fig.5.6 shows the photolysis curves of primary (p 0), secondary (p 5-8) and immortalized (p 30) RF61-fibroblasts after 3 h of repair. From these curves, and the corresponding curves for 24 h of repair, the number of patches and their average size were obtained. Table 5.1 shows that the mean repair patch size is not significantly different for the three cell populations or for the two repair periods studied. The number of repair patches, however, is substantially higher in the secondary cells (Table 5.1). The differences in the number of repair patches suggest that secondary cells have an initial rate of DNA repair synthesis that is about twice as high as that of primary or immortalized cells (Table 5.1). For immortalized cells no accurate BrdU photolysis data on the 24-h level could be obtained. This was due to the relatively high proliferative activities of such cells, which resulted in increased semi-conservative incorporation of BrdU. Apparently, this made the opposite DNA strand extra sensitive to background breaks which made it very difficult to accurately determine the plateau level of the BrdU photolysis curve.

Thus, the BrdU data showed the same trend as the results obtained with UDS (Fig.5.3): an increase in DNA repair synthesis from primary to secondary cells, followed by a decrease upon development into an immortal cell line. The latter is more clearly indicated by the BrdU data than by the pattern of UDS. This discrepancy may be explained by the generally larger (and varying) DNA content of the immortal cell lines. Tetraploid and hypertetraploid cells were observed as early as p 26 (results not shown). In these cells, therefore, determinations of UDS per nucleus can be expected to indicate relatively high levels of DNA repair synthesis when compared to the BrdU photolysis method, which provides data per amount of DNA.

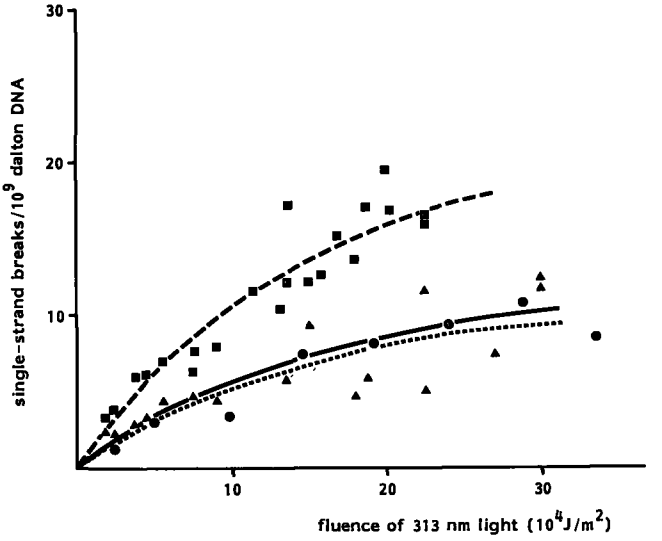


Figure 5.6: BrdU photolysis curves of primary (▲), secondary (■) and immortalized (●) rat fibroblasts (RF61) after 4.6 J/m² of UV and 3 h of repair. Each point indicates the number of single-strand breaks above the number of background breaks (breaks in the non-BrdU containing control cells). Each curve represents the results of 2 independent experiments; the lines were drawn according to the equation describing photolysis kinetics, which had been fitted to the experimental points.

### 5.3.4 Effect of thymidine pool size and thymidine kinase activity

A common disadvantage of DNA repair synthesis assays based on the incorporation of nucleotide analogues, is their dependence on factors such as thymidine pool size and thymidine kinase (TK) activity. It has been shown that in exponentially dividing cell populations both TK activity and thymidine pool sizes are much higher than in confluent populations (Olashaw et al., 1983). In order to asses if such variations in pool size and TK activity would influence the DNA repair synthesis endpoints used in this study, we determined UDS in exponentially dividing cells and in cells that were allowed to grow confluent or rendered quiescent by means of serum starvation. Table 5.2 shows that the UDS levels are about the same in these three secondary cell populations, although the mitotic index varies from the regular 70 % in exponentially dividing cells to less than 10 % in confluent cells. With respect to TK activity our data

TABLE 5.1 NUMBER AND SIZE OF REPAIR PATCHES IN PRIMARY, SECONDARY AND IMMORTALIZED RAT FIBROBLAST POPULATIONS AFTER 4.6 J/m² OF UV

| Cell population | postirradiation time | number of patches per $10^9$ mw DNA | number of nucleotides per repair region |
|---|---|---|---|
| Primary (p0) | 3 h | 12 ± 2 | 69 ± 15 |
| | 24 h | 36 ± 4 | 46 ± 5 |
| Secondary (p5-p8) | 3 h | 25 ± 3 | 56 ± 5 |
| | 24 h | 51 ± 5 | 48 ± 5 |
| Immortal (p30) | 3 h | 11 ± 2 | 73 ± 15 |

All data are derived from photolysis curves as shown in Fig. 5.6.
The errors (SD) were estimated from the photolysis curves of at least 2 experiments.
For immortalized cells, no 24-h data were available (see text).

TABLE 5.2 UDS AND THYMIDINE KINASE ACTIVITY IN RAT FIBROBLASTS

| Cell population | UDS[a] (grain area/nucleus) | S-phase cells[b] (%) | TK activity[c] (cpm/$10^5$cells) |
|---|---|---|---|
| Primary (p0) | 414 ± 21 | 90 ± 5 | 260 ± 100 |
| Secondary (p5-p8) | | | |
| proliferating | 859 ± 40 | 79 ± 8 | 370 ± 100 |
| confluent | 919 ± 28 | 9 ± 3 | -10 ± 100 |
| quiescent | 898 ± 100 | 18 ± 5 | ND |
| Immortal (p30) | 752 ± 35 | 97 ± 2 | 1870 ± 100 |

a UDS was determined over 24 h after a UV dose of 10.3 J/m².
b The percentage of S-phase cells was determined over 24 h.
c TK activities were determined from linear incorporation curves constructed from 3 time points, 0, 10 and 20 min.

All data are derived from at least two experiments.
The experimental errors (SD) were estimated. ND = not determined.

are essentially the same as those of Olashaw et al. (1983); no TK activity was detected in confluent cells, whereas this activity is clearly present in exponentially dividing cells, both secondary and primary (Table 5.2). In the immortal cell lines TK activity appeared to be much higher than in secondary cells whereas DNA repair synthesis in these cells is lower. We conclude from the data in Table 5.2 that in rat fibroblasts neither variations in thymidine kinase activity nor pool size differences have any significant influence on UDS. This may be due to the high, probably saturating, concentrations of radioactive thymidine used in this assay.

### 5.3.5 Removal of pyrimidine dimers

Recently we have shown that, although rat fibroblasts are quite capable of performing DNA repair synthesis, they almost lack the capacity to remove pyrimidine dimers (Vijg et al., 1984; Chapter 3). An extension of these data is provided in Table 5.3, which shows that, after a dose of 4.6 J/m$^2$ and over a repair period of 24 h, neither primary (p 0), secondary (p 5) nor immortal (p 31) rat skin fibroblasts remove more than about 20% of the dimers originally induced. Since Peleg et al. (1977) and La Belle and Linn (1984) demonstrated that embryonic mouse cells at the beginning of their in vitro lifespan were able to remove dimers, we considered it of interest to investigate whether primary

TABLE 5.3 PERSISTENCE OF ESS IN VARIOUS RAT FIBROBLAST POPULATIONS AFTER 4.6 J/m$^2$ OF UV

| Cell population | postirradiation time | number of ESS per $10^9$ mw DNA | percentage of removal |
|---|---|---|---|
| (A) Embryonic primary | 0 h | 207 ± 6 | |
| | 24 h | 183 ± 5 | 12 ± 4 |
| (B) Adult primary | 0 h | 240 ± 8 | |
| | 24 h | 198 ± 12 | 18 ± 6 |
| secondary | 0 h | 253 ± 12 | |
| | 24 h | 198 ± 7 | 22 ± 6 |
| immortalized | 0 h | 227 ± 14 | |
| | 24 h | 195 ± 2 | 14 ± 6 |

The errors (SD) were estimated from at least 2 experiments.

embryonic rat fibroblasts possessed the same capacity. However, as shown in Table 5.3 also fibroblasts from rat embryos (15 days gestation) were unable to remove a substantial number of pyrimidine dimers.

## 5.4 Discussion

The results obtained in the present study allow some conclusions with respect to the activity of two steps in the DNA excision repair pathway, viz., pyrimidine dimer removal and repair synthesis, during passaging of rat skin fibroblasts in vitro. The persistence of ESS in primary (adult and embryonic), secondary as well as immortalized cells suggests that in all of these cell populations dimer removal occurs very slowly. This finding is not in agreement with the data of Peleg et al. (1977) and La Belle and Linn (1984), who showed that at least early passage mouse embryo cells do remove dimers. This discrepancy is not likely to be due to methodological differences (both Peleg et al. and La Belle and Linn used a chromatographical method for the determination of pyrimidine dimers, whereas we applied the UV-endonuclease enzymatic method), since we have checked our UV-endo data by HPLC analysis and still found dimers to be persistent in primary embryonic fibroblasts (results not shown).

In contrast to their low capacity to remove dimers, rat fibroblasts are well able to perform DNA repair synthesis at any stage of their in vitro lifespan, as was indicated both by UDS and by the appearance of BrdU containing repair patches. The observed increase in both the initial rate and the end level of DNA repair synthesis in rat fibroblasts over the first 5-8 passages was found to be associated with the differentiation of primary, spindle shaped cells into large pleiomorphic ones. In this context it is of interest that in some cases alterations in DNA repair activities were found to be associated with differentiation. For instance, human peripheral blood lymphocytes have been shown to undergo a marked increase in their capacity to perform DNA repair synthesis upon phytohemagglutinin (PHA) stimulation (Lavin and Kidson, 1977). This has been interpreted in terms of a possible role of DNA repair systems in the differentiation of the numerous immune system functions (Kidson, 1978).

For rat fibroblasts a detailed morphological study made it likely that in vitro these cells differentiate via one or more mitotically active stages into non-dividing cells (Kontermann and Bayreuther, 1979). Our data are in agreement with such a sequence of events. In addition, they indicate that cell density is an important factor in determining how rapid, in terms of the number of generations, terminal differentiation occurs. This can be deduced

from Fig.5.1, showing that only thinly inoculated cultures undergo a growth crisis, whereas densely inoculated cultures do not show a significant decrease in the percentage of cells in S-phase. The morphological data clearly show that, like in thinly inoculated cultures, also in the densely inoculated ones differentiation occurs (Fig.5.2). However, in the latter case this process does not lead to a growth crisis. We conclude that in densely inoculated populations terminal differentiation requires more generations, allowing immortal cells that have arosen to take over the population gradually, without any significant growth inhibition, until a permanent cell line results.

Considering our results on DNA excision repair in the rat fibroblast cell system, it can be concluded that the large mitotically active cells have significantly higher levels of DNA repair synthesis than primary, terminally differentiated or immortalized cells. Analogous to the situation in PHA-stimulated lymphocytes, DNA excision repair levels may be higher in differentiating fibroblasts (secondary cells) than in cells just removed from their "resting" state (primary cells).

With respect to terminally differentiated cells our data indicate that the decrease in UDS over 3 h (initial rate) is much more pronounced than that over 24 h (end level). A comparable difference in kinetics was found in a study on UDS in fibroblasts taken from old and young rats, that is, a small age-related decrease in UDS over the first 3 h and no change in the 24-h levels (Vijg et al., 1985; Chapter 4). In view of the present results the possibility must be taken into account that the age-related decrease in the initial rate of UDS observed in that study simply reflects a slight increase in the rate at which cells from old animals terminally differentiate. Although in the present study this was not systematically investigated, others have shown that for human fibroblasts the in vitro lifespan of cells taken from old individuals is slightly but significantly shorter than that of cells from young individuals (Martin et al., 1970).

The relatively low rate of UDS in the large terminally differentiated cells as compared to that in its mitotically active counterparts of about the same morphology may also reflect a more general phenomenon. It was demonstrated that terminally differentiated cells are generally characterized by low DNA repair activities (Hahn et al., 1971; Wheeler and Wierowski, 1983). The possibility that the low rate of UDS in terminally differentiated fibroblast populations reflects a general deterioration of cellular functions with a stochastic character instead of being a programmed event is unlikely in view of the absence of cells that were unable to perform UDS. Moreover, the variation in UDS among terminally differentiated cells was not found to be significantly

larger than that in most primary or immortalized cell populations (results not shown).

The observation of a relatively low excision repair activity in immortalized cells is in agreement with findings of others (Elliott and Johnson, 1985). With regard to the initial rate of DNA repair synthesis these cells are very similar to the primary populations, which also predominantly consist of small spindle shaped cells. In this respect it is important to realize that immortalization of rat fibroblasts in vitro appears to occur before or during the early stages of terminal differentiation. This was demonstrated in experiments in which immortal cell lines were obtained from clones of small spindle shaped cells without having gone through the changes in cell morphology that are so characteristic for rat fibroblast cell cultures during passaging in vitro (unpublished observations). The fact that most of these clones had a spindle shaped morphology strongly suggests that immortalization occurs in vitro with a frequency that is highest in the small spindle shaped cells and becomes smaller at every step in the pathway towards terminal differentiation. This is in agreement with recent data on immortalization of Chinese hamster cells (Kraemer et al., 1986). Thus, our data indicate that cells that escape terminal differentiation by entering the process of immortalization maintain both their morphology and DNA repair characteristics, in contrast to cells that do undergo terminal differentiation.

In conclusion, the variations in DNA repair synthesis observed in fibroblasts during passaging in vitro are in our opinion best explained in association with a differentiation process instead of being a reflection of cellular aging. Therefore, fibroblasts of various passages are not a good model system for studying the possible relationship between DNA repair and the aging process. Moreover, in view of the fact that DNA repair in somatic cells can undergo such rapid changes upon establishment in culture, it is advisable to direct aging studies in this field towards the analysis of repair processes in vivo (Vijg et al., 1986a).

# CHAPTER 6

## DIFFERENCES IN PYRIMIDINE DIMER REMOVAL BETWEEN RAT SKIN CELLS IN VITRO AND IN VIVO*

### 6.1 Introduction

The persistency of UV-induced pyrimidine dimers in cultured rodent cells is well documented (Lohman et al., 1976; Ganesan et al., 1983; Takebe et al., 1983; Yagi et al., 1984; Vijg et al., 1984). This persistency is not likely to be due to an inherent DNA repair defect of rodent cells, as can be deduced from data on the survival of such UV-irradiated cells, which is not lower than that of human cells under comparable conditions (Ganesan et al., 1983; Yagi et al., 1984; Chapter 3). In addition, the capacity of rodent cells to perform unscheduled DNA synthesis over a given time period is not dramatically lower than that of human cells; consequently, it appears too high to match the low number of dimers removed (Lohman et al., 1976; Yagi et al., 1984; Vijg et al., 1984). Interestingly, it has been shown by Peleg et al. (1977), and more recently by La Belle and Linn (1984), that early passage embryonic mouse cells remove pyrimidine dimers well. These findings suggest that in rodents the excision repair pathway(s) similar to those via which UV damage is repaired in human cells, can be activated but are not always fully utilized, possibly due to changes in the expression of specific DNA repair genes during embryonic development (Peleg et al., 1977).

Recently we showed that UV-induced pyrimidine dimers in early passage fibroblasts from rat embryos were as persistent as those in cells from adult rats (Vijg et al., 1986b). In this regard it is conceivable that rat fibroblasts lose their capacity to remove dimers upon establishment into culture. In order to test this possibility it is necessary to measure the induction and removal of pyrimidine dimers in cells in vivo, in comparison to the same cell type cultured in vitro. In principle, such a comparative study can be performed by using the UV-endonuclease enzymatic method (Paterson et al., 1973; Chapter 2). However, a serious disadvantage of this method is its dependence on radio-

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* Parts of this work have been submitted for publication by E. Mullaart, P.H.M. Lohman and J. Vijg.

actively labelled DNA for the analysis of the breaks induced by the enzyme, which involves centrifugation through alkaline sucrose gradients. This limits its application to growing cells cultured in vitro, the DNA of which can be labelled during S phase with radioactive precursors. Modifications that circumvent the need of detection by radioactivity and therefore allow application of the alkaline sucrose gradient centrifugation technique to non-dividing cells or freshly isolated tissues, are based on the detection of DNA in the gradient fractions by fluorescent staining (Ono and Okada, 1973; Brash and Hart, 1983). Unfortunately, these modifications made the method rather time-consuming, because the DNA in each gradient fraction has to be precipitated, stained and spectrophotometrically quantified. Furthermore, the sensitivity of these non-radioactive detection methods appears to be low.

Recently, a rapid and sensitive method for the determination of non-radioactivity labelled alkaline sucrose gradient DNA profiles was developed (Vijg et al., 1986c). It is based on the fractionation of gradients in the wells of plastic microtiter plates and the subsequent covalent labelling of DNA adsorbed to the walls by reaction of the guanines with N-acetoxy-2-acetylaminofluorene (N-AcO-AAF). Then the treated DNA is quantified by means of an enzyme-linked immunosorbent assay (ELISA), with specific antibodies of high affinity towards dG-AAF. The method was found suitable for the accurate and sensitive detection of pyrimidine dimers induced in vitro as well as in vivo in rat skin cells by irradiation with UV. Further improvement was obtained when antibodies with a high affinity for DNA became available, which made the direct immunochemical detection of DNA possible without the necessity of prelabelling.

The availability of the above described method allows an unbiased comparison of the repair of pyrimidine dimers in skin cells in vitro with that in vivo. Here we show that epidermal cells in vivo repair pyrimidine dimers rapidly, whereas in the same cell type in vitro these UV-induced lesions are as persistent as in cultured fibroblasts.

## 6.2 Materials and Methods

### 6.2.1 Animals

The rats used in this study were 6 and 36-month old female Wistar-derived WAG/Rij rats.

### 6.2.2 Cell isolation and culture

Rat epidermal keratinocytes were isolated and cultured according to Rheinwald and Green (1975) with some modifications. A rat was narcotized with ether and after shaving a part of its back with a razor blade in 70% alcohol, 10-mm diameter skin biopsies were taken, cut into small pieces and incubated overnight at 4°C, floating (with the horny layer up) on the surface of 0.25% trypsin, 5 mM EDTA in PBS. After this period the epidermis was removed. A cell suspension was obtained by thorough disaggregation by pipetting. About 250000 cells were seeded in a 6-cm tissue culture dish (Greiner) on top of a feeder layer of about 750000 rat fibroblasts, plated 1 day in advance after having received a lethal dose of 3 krad of gamma radiation. The culture medium used was the complete DMEM described in Chapter 3, containing 0.1 nM cholera toxin, 0.4 ng/ml hydrocortisone and 10 ng/ml epidermal growth factor (added to the cultures after 2 days). The medium was changed 3 times a week. After 5-7 days the cells were used for the experiments.

Rat fibroblasts, obtained from skin biopsies (Vijg et al., 1984; Chapter 3), were used between passage 5 and 10. They were cultured as described in Chapter 3 and used for the experiments as an exponentially dividing population in 6-cm petri dishes.

### 6.2.3 Determination of pyrimidine dimers after UV-irradiation in vitro

Irradiation of fibroblasts with UV-C was as described in Chapter 3. After irradiation, the cells were incubated with fresh medium for periods from 0 to 24 h. Then, the fibroblasts were rinsed with PBS, drained, immediately frozen on dry ice and stored at -70°C for not more than 3 days. Cultured keratinocytes were rinsed with PBS and irradiated (on ice) with near UV (as described in the next paragraph) through a Schott WG 305 filter (mimicking the horny layer of the skin) for 6 min. During irradiation the cells were covered by a thin layer of PBS containing 1 mg/ml glucose. To remove the fibroblast feeder layer from the epidermal keratinocytes, these cells were rinsed once with PBS. Subsequently, PBS containing 0.02 % EDTA and 0.1 % glucose was added. The feeder layer was removed by pipetting (complete removal was evident from microscopic observation) and the cells were frozen on dry ice like the fibroblasts. Double labelling experiments on fibroblasts and epidermal keratinocytes were performed essentially as described in Chapter 3 for the experiments on BrdU photolysis. The keratinocytes were labelled with $^{14}C$ and

the fibroblasts with $^{3}H$. After 3 days of labelling and one day of incubation in non-radioactive medium, the medium was removed and the cells were washed with ice cold PBS. Subsequently the cells were irradiated with UV-B and allowed to repair as described above for keratinocytes. The procedures for lysis of the cells, DNA isolation and determination of ESS were essentially as described earlier (Paterson et al., 1973; Wade and Lohman, 1980) with one modification. This involved the lysis of the keratinocytes, which was accomplished by incubation of the cells, still attached to the petri dish, in the SDS lysis buffer described in the next paragraph for 1 h at 37°C in the presence of 0.5 mg/ml proteinase K under continuous rocking.

### 6.2.4 Determination of pyrimidine dimers after UV-irradiation in vivo

For UV irradiation in vivo, a rat was narcotized with ether, shaved and irradiated with UV-B (light of the wavelength range 290-320 nm) for various periods of time, with a Philips TL-20/12 fluorescent sunlamp at a fluence rate of 11 $W.m^{-2}$. After each time interval a 10-mm diameter skin biopsy was taken and kept in ice-cold PBS for no longer than 3 h. For measuring the rate of DNA repair, a second and a third biopsy were taken at 3 and 24 h after irradiation. During the repair periods, the rats were kept in the dark to exclude the possibility of photoreactivation repair. Epidermal keratinocytes were isolated as described in Section 6.2.2, by overnight floating on trypsin at 4°C. The cell suspensions obtained were lysed in 0.5% (w/v) SDS, 150 mM NaCl, 10 mM EDTA and 20 mM Tris (pH 7.6), followed by a 3-h incubation period at 55°C in the presence of 0.5 mg/ml proteinase K under continuous rocking. DNA isolation was as described earlier (Paterson et al., 1973; Wade and Lohman, 1980). For the determination of pyrimidine dimers, these were first converted into single-strand DNA breaks by incubating 50-100 ng of DNA in 80 µl with 10 µl of crude M.luteus extract for 30 min at 37°C, in the presence of 10 µl of tRNA (1 mg/ml) to suppress aspecific nuclease activity. After the incubation period, the sample was treated with proteinase K (1 mg/ml) for 1 h at 37°C and subsequently extracted once with 2 volumes of phenol saturated with 0.2 M Tris-HCl, pH 7.6, followed by overnight dialysis against UV-endo buffer (Wade and Lohman, 1980) to remove the phenol. The DNA was then denatured by addition of 100 µl of 1 M NaOH, 0.01 M EDTA. Subsequently, the mixture was carefully layered onto a low-salt alkaline sucrose gradient (5-20% w/v, 0.5 M NaCl, 0.2 M NaOH, 0.01 M EDTA) and centrifuged at 40000 rpm in a Beckman SW60Ti rotor for 80-170 min (depending on the number of breaks expected) at 20°C.

### 6.2.5 Gradient fractionation and detection of DNA

The procedures for fractionation, liquid-scintillation counting and computer analysis of the DNA distributions obtained with radioactively labelled cells have been described (Wade and Lohman, 1980). Non-radioactive detection of DNA profiles was essentially as described earlier (Vijg et al., 1986c), but with some modifications. Alkaline sucrose gradients containing DNA were centrifuged as described above, fractionated into 96-well polystyrene microtiter plates (Greiner) at 8 drops (about 200 µl) per well, which were neutralized by adding 0.25 volume (60 µl) of neutralization buffer (1.3 M $KH_2PO_4$, 1.7 M $Na_2HPO_4$, pH 6.9). Directly thereafter 100 µl was taken out of each well and equally divided over the 2 corresponding wells of 2 new microtiter plates, which had previously been coated with poly-L-lysine (by overnight incubation at 4°C with 1 µg/ml poly-L-lysine in PBS). Subsequently, the DNA was allowed to adsorb to the coated surface of the wells, overnight at room temperature. The amount of DNA in each well was quantified by means of a direct ELISA (Engvall, 1980) with a mouse monoclonal antibody with a high affinity towards DNA. The absorbance data were obtained with a multichannel photometer (Titertek Multiskan, Flow) and analysed with respect to the DNA distribution in the original gradient by using a modification of our computer program for the analysis of radioactivity distributions in sucrose gradients (Wade and Lohman, 1980).

## 6.3 Results

When skin is irradiated with ultraviolet light (UV), the radiation will not penetrate deeply. The local structure in different skin regions determines to a large extent which cell types are being damaged. The thin upper layer of epidermal keratinocytes is most susceptible to the genotoxic effects of UV, whereas the DNA of fibroblasts - located much deeper in the skin - is not likely to be severely damaged. Thus, epidermal keratinocytes are the relevant cells in the skin for studies with respect to the induction and removal of UV-induced pyrimidine dimers in vivo.

Unfortunately, most studies on dimer removal in mammalian cells, including our own (Vijg et al., 1984), have been performed with cultured fibroblasts. It was unknown whether the well documented persistency of UV-induced pyrimidine dimers in this cell type would also be a characteristic of cultured keratinocytes. In view of the intended comparison between the in vivo and the in vitro situation we were obliged to investigate this question. For an optimal

comparison of dimer removal in fibroblasts with that in keratinocytes, these cells were separately isolated from a rat skin biopsy and subsequently metabolically labelled with $^{3}H$-thymidine and $^{14}C$-thymidine, respectively. The cells were UV-irradiated and DNA was isolated, immediately thereafter or after a certain repair period. During this period the cells were kept in the dark to exclude the possibility of photoreactivation repair. DNAs, isolated from identically treated keratinocyte and fibroblast samples, were mixed and the pyrimidine dimer contents were determined as described in Materials and Methods. In this way the number of dimers could be compared on the basis of the two distinguishable sedimentation profiles in the same gradient.

Figure 6.1 clearly shows that there is no difference in dimer removal between fibroblasts and keratinocytes after irradiation with UV-B. In neither of the two cell types was there any significant repair over the first 3 h, while after 24 h only 20% of the dimers was removed. These DNA repair time courses were completely identical to that found for rat fibroblasts after irradiation with UV-C (Fig.6.1).

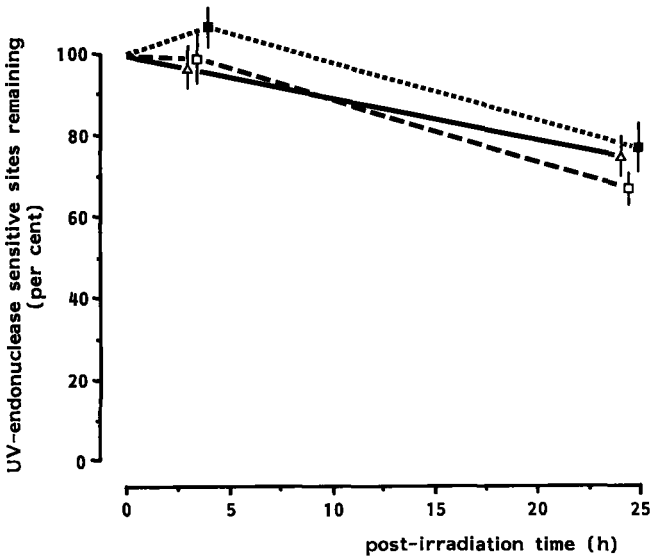



Figure 6.1: Time course of in vitro repair of ESS in rat epidermal keratinocytes (□) in comparison with that in rat fibroblasts (■). The fractional disappearance of ESS from DNA after exposure of the cells to 4000 J/m² of UV-B has been plotted. Under the experimental circumstances such a dose induces about 180 ESS. A control experiment with rat fibroblasts irradiated with 4.6 J/m² of 254-nm UV has been included (Δ).

In order to accurately quantify the number of pyrimidine dimers present in the DNA of epidermal cells at various time points after irradiation of rat skin in vivo, we also wished to apply the UV-endonuclease method. However, an impediment for the application of this method in in vivo studies was its dependency on radioactively labelled DNA for the analysis of sedimentation profiles in sucrose gradients. Recently, this problem was solved by the development of a rapid and sensitive immunochemical procedure for the quantitation of DNA in such gradients, that can be applied on profiles of non-radioactive DNA (Vijg et al., 1986c). With this technique, about 1-2 ng of DNA can still be detected. Originally, DNA was detected via an enzyme-linked immunosorbent assay of AAF, covalently bound to guanine residues. When during the course of this study antibodies became available that allowed the direct detection of DNA, the prelabelling with AAF could be omitted. The results obtained when these antibodies were used for detecting DNA in alkaline sucrose gradients, without previous treatment with AAF, were not significantly different from those described for the AAF system (unpublished).

Using this modified UV-endonuclease assay, we determined the induction of pyrimidine dimers in the DNA of epidermal keratinocytes irradiated in vivo with UV-B. As shown in Fig. 6.2, a linear dose dependency was observed. The

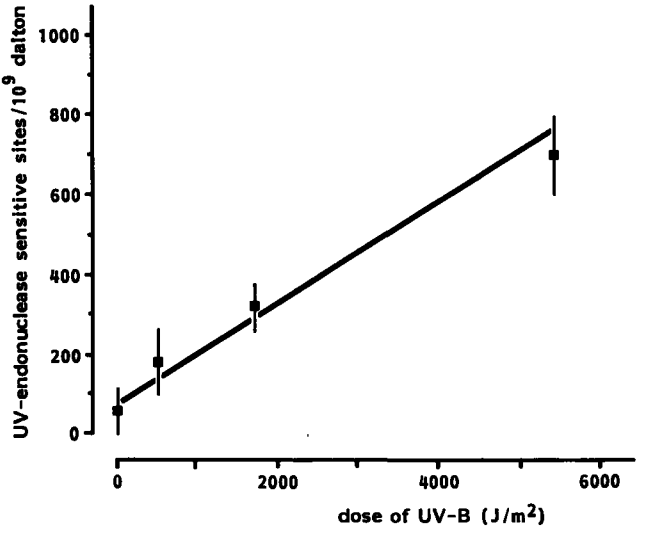



Figure 6.2: Dose response curve for ESS induction by UV-B in rat skin in vivo. Points indicate the mean of 3 determinations. The errors were estimated from the standard deviations.

detection limit was about 500 J/m² of UV-B, which is relevant for human exposures as it is the equivalent of 1-2 times the minimal erythemal dose.

Subsequently, we determined the number of pyrimidine dimers in rat epidermal skin DNA of 6-, and 36-month-old rats, immediately after exposure to 4000 J/m² of UV-B and at 3 and 24 h thereafter. During the repair periods the animals were kept in the dark. After 3 h of repair, a substantial shift of the DNA distribution in the sucrose gradient towards a higher molecular weight was seen (Fig.6.3), which clearly demonstrated that rat epidermal keratino-

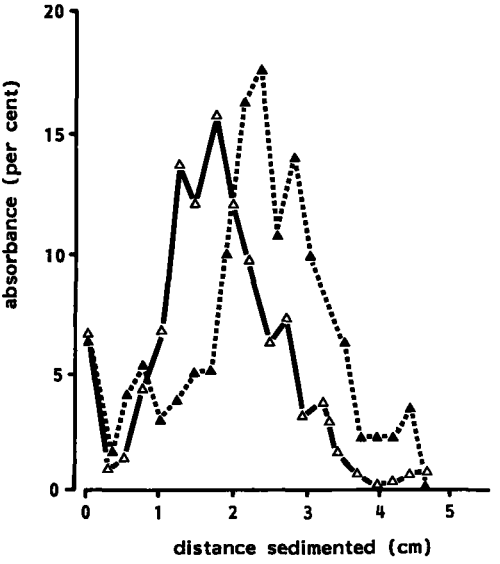



Figure 6.3: Immunochemically detected profiles of rat skin DNA after treatment with M.luteus extract and centrifugation in alkaline sucrose gradients. DNAs were isolated from a skin biopsy at 0 h (closed line) and at 3 h (broken line) after irradiation in vivo with 4000 J/m² of UV-B.

cytes in vivo are well able to remove pyrimidine dimers over this short time interval. The increase in molecular weight corresponds to 50-60 % removal.

Although the removal of pyrimidine dimers in the epidermis was initially rapid, about 30% of the dimers originally induced were still present at 24 h after irradiation (Fig.6.4). We were able to confirm the persistency of a considerable fraction of the lesions induced, by using a non-quantitative

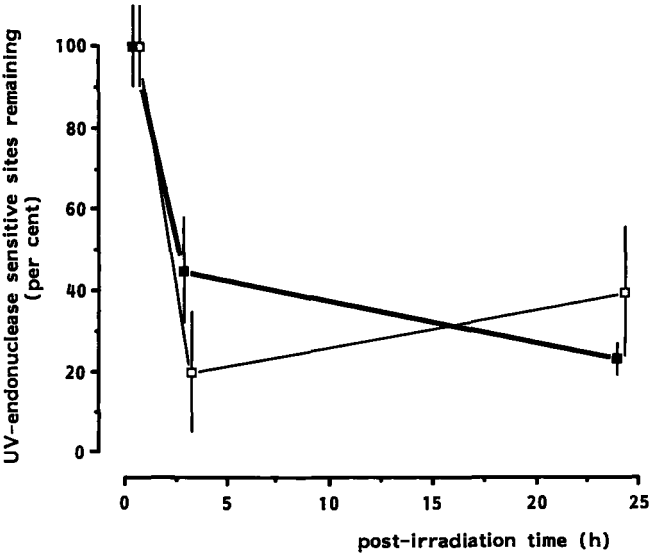



Figure 6.4: Time course of in vivo repair of ESS in rat skin. The fractional disappearance of ESS from DNA after exposure of rat skin to 4000 J/m² of UV-B, which induces about 600 ESS, has been plotted. Points indicate the mean of triplicate determinations. Closed symbols, 6-month old rats; Open symbols, 36-month old rats.

immunofluorescence assay on cryostat sections of rat skin at various time points after irradiation with UV-B. With an antibody specific for thymine dimers we could still detect a considerable number of lesions as late as 48 h after irradiation (results not shown). Figure 6.4 does not indicate any significant differences in the capacity of young and old animals to remove UV-induced pyrimidine dimers.

## 6.4 Discussion

In the present study we have shown that rat epidermal keratinocytes in vivo are able to remove 50-60% of UV-induced pyrimidine dimers by dark repair within 3 h, whereas in the same cell type cultured in vitro no significant removal was observed over this time period. Although we are not aware of any previous studies in which such a direct comparison has been made, this result is not completely unexpected. Indeed, Peleg et al. (1977) already showed that rodent cells have the potential capacity to rapidly remove UV-induced pyrimi-

dine dimers. In addition, data from Sutherland et al. (1980) and D'Ambrosio et al. (1981) indicated a more rapid removal of pyrimidine dimers from human skin in vivo than that found by numerous others, including ourselves (Chapter 3) for human fibroblasts in culture. According to these data, in human skin about 50% of the dimers induced are removed within 1 h, while 24 h after irradiation virtually all dimers have disappeared, with repair kinetics composed of rapid initial phase followed by a much slower later phase.

On the basis of our study we may now definitely conclude that the rate of DNA repair, measured as the removal of UV-induced ESS, differs between the in vitro and the in vivo situation. It appears that mammalian cells are able to suppress the utilization of the DNA repair pathway responsible for the removal of pyrimidine dimers as a consequence of their transfer into culture, possibly in response to the greatly altered circumstances these cells have to deal with during active replication in vitro, as compared to the relatively "resting" state in the skin.

Thusfar, it is unknown whether other repair systems follow the same pattern, or - on the contrary - may become activated. Even if our findings could be extended to other DNA repair systems, other forms of DNA damage and other cell types, this would still not be indicative of a decreased capacity to efficiently deal with DNA damage in cells in vitro as compared to the in vivo situation. As yet the biological consequences of rapid or slow removal of DNA lesions are far from clear. Before conclusions can be drawn it is necessary to obtain insight into the nature of the relationship between induced DNA damage, DNA repair processes and biological phenomena such as the induction of mutations, cancer and aging. The major implication of our present findings is that one should be cautious with the extrapolation of data on DNA repair responses obtained with cultured cells to biological phenomena in the living organism.

# CHAPTER 7

## SUMMARY AND GENERAL DISCUSSION

### 7.1 DNA Repair and Maximum Lifespan

In the introduction to this thesis the possibility was considered that the characteristics of an organism's DNA repair responses ultimately determine its maximum attainable lifespan. In research on aging much attention has been paid to differences in DNA repair processes between species differing in their maximum lifespan. The observation that cells of long-lived species generally have a higher level of UV-induced DNA repair synthesis than those of short-lived ones was initially made by Painter and Cleaver in the late sixties. These investigators were the first to compare several kinds of cultured mammalian cells with respect to DNA repair parameters (Painter and Cleaver, 1969). With the exception of cells derived from XP patients, all kinds of cells showed DNA repair synthesis upon treatment with the genotoxicant ultraviolet light (UV). However, cells of human origin or those from the cow exhibited the phenomenon to a greater extent than cell lines derived from rodents.

In 1974 a strong log-linear correlation was reported between the amount of UV-induced unscheduled DNA synthesis (UDS) in cultured fibroblasts and maximum lifespan, for the seven mammalian donor species, shrew, mouse, rat, hamster, cow, elephant and man (Hart and Setlow, 1974). Although the correlation reported by Hart and Setlow further extends the early findings of Painter and Cleaver, it seems highly speculative to interpret it as evidence for "DNA repair" as a longevity-assurance system. The longevity-assurance theory states that animal species with a longer maximum lifespan are genetically equipped with more efficient systems for the protection, regulation and repair of the essential information molecules in the organism. The findings of Hart and Setlow received much publicity (see for instance, Kahn, 1985) and "DNA repair" (usually considered synonymous to UV-induced UDS) was adopted by many gerontologists as a biomarker for the rate of aging. Subsequent findings of Hart et al. suggested a correlation between lifespan and DNA repair synthesis also among species with less difference in their taxonomical position. They reported a 2.2 times higher rate of repair synthesis in cells of the whitefooted mouse (Peromyscus leucopus) than in those of the house mouse

(Mus musculus). The latter species has a 2.5 times shorter mean lifespan (Hart et al., 1979). The same type of relationship was reported by Paffenholz (1978) for UV-induced DNA repair synthesis in 3 inbred strains of mice with different mean lifespans. Walford and Bergmann (1979) even claimed to have found such a correlation among congeneic mouse strains, differing only in their MHC haplotype. They concluded that longevity was associated with specific MHC haplotypes. Cells from mice with a "long-lived" MHC haplotype had high levels of UDS. On the basis of these findings the authors reasoned that the MHC and DNA repair were interrelated longevity assurance systems.

On the other hand there were also reports on less perfect correlations. Kato et al. (1980) failed to find a correlation between maximum lifespan and DNA repair synthesis in cells derived from 34 mammalian species, and data from Francis et al. (1981) on 21 species suggested only a slight linear correlation between maximum lifespan and DNA repair synthesis. Furthermore, no difference in repair synthesis was observed between cells derived from a congeneic pair of short-lived and long-lived mice (Collier et al., 1982). Interestingly, we did not find any difference between UV-induced UDS in skin fibroblasts from male WAG/Rij rats and that in cells from females, in spite of a difference in maximum lifespan of almost a factor of two (unpublished results). Recently, Turturro and Hart (1984) explained the conflicting data on inter-species differences in DNA repair synthesis in terms of certain shortcomings in the experimental design of the studies that failed to show the log-linear correlation reported earlier by Hart and Setlow.

Whatever the precise characteristics of a putative interrelationship of UV-induced DNA repair synthesis in cells from various species may be, a correlation of "DNA repair capacity" with lifespan can not directly be inferred from it for the simple reason that there is no experimentally approachable endpoint for "DNA repair capacity". Since there are so many different DNA repair pathways their utilization should not necessarily be the same for all species. In other words, there may be mechanistic differences in the way cells from different species remove damage induced in their DNA. Furthermore, in view of the limitations of the present methods for studying DNA repair (see Chapter 2) it is necessary to employ a variety of methods to enable the identification and accurate quantification of each step in the DNA repair pathway of interest. In addition, the possibility should be taken into account that certain DNA repair characteristics of cells in culture vary with donor age, during their transfer from the in vivo to the in vitro situation or during subculturing. Characteristics of the in vivo situation such as hormonal activities, specific cell to cell contacts, metabolic conditions and the status of

differentiation may influence repair activities of the cells under study and could easily change with age and/or become lost in culture. In this thesis some of the above mentioned sources of variation were studied.

## 7.2 DNA Excision Repair in Human and Rat Fibroblasts in Culture

It is generally assumed that UV-induced DNA repair synthesis, measured for instance as UDS, reflects the removal of pyrimidine dimers, the main UV lesion. In 1976, Lohman et al. showed that interspecies differences in DNA repair synthesis can be misleading, by demonstrating that UV-irradiated established hamster cells were well able to perform DNA repair synthesis, but failed to remove pyrimidine dimers. In addition, it was shown that human cells not only perform DNA repair synthesis but also show a great capacity for removal of pyrimidine dimers. The authors emphasized that interspecies differences in the intrinsic capacity to restore the integrity of DNA are not necessarily reflected in the ability to perform DNA repair synthesis, or vice versa (Lohman et al., 1976).

To obtain insight in the relation between lesion removal and repair synthesis, we studied UV-induced excision repair in normal (untransformed) rat and human fibroblasts. By using an enzymatic method (the UV-endonuclease method) for monitoring the removal of pyrimidine dimers and the so-called bromodeoxyuridine (BrdU) photolysis assay for determining the number and average size of repair patches, in principle we covered all but the initial (recognition) steps of the excision repair pathway (see Chapter 2). The results obtained are described in Chapter 3 and allowed the following conclusions.

1. The average repair patch size remains fairly constant over the whole repair period.
2. The repair patch size is not different for the two species.
3. After the same UV dose and repair period, less repair patches are present in rat cells than in human cells.
4. After prolonged repair periods, some dimer removal can be observed in rat cells. Relative to human cells, the difference in dimer removal is substantially larger than the difference in repair synthesis, both at 3 and 24 h after irradiation (confirming the discrepancy between dimer removal and DNA repair synthesis reported by Lohman et al., 1976).
5. In both species, DNA repair synthesis seems to proceed considerably faster than dimer removal, as is most obvious in rat cells; at 3 h after irradiation no significant dimer removal had occurred, although about 24 repair patches per $10^9$ molecular weight of DNA were already created.

In view of these findings we suggest that in rat cells excision repair takes place primarily according to a mechanism in which DNA repair synthesis precedes dimer removal. A model for such an hypothetical mechanism has been presented in Chapter 3. Whatever the nature of the observed differences in excision repair between rat and human cells may be, it can be concluded from this study that interspecies differences in DNA repair processes are more complicated than one might assume.

Another aspect of DNA excision repair in rodent cells that deserves attention is their relatively high survival upon UV irradiation. The survival characteristics of rodent cells, in terms of their colony-forming capacity, do not differ from those of human cells, in spite of their poorer capacity of dimer removal and repair synthesis. This has been shown for mouse fibroblasts (Ganesan et al., 1983; Yagi et al., 1984) and also for the rat skin fibroblasts used in our study (Chapter 3). In view of their identical survival characteristics it seems highly unlikely that the observed differences in DNA excision repair between human and rodent cells reflect a difference in "repair capacity". Instead, the utilization of different DNA repair pathways could be fundamental to the above described discrepancy. The characteristics of the complex of DNA repair activities in rodents could then be such that the survival of their actively dividing cells is comparable to that of human cells, but at the cost of (more) mutations. In other words, the balance of error prone versus error free DNA repair activities might be higher in rodent than in human cells. Consequently, the frequency of genetic changes in rodent cells would be higher than in those of humans. This is in keeping with findings of Britten (1986), indicating a higher rate of DNA sequence evolution of rodents in comparison to primates. The observed differences in mutation rate were probably not due to differences in generation time and/or population history or selection. Instead, evolutionary changes in DNA repair were considered as the most likely explanation for this phenomenon.

## 7.3 DNA Excision Repair in Rat Skin Cells In Vitro and In Vivo

The above described differences in repair characteristics between human and rodent cells in culture illustrate the limited significance of studies aiming at a correlation of species lifespan with only one DNA repair parameter. In our studies on one cell type, which is easy to handle in culture, in combination with a model genotoxic agent such as UV, we were not able to get more than limited information about the nature of interspecies differences in DNA repair. But even if the mechanism(s) of excision repair under such standardized

conditions were well understood, the problem remains that repair characteristics appear to change during culturing of primary cells in vitro or during aging in vivo. By studying UV-induced DNA repair synthesis in rat fibroblasts both in relation to donor age (Chapter 4) and during in vitro passaging (Chapter 5) we showed that while undergoing terminal differentiation (also termed in vitro senescence) these cells underwent a 50% reduction in their capacity to perform DNA repair synthesis. Simultaneously we showed that during in vivo aging, repair synthesis in the same cells decreased by about 16% (Chapter 4). However, upon establishment into culture, primary rat fibroblasts of old donors reached growth crisis earlier than those of young donors (unpublished results). The relatively low average level of repair synthesis in fibroblast populations from old rats could therefore be due to the inclusion in the UDS determinations of some terminally differentiated cells, instead of reflecting a true age-related decrease in DNA repair activity.

Related to the variation observed during passaging in vitro is the possibility that DNA repair characteristics of cells change during their transfer from the in vivo to the in vitro situation. In 1977, Peleg et al. reported that primary mouse embryonic fibroblasts excise dimers well. However, after a number of passages in vitro they lost this capacity. This could reflect the loss of dimer-removing capacity upon bringing cells into culture. It is almost needless to emphasize the importance of such potential variation for the subject of this thesis. After all, aging of mammals is in the first place an in vivo phenomenon. To address this question, reliable techniques, unbiased by differences in cell morphology or animal physiology, are required for comparing DNA repair activities in different cells and tissues in vivo and in vitro. Recently, we have adapted the UV-endo method for the detection of pyrimidine dimers to the application on skin cells in vivo (Vijg et al., 1986c). Chapter 6 describes the application of this method in the study of DNA repair of UV damage in rat skin in vivo.

We showed that rat epithelial cells in vivo remove UV-induced pyrimidine dimers much faster than do the same cells in vitro. Whereas with fibroblasts or epidermal cells in vitro we never observed any removal within three hours, epidermal cells in vivo have already removed more than 50% of the damage over this time period. Interestingly, a substantial part of the damage is persistent; at 24 h after irradiation about 30% of the induced lesions can still be found. No differences in DNA repair kinetics were found between old and young rats.

Our observation that rat epidermal cells in vivo are well able to remove pyrimidine dimers, whereas such cells in vivo are not, clearly demonstrates that rats are genetically equipped with the excision repair pathway via which

pyrimidine dimers can be rapidly removed. Whether this pathway is identical to the one human cells in culture utilize remains to be seen. Indeed, it is well conceivable that excision repair can proceed via multiple pathways. The possibility that the utilization of such pathways depends on the cell type, the species and the environmental constraints the cells are subject to, is a fascinating area to explore further. Especially the rationale for the utilization of different DNA repair pathways by different species is an important aspect of research on aging. One might speculate that the set of genetically expressed repair pathways and possible switches therein is the resultant of a balance between such factors as the generation time of the cell (for some cells this means the total lifespan of the organism) and the amount of DNA sequence changes that can be tolerated. However, before any realistic conclusions can be drawn about the possible role of a multitude of more or less error-prone DNA repair pathways in the aging process it is necessary to develop techniques that allow to accurately assess the frequency and molecular nature of DNA sequence changes in various organs and tissues of aging organisms.

## 7.4 Recapitulation and Conclusions

The purpose of the research described in this thesis was to further investigate the possible role of DNA repair in the aging process in mammals. Two main lines of research were explored, viz., the putative correlation of DNA repair with species lifespan and the possible occurrence of changes in DNA repair during cell aging. The possibility that differences in DNA excision repair between cells of different species might be fundamental to the well documented interspecies variation in maximum lifespan was investigated by studying various steps in UV-induced DNA excision repair in rat and human fibroblasts, using a number of different techniques. Possible changes in DNA repair during cell aging were studied by repeated determinations of UV-induced DNA repair synthesis in fibroblasts from inbred rats of various ages. In addition, the possibility was investigated that rat skin cells would undergo changes in DNA excision repair during subculturing and upon their transfer from the in vivo to the in vitro situation. The results obtained can be summarized as follows.

1. In cultured rat and human fibroblasts there is a lack of correspondence between the removal of pyrimidine dimers and the number of newly synthesized patches of DNA; especially for rat cells we found evidence that repair synthesis can, at least in part, precede dimer removal.
2. There are no considerable changes in UV-induced DNA repair synthesis

in rat fibroblasts in vitro as a function of donor age.

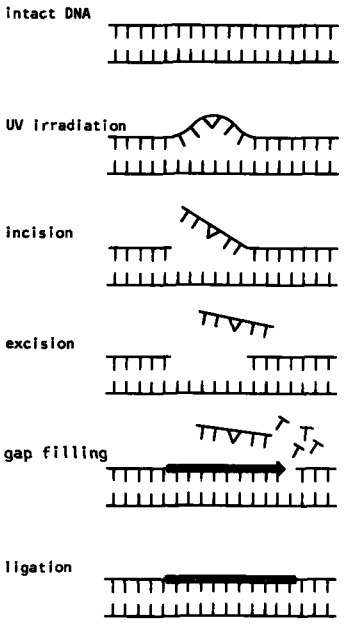
3. UV-induced DNA repair synthesis undergoes a 50% increase during the first stages of terminal differentiation in vitro, followed by a 50% decrease after the cessation of overall replicative ability; during immortalization, however, no changes in DNA excision repair were observed.
4. Epidermal keratinocytes in vivo, in both young and old rats, repair UV-induced pyrimidine dimers rapidly although not completely; in vitro, however, UV-induced pyrimidine dimers are as persistent as they are in rat fibroblasts in culture.

From these results three major conclusions can be drawn:

a. mammalian cells can utilize more than one pathway to remove pyrimidine dimers by excision repair.
b. mammalian cells can suppress the utilization of a DNA repair pathway.
c. mammalian cells do not undergo major changes in UV-induced DNA excision repair during aging.

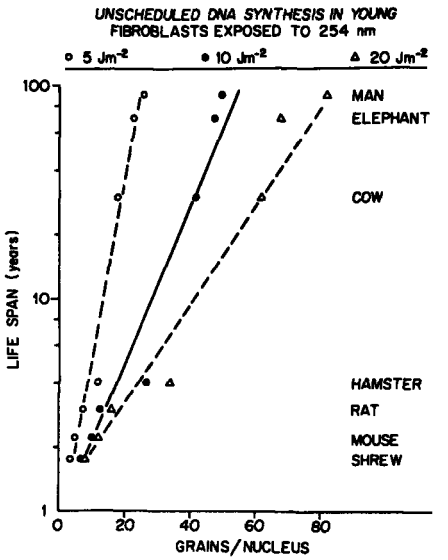
# SAMENVATTING

Het onderwerp van dit proefschrift heeft betrekking op de mogelijke rol van DNA-herstelsystemen in het verouderingsproces. Met name aan de mogelijke relatie tussen de zogenaamde "DNA-herstelcapaciteit" van een diersoort en diens maximale levensduur is binnen het verouderingsonderzoek veel aandacht besteed. Tegen het einde van de zestiger jaren werd door de Amerikaanse onderzoekers Painter en Cleaver waargenomen dat gekweekte huidcellen afkomstig uit (langlevende) koeien of mensen na bestraling met ultraviolet licht (UV) meer DNA-herstelsynthese per tijdseenheid vertoonden dan cellen uit (kortlevende) knaagdieren. Bij DNA herstelsynthese worden nieuwe stukjes DNA ingebouwd ter vervanging van de door het UV beschadigde gedeelten (Fig.1). In het algemeen wordt aangenomen dat de snelheid waarmee deze inbouw plaats vindt een maat vormt voor de activiteit van het DNA-herstelsysteem dat de reparaties uitvoert.




Figuur 1:
Schema van de wijze waarop o.a. zoogdiercellen verondersteld worden DNA-beschadigingen te verwijderen via excisieherstel. Na beschadiging van het DNA, bij voorbeeld door ultraviolet licht (UV), wordt het stukje DNA dat de schade bevat eruit geknipt, en door middel van zogenaamde "DNA herstelsynthese" vervangen door een nieuw stukje.

In 1974 rapporteerden twee andere Amerikaanse onderzoekers, Hart en Setlow, hun resultaten over de UV-geïnduceerde DNA-herstelsynthese in cellen van zeven verschillende zoogdieren: spitsmuis, muis, rat, hamster, koe, olifant en mens. Een gedeelte van deze resultaten is weergegeven in Fig. 2. Daaruit blijkt dat de hoeveelheid DNA-herstelsynthese in de met UV bestraalde cellen van de verschillende diersoorten log-lineair gecorreleerd is. Dit betekent dat, hoe langer de levensduur van de betreffende diersoort is, hoe meer DNA-herstelsynthese per tijdseenheid plaats vindt.




*Figuur 2:*
De door Hart en Setlow in 1974 gepubliceerde correlatie tussen UV-geïnduceerde DNA herstelsynthese in gekweekte huidfibroblasten ("grains/nucleus" op de x-as) en de logaritme van de levensduur ("life span" op de y-as). Duidelijk is te zien dat voor 3 doses UV (5, 10 en 20 Joules per $m^2$) de hoeveelheid herstelsynthese (over 13 uur) groter is in lang levende dieren (mens, olifant, koe) dan in kort levende dieren (knaagdieren).

Deze bevindingen kregen veel publiciteit. Immers, zij leken de theorie te ondersteunen dat bepaalde cellulaire funkties de lengte van het leven bepaalden. Het "DNA herstel" werd door veel gerontologen direkt beschouwd als één van de belangrijkste van deze funkties. De verwachtingen omtrent verdergaand onderzoek op dit gebied waren dan ook hooggespannen. Ongelukkigerwijs resulteerden veel andere studies vaak in een veel minder fraaie correlatie. Hoewel met name Hart nog een aantal resultaten publiceerde die mooi in de theorie pasten, bleek uit onderzoekingen van andere Amerikaanse en van Japanse onderzoekers aan veel meer diersoorten dat, gemiddeld genomen, cellen van langlevende dieren inderdaad meer DNA-herstelsynthese vertoonden, maar dat dit, zelfs binnen de zoogdieren, lang niet altijd op hoeft te gaan. Bij de lagere soorten dan zoogdieren kon zo'n correlatie helemaal niet worden aangetoond. Buiten Hart zelf, die alle negatieve resultaten consequent weet te

verklaren uit fouten in de experimentele opzet etc., is er dan ook vrijwel geen ervaren molekulair-biologisch onderzoeker die nog verondersteld dat er een simpele relatie is tussen UV-geïnduceerde DNA-herstelsynthese in de huidcellen en levensduur. De echte reden hiervoor is niet zozeer het al of niet gecorreleerd zijn van DNA-herstelsynthese met levensduur, maar meer het toegenomen begrip omtrent de complexiteit van DNA herstelprocessen. Het is op dit moment wel duidelijk dat er veel verschillende manieren zijn om beschadigingen van het DNA te herstellen. Het is daarom heel goed mogelijk dat cellen van de ene diersoort de voorkeur geven aan het gebruik van het ene systeem, terwijl cellen van andere diersoorten preferentieel weer andere systemen gebruiken. Deze complexiteit kan zich heel goed voortzetten binnen het individu. Een levercel zou DNA schade bijvoorbeeld op een geheel andere manier kunnen verwijderen dan een huidcel. Verder is het niet onwaarschijnlijk dat zich veranderingen voordoen in het gebruik van DNA-herstelsystemen *tijdens en na het in kweek brengen van cellen.*

Op basis van het bovenstaande zal duidelijk zijn dat de "DNA-herstelcapaciteit" van een organisme niet zo gemakkelijk experimenteel benaderbaar is. In dit proefschrift zijn metingen verricht aan DNA-herstelprocessen in met UV bestraalde huidcellen van mensen en ratten met gebruikmaking van verschillende methoden. Daarbij is getracht inzicht te verkrijgen in de mogelijke invloed op de DNA-herstelaktiviteiten van een aantal variabelen, waaronder de leeftijd van de donor, de tijd gedurende welke de (delende) huidcellen gekweekt werden en het kweken zelf. Met betrekking tot dit laatste is vergelijkend onderzoek gedaan aan herstel van UV-schade in cellen in het intakte dier en in cellen van hetzelfde type na het in kweek brengen. Door met behulp van verschillende methoden verschillende stappen in het herstelproces te meten is tevens getracht het boven beschreven verschil in DNA-herstelsynthese tussen kortlevende ratten en langlevende mensen wat nader te karakteriseren.

Tijdens het gehele onderzoek werd gebruik gemaakt van UV als schadeinducerend agens, omdat dit a) goed is te doseren en b) tot het best gekarakteriseerde genotoxische agens behoort. Voor de mens komt daar dan bij dat meer kennis omtrent het UV-schademetabolisme in de huid van belang is in verband met de mogelijke rol van UV bij huidveroudering en het ontstaan van huidtumoren. Behoudens het gedeelte van het proefschrift dat handelt over verschillen in DNA-excisieherstel tussen humane en rattecellen in vitro (Hoofdstuk 3), zijn alle andere studies verricht aan ingeteelde ratten. Dit is niet zo verwonderlijk gezien de belangrijke rol van knaagdieren als modelsystemen voor het onderzoek naar veroudering en kanker. Het gebruik van ingeteelde ratten, gehouden onder identieke condities, beperkt de interindivi-

duele variabiliteit (de belangrijkste storende faktor bij verouderingsonderzoek aan mensen) tot een minimum. Verder zijn experimenten met mensen om voor de hand liggende redenen nu eenmaal moeilijker uitvoerbaar dan met proefdieren.

Na de algemene inleiding waarin de aard van het verouderingsproces wordt bediscussieerd, alsmede de mogelijke rol van "DNA-herstel" daarin, biedt Hoofdstuk 2 een overzicht van de gebruikte (en tevens ook van andere mogelijke) methoden voor het meten van DNA-excisieherstel. Hoofdstuk 3 handelt over experimenteel onderzoek aan UV-geïnduceerd DNA-herstel in huidfibroblasten van mens en rat. Het betreft hier in feite een uitgebreide analyse van een fenomeen dat reeds in 1976 door Lohman werd gesignaleerd en gepresenteerd in een kort verslag in Mutation Research. Daarin wordt beschreven dat gekweekte hamstercellen na bestraling met UV goed in staat zijn tot DNA-herstelsynthese. Echter met behulp van een op zijn laboratorium opgezette methode voor het meten van de door het UV aangerichte beschadigingen toonde Lohman aan dat de gevonden herstelsynthese niet gekoppeld was aan enige noemenswaardige verwijdering van de belangrijkste UV-schade, pyrimidine dimeren. Dit is zeer verwonderlijk aangezien herstelsynthese in het algemeen geacht wordt op te treden nadat de schade verwijderd is (Fig.1). Andere gegevens afkomstig van het laboratorium van Lohman lieten zien dat gekweekte humane cellen wel degelijk in staat waren tot verwijdering van de schade.

*Om nu wat meer inzicht te verkrijgen in de relatie tussen schadeverwijdering* en herstelsynthese zijn humane en rattecellen steeds in één experiment vergeleken, zowel wat betreft DNA-herstelsynthese als schadeverwijdering. Schadeverwijdering werd gemeten zowel met behulp van de zogenaamde UV-endo methode als met behulp van HPLC analyse (beide technieken staan beschreven in Hoofdstuk 2). Herstelsynthese werd gemeten als de mate van inbouw van radioactieve thymidines per tijdseenheid. Dit wordt ook wel "unscheduled" DNA-synthese (UDS) genoemd, ter onderscheiding van de "scheduled" DNA-synthese, zoals die optreedt vlak vóór de celdeling. Daarnaast werd met behulp van nog een andere methode, de BrdU-photolyse methode, de grootte van de gemiddelde "herstel patch", het stukje beschadigd DNA dat vervangen is, alsmede het aantal van deze patches gemeten. Het aantal patches, vermenigvuldigd met de gemiddelde grootte daarvan, zou dus eigenlijk dezelfde relatieve waarden op moeten leveren als de boven beschreven UDS waarden. Echter de BrdU-photolyse methode is veel specifieker dan de UDS assay, hetgeen van essentieel belang is bij een vergelijkend onderzoek naar excisieherstel.

De verkregen resultaten hebben tot de volgende conclusies geleid:

1. Tijdens het proces van herstelsynthese, dat bij de gebruikte dosis in beide celtypen grofweg 24 uur duurt, blijft de gemiddelde grootte van de herstelpatches, die steeds gecreëerd worden, gelijk.
2. De gemiddelde patchgrootte in het DNA van humane en rattecellen verschilt niet.
3. Na eenzelfde UV-dosis en herstelperiode zijn er minder patches gevormd in het DNA van de rattecellen dan in dat van humane cellen; echter na 24 uur is dit verschil veel kleiner dan na 1½ tot 3 uur.
4. Schadeverwijdering in rattecellen wordt pas gevonden tussen 3 en 24 uur; na 3 uur is nog geen verwijdering opgetreden. Het verschil in dimeerverwijdering tussen humane en rattecellen is significant groter dan het verschil in herstelsynthese. De discrepantie tussen dimeerverwijdering en DNA-herstelsynthese zoals voor het eerst gevonden door Lohman is hiermee bewezen
5. In cellen van zowel mens als rat lijkt DNA herstelsynthese sneller te gaan dan dimeerverwijdering. In rattecellen is dit het duidelijkst; 3 uur na UV-bestraling zijn nog geen dimeren verwijderd, terwijl reeds 24 herstelpatches per $10^9$ dalton DNA zijn gecreëerd.

Op basis van onze bevindingen lijkt het er sterk op dat in ieder geval in rattecellen, maar wellicht ook voor een deel in die van de mens, DNA-herstelsynthese vooraf gaat aan dimeerverwijdering. Een model voor zo'n hypothetisch mechanisme is gepresenteerd aan het eind van Hoofdstuk 3. De belangrijkste algemene conclusie uit boven beschreven werk is dat de gevonden verschillen in DNA-herstel tussen humane en rattecellen niet noodzakelijkerwijs duiden op een verschil in DNA herstelcapaciteit tussen de twee diersoorten. Het is zeer goed mogelijk dat knaagdiercellen preferentieel gebruik maken van een DNA excisieherstelsysteem dat, hoewel potentieel mogelijk ook bij de mens aanwezig, daar toch niet de voorkeur geniet.

Dat knaagdiercellen niet noodzakelijkerwijs een inferieur DNA herstelsysteem hebben, blijkt ook uit hun vermogen tot klonale uitgroei na UV bestraling. Zoals Fig. 3.1 laat zien, hebben humane en rattecellen dezelfde mate van overleving (klonale uitgroei).

Terwijl Hoofdstuk 3 gewijd is aan verschillen in DNA-excisieherstel tussen humane en rattehuidfibroblasten gaan de overige 3 hoofdstukken uitsluitend over rattecellen. Hoofdstuk 4 beschrijft een serie metingen van UV-geïnduceerde UDS in huidfibroblasten van ratten van verschillende leeftijd. Hoewel het oorspronkelijk de bedoeling was een aantal ratten gedurende hun leven te vervolgen door met bepaalde tussenpozen een meting te doen aan fibroblasten

geïsoleerd uit een huidbiopt, bleek al gauw dat de interindividuele verschillen in UDS zeer gering waren. Opmerkelijk was de kleine, doch significante leeftijdsgeassocieerde daling in UDS. Dat deze daling wellicht geen echte afname in UDS vertegenwoordigde, maar meer een secundair effekt, bleek uit de vervolgstudie waarbij gekeken werd naar UV-geïnduceerd excisieherstel in rattefibroblasten tijdens het kweken in vitro.

Reeds door anderen werd beschreven dat gekweekte huidfibroblasten morfologische veranderingen ondergaan alvorens in een z.g. "groeicrisis" terecht te komen. Humane fibroblasten bereiken zo'n groeicrisis pas na ca. 60 populatieverdubbelingen en komen er dan nooit meer uit. Veel onderzoekers beschouwen deze afname in proliferatieve capaciteit, die geassocieerd is met een serie morfologische veranderingen, als representatief voor het cellulaire verouderingsproces zelf. Op basis hiervan zou men kunnen zeggen dat wij bij de rat zowel naar in vivo als in vitro veroudering hebben gekeken (zie ook Fig.1.4).

Metingen van UV-geïnduceerd DNA excisieherstel in rattefibroblasten tijdens het kweken in vitro laten in eerste instantie een snelle stijging zien die geassocieerd bleek met het overgaan van de primaire populatie van smalle spoelvormige cellen in een populatie van grote pleiomorfe cellen die nog steeds actief deelden. In een dun opgezette populatie cellen vond uiteindelijk terminale differentiatie (groeicrisis) plaats, d.w.z. de meeste cellen verloren hun vermogen tot delen en weerhielden door het grote oppervlak dat ze innamen, de weinige overgebleven kleine cellen van uitgroei. Echter, doorgaan met populatieopsplitsingen leidde tot het selectief uitgroeien van deze "onsterfelijke" cellen. Het bleek dat de terminaal gedifferentieerde cellen een UDS-niveau hadden dat de helft lager was dan dat van de actief delende grote pleiomorfe cellen en dus gelijk aan dat van de primaire cellen.

Onsterfelijke cellijnen konden veel gemakkelijker worden verkregen door de kweek gewoon wat dichter op te zetten. In dat geval bleven er steeds zoveel potentieel onsterfelijke cellen in de populatie dat een groeicrisis in het geheel niet waarneembaar was. De reden hiervan is waarschijnlijk dat bij lage celdichtheid het terminale differentiatieproces veel sneller gaat. Dit werd gesuggereerd door microscopische waarnemingen. De resultaten uit dit onderzoek verkregen, werden geïnterpreteerd in de context van het terminaal differentiëren van rattefibroblasten in kweek. Gesteld werd dat rattefibroblasten na isolatie uit de huid en stimulatie tot deling blijkbaar in staat zijn tot een hogere UDS respons dan in hun "rustende" toestand in de huid, of kort na isolatie. Echter na terminale differentiatie is de UDS respons weer terug in z'n oude toestand. Deze interpretatie is deels gebaseerd op parallellen met UDS in andere celtypen waarbij lage waarden in het algemeen werden geassocieerd

met (terminaal) gedifferentieerde cellen en een snelle toename in UDS met proliferatie en differentiatie. In dit verband is het verleidelijk om te speculeren over de mogelijkheid dat een goed DNA-herstel van essentieel belang is in prolifererende en differentiërende cellen met hun vele genen die afwisselend aan en uit worden gezet, en omgekeerd dat in terminaal gedifferentieerde of rustende cellen de DNA-herstelactiviteiten flink teruggeschroefd kunnen worden. Echter, zo'n simplistische denkwijze was al eens eerder toegepast naar aanleiding van UDS-metingen in cellen van kortlevende en langlevende dieren. Aardig dus om de gedachten te bepalen en wellicht een uitgangspunt voor nader onderzoek. Voorlopig kunnen we volstaan met de conclusie dat UV-geïnduceerde excisieherstelprocessen behoorlijk kunnen variëren tijdens het kweken. Hoewel onbewezen, is het niet onwaarschijnlijk dat de geringe leeftijdsgeassocieerde afname in UDS zoals beschreven in Hoofdstuk 4, volledig te wijten is aan een hoger percentage terminaal gedifferentieerde cellen in kweekjes van oude dieren. Inderdaad was ons herhaalde malen gebleken dat fibroblasten uit oude ratten iets langzamer groeiden dan cellen uit jonge dieren.

Op basis van de resultaten vermeld in de Hoofdstukken 4 en 5 kunnen we concluderen dat het niveau van UV-geïnduceerde DNA herstelsynthese in geïsoleerde rattehuidfibroblasten opmerkelijk stabiel is tijdens veroudering, maar onderhevig aan snelle veranderingen tijdens terminale differentiatie in kweek. Tijdens immortalisatie, wat door velen beschouwd wordt als een voorwaarde voor neoplastische transformatie, lijkt UDS opmerkelijk stabiel. Zoals ook beschreven in Hoofdstuk 5 lijkt immortalisatie bij voorkeur op te treden in de spoelvormige primaire cellen en met een veel minder hoge frequentie tijdens de verschillende stadia van terminale differentiatie; slechts weinig onsterfelijke cellijnen met een morfologie zoals die van de grote pleiomorfe cellen konden worden verkregen. De meeste onsterfelijke cellijnen waren spoelvormig, zoals de normale primaire cellen, en hadden hetzelfde niveau van DNA-herstelsynthese na UV-bestraling.

De boven beschreven resultaten zijn redelijk in overeenstemming met bevindingen van andere onderzoekers zoals vóór, vrijwel gelijktijdig of na ons gepubliceerd. Echter, zij wijken op één belangrijk punt af. Terwijl Amerikaanse en Israëlische onderzoeksgroepen onafhankelijk van elkaar aantoonden dat primaire fibroblasten afkomstig uit muize-embryo's zeer goed in staat waren UV-schades te verwijderen (i.t.t. late passagecellen waarin ook zij persistentie van dimeren vonden) kon dit niet door ons worden bevestigd. Noch primaire cellen uit volwassen ratten, noch die uit embryo's bleken in staat om over een tijdsbestek van 24 uur meer dan 20% van de geïnduceerde pyrimidine dimeren

te verwijderen (Hoofdstuk 5). Dit laatste werd door Japanse onderzoekers bevestigd voor wat betreft de muizestam waar zij mee werkten, maar is door hen nooit gepubliceerd (H. Takebe, persoonlijke mededeling).

Om de mogelijkheid te onderzoeken of rattehuidcellen wellicht de capaciteit tot UV schadeverwijdering verliezen tijdens het in kweek brengen, bleek het noodzakelijk de UV-endo methode voor de detectie van pyrimidine dimeren dusdanig te modificeren dat het gebruik ervan niet langer beperkt was tot de in vitro gekweekte cellen. Hoofdstuk 6 beschrijft de aangebrachte modificaties, alsmede de toepassing van de techniek bij de bestudering van in vivo herstel van UV-schades in rattehuidcellen. Omdat het gebruikte UV niet ver genoeg de huid in straalt zijn deze experimenten uitgevoerd met epidermale huidcellen in plaats van met fibroblasten waar al het in vitro werk mee gedaan was. In dit verband werd eerst nagegaan of pyrimidinedimeren ook in epidermale cellen persistent waren. Dit bleek inderdaad het geval. Echter, 3 uur na UV-bestraling van de geschoren huid van een levende rat bleek reeds de helft van de geïnduceerde schade verdwenen. Dit is tamelijk opmerkelijk, gezien het feit dat met fibroblasten of epidermale cellen in vitro over zo'n korte tijd nog nooit significante verwijdering was aangetoond. Echter, 24 uur na bestraling bleek toch nog tenminste 30% van de geïnduceerde schade aanwezig. Met behulp van een antilichaam tegen thymidinedimeren werd aangetoond dat zelfs na 48 uur nog DNA schade in de epidermis aanwezig was. Dit suggereert dat tenminste een deel van de geïnduceerde schade persistent is.

UV-schadeverwijdering uit bestraalde epidermis van oude ratten bleek niet significant af te wijken van dat in jonge beesten. Echter, gezien het geringe aantal dieren (twee) en de vrij grote experimentele fout kan hieruit hoogstens geconcludeerd worden dat er geen grote leeftijdsgeassocieerde verschillen zullen zijn.

De belangrijkste conclusie met betrekking tot de resultaten beschreven in Hoofdstuk 6 is ongetwijfeld dat de UV-schadeverwijderingsstap in het excisieherstelproces sterk verschilt tussen de in vivo en de in vitro situatie. Blijkbaar zijn rattecellen in staat om na het in kweek brengen hun DNA-herstelpatroon drastisch te wijzigen. Omtrent de noodzaak hiervan kan alleen maar gespeculeerd worden. Het is echter niet onmogelijk dat in snel delende gekweekte cellen het accent meer op andere DNA-herstelprocessen wordt gelegd, meer aangepast aan de nieuwe omgeving. Eens te meer is dit een illustratie van de complexiteit van DNA-herstel.

Teruggaand naar het begin van deze samenvatting kan gesteld worden dat de in dit proefschrift beschreven onderzoekingen bepaald geen spectaculair nieuw licht werpen op de mogelijke rol van "DNA-herstel" in het verouderings-

proces. Een door veel auteurs beschreven verminderd vermogen tot DNA-herstel in cellen van oude dieren is hier niet gevonden. Integendeel, de hier bestudeerde DNA-herstelresponses zijn opmerkelijk stabiel in relatie tot de leeftijd. De enorme complexiteit van zelfs een schijnbaar simpel proces als de verwijdering van pyrimidinedimeren doen het ergste vrezen voor om het even wat voor correlatie tussen een DNA-herstelrespons van een cel en de maximale levensduur van het donordier. Al met al dus niet erg hoopgevend voor de "DNA repair theories of aging", zoals tot nog toe geformuleerd. Echter, zoals uit vrijwel alle metingen van DNA-herstelresponses, inclusief de onze, blijkt is het herstel van DNA-beschadigingen vrijwel nooit compleet. Zelfs in UV-bestraalde gekweekte humane fibroblasten duurt het erg lang voor er geen dimeren meer detecteerbaar zijn. Hoewel dit deels nog te wijten zou kunnen zijn aan de tamelijk kunstmatige situatie en de (altijd) hoge UV dosis, anders is het in de experimenten beschreven in Hoofdstuk 6. De situatie daar is betrekkelijk natuurlijk (de in vivo situatie) en de dosis zeker niet te hoog. Op een zonnige dag doet vrijwel elk mens wel zo'n dosis op. Toch blijkt een groot deel van de aangebrachte schade persistent. Een soortgelijke bevinding werd gedaan buiten het kader van dit proefschrift. Ratten die intraperitoneaal werden ingespoten met het carcinogeen 2-acetylaminofluoreen bleken tenminste 21 dagen later nog DNA schades te bevatten (ook hier werden géén leeftijds-geassocieerde verschillen gevonden in de mate van DNA-herstel). Gezien de dagelijkse blootstelling van levende organismen aan carcinogene chemicaliën - dat door velen wel als kenmerkend voor deze tijd wordt beschouwd - is ook dit een betrekkelijk natuurlijke situatie. Echter, er zijn aanwijzingen dat bij-producten van de ademhaling, de lichaamswarmte zelf, alsmede de suiker in onze voeding al meer DNA-schades induceren dan welk ten onrechte in het milieu geraakt landbouwgif dan ook. Het is daarom zeker niet onwaarschijnlijk dat bepaalde typen DNA-schades zich ophopen tijdens veroudering, wellicht selectief in bepaalde gebieden van het genoom. Vóór het bestuderen van een groot aantal DNA-herstelprocessen in verschillende celtypen in relatie tot de leeftijd lijkt het daarom zaak eerst pogingen in het werk te stellen zo'n mogelijke ophoping van "spontane" DNA-schades in het verouderend genoom vast te stellen. De recente ontwikkeling van uiterst gevoelige en specifieke methodes voor de detectie en kwantificering van DNA-schades maakt dit mogelijk.

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# CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 19 juni 1954 te Rotterdam. Na het behalen van het diploma MULO in 1971 werd een aanvang gemaakt met de studie HBO medische microbiologie die in 1975 werd afgerond met het behalen van het diploma HBO-A. Na twee jaar als analist te hebben gewerkt, één jaar op het klinisch chemisch laboratorium van het Holy Ziekenhuis te Vlaardingen en één jaar op de afdeling Immunohaematologie van het Academisch Ziekenhuis te Leiden, werd in 1977 aangevangen met de studie biologie aan de Rijksuniversiteit te Leiden (op basis van een colloquium doctum). Het kandidaatsexamen werd afgelegd in 1980 en het doctoraalexamen in 1982.

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